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Olive Oil

Constituents, Quality, Health Properties and Bioconversions

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OLIVE OIL – CONSTITUENTS, QUALITY, HEALTH PROPERTIES AND BIOCONVERSIONS

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Meet the editor

Dr Dimitrios Boskou received his Diploma and Doctor's degree in Chemistry from Aristotle University of Thessaloniki (Hellas), his PhD from the University of London, UK, and the degree of Doctor of Science from the School of Chemistry, Aristotle University. He served as a lecturer, an associate professor, a professor, and Head of the Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University (1970-2006). From 1986 to 1998, he was a member of the Commission on Oils, Fats, and Derivatives of the International Union of Pure and Applied Chemistry (IUPAC). Between 1995 and 2005 he served as a member of the Supreme Chemical Council in Athens. Since 1995, he has been a member of the Scientific Committee for Food of the European Commission and Expert of EFSA (Food Additives Panel). He has published more than 85 papers and reviews, he is the author and editor of six books, and is a lead author of more than 10 chapters in books related to oils and fats, natural antioxidants, and olive oil chemistry. He is a contributor to international scientific encyclopedias and the Lexicon of Lipid Nutrition, a joint IUPAC/IUNS work.

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Preface

Olive oil is an integral component of the dietary pattern known as 'Mediterranean diet', which was first acknowledged almost 40 years ago. Over the years, many investigations (both epidemiological and laboratory) indicated that this diet may be associated with lower levels of systematic inflammation, and lower rates of diseases such as cardiovascular, coronary heart disease, certain types of cancers, diabetes, and others. As a result, olive oil, a staple food for thousands of years for the inhabitants of the Mediterranean region, is now becoming popular among consumers all over the world.

The health effects of olive oil are attributed to its high content of monounsaturated fatty acids and the presence of some minor components, which have become the subject of intensive research over a short period of time. Efforts focus mainly on minor constituents of virgin olive oil with biological importance or on those which affect the organoleptic properties and contribute to its remarkable oxidative stability. Further research is expected to provide new insight into the role of each class of olive oil minor constituents, possible synergism and the magnitude of the contribution of the various bioactive ingredients to the overall positive heath impact in fighting disease.

This book presents some important aspects of the current state of the art in the chemistry, analysis and quality assessment of olive oil and its minor constituents, extraction of olive oil from the fruits, water treatment, and innovative approaches for the production of olive oil based products. It also discusses bioavailabilIty and pharmacological and other properties of bioactive ingredients in the light of new evidence for the composition of olive oil. It also covers also some aspects related to biotechnology and other technologies to retain optimum levels of such bioactive ingredients in the various olive oil forms and to protect the environment from olive mills waste products.

The book, composed of monographic chapters, is organized in five parts.

Part 1 "**Olive Oil Composition, Analysis and Quality"** discusses broadly non-volatile and volatile components related to flavor (chapters 1and 2), analysis and quality assessment methods (chapters 3-8,10), and traceability of origin (chapter 9). Chapter 11 is an extensive presentation of olive oil produced in Australia - a new country where olive tree was first introduced only two centuries ago and its systematic

cultivation is very recent. Chapter 12 examines olive oil from the point of view of consumers and analyzes the tendencies and preferences in relation to quality and other attributes. Important topics covered in this part are:

Biosynthesis of volatiles
Effect of agronomic and other factors such as storage on quality characteristics
Taste receptors and bitterness perception
Conventional methods of analysis and innovative approaches for the determination of trace metals, organoleptic characteristics, and the detection of sensory defects

Part 2"Olive Oil Extraction and Waste Water Treatment" describes biotechnological and other methods to improve recovery of olive and olive pomace oil and treatment of mill wastes (chapters 13-17). Chapter 13 proposes an improved hydrothermal treatment to obtain a higher level of microcomponents with biological value in olive pomace oil. Chapters 14-16 are presentations related to genetic improvement of olives, microbial biotechnology applications in olive oil industry, enzymatic extraction, green technology and bioremediation. Specific topics analyzed are: treatments of the solid wastes and wastewaters from the two and three phase extraction systems; anaerobic digestion processes ,energy recovery; production of value added products by microorganisms using oil mills waste as substrate.

Part 3 "Bioavailability and Biological Properties of Olive Oil Constituents" presents the chemistry, metabolism, bioavailability (and the different endogenous and exogenous variables involved) and properties of important bioactive compounds such as hydroxytyrosol, oleocanthal, other polar phenols and carotenoids, present in olive oil (chapters 18-21). Emphasis is given to recent research related to anti-inflammatory actions, the role that these compounds may have in the clinical treatment of chronic disease, as well as the possible use of preparations based on olive oil constituents as therapeutic agents. Chapter 22 deals with fatty acids and sensitive neurons involved in the regulation of energy and glucose homeostasis. The research aims at identifying novel pharmacological targets for the prevention and treatment of diabetes and obesity.

Part 4 "Innovative techniques for the production of olive oil based products" covers topics such as replacement of animal in meat products by olive oil to obtain products rich in monounsaturated fatty acids (a healthier fatty acid profile, enzymatic production of structured olive oil triacylglycerols and applications in the cocoa butter equivalents and neutraceuticals industry (chapters 23-25). Chapter 26 focuses on the role olive oil may play as an inducer of lipase production. Chapter 27 deals with the incorporation of olive oil into phospholipid membranes of liposomes carrying active cytotoxic agents, in particular, photosensitizers. It reports also on the use of these totally natural and biocompatible olive oil-containing liposomes in ointments and creams for application on skin areas contaminated with bacteria.

Part 5 "Regional studies" contains chapter 28 that discusses olive cultivation and olive oil production in Albania. The chapter is an agro-economic study analyzing the structural and constitutional reforms, which followed the transition from a centrally planned to a market economy, and the impact on the growth and perspective of olive oil sector in this country.

It is hoped that this book will serve as useful source of knowledge recently accumulated and as a comprehensive reference for a broad audience, mainly food scientists, biotechnologists, nutritionists, pharmacologists, researchers in Biosciences, olive growers, olive oil producers, but also members of the general public and consumers who are looking to extract health benefits from the diet of the people living in the countries surrounding the Mediterranean Sea.

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Part 1

Olive Oil Composition, Analysis and Quality

1

Volatile and Non-Volatile Compounds of Single Cultivar Virgin Olive Oils Produced in Italy and Tunisia with Regard to Different Extraction Systems and Storage Conditions

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1. Introduction

Virgin olive oil has a fundamental role in the markets of alimentary oils because of its unique aroma, its stability and its healthy benefits. In this chapter the attention will be focused on Tunisian and Italian single cultivar olive oils.

The oils under investigation were produced by different extraction systems and characterised for their volatile and non-volatile compounds (Benincasa et al., 2003; Cerretani et al., 2005; Garcia et al., 1996). It is well known that volatile and non-volatile components of products of plant origin are dependent on genetic, agronomic and environmental factors. There are few reports (Angerosa et al., 1996, 1998a, 1998b, 1999; Morales et al., 1995; Solinas et al., 1998) on the evaluation of the relationships between the aroma components of virgin olive oil with the metabolic pathways and varietal factors. Olive ripening process and, to some extent, the fruit growing environment, affect also the composition of the volatile compounds of the oil (Aparicio & Morales, 1998; De Nino et al., 2000; Guth & Grosh, 1993; Montedoro & Garofalo, 1984; Morales et al., 1996). Volatile and non-volatile compounds are retained by virgin olive oils during their mechanical extraction process from olive fruits (*Olea europaea* L.). Non-volatile compounds such as phenolic compounds stimulate the tasting receptors such as the bitterness perception, the pungency, astringency and metallic attributes. Instead volatile compounds, stimulating the olfactive receptors, are responsible for the whole aroma of the virgin olive oil. The chromatograms of volatile compounds of olive oils were obtained by solid phase micro extraction-gas chromatography/mass spectrometry (SPME-GC/MS) (Hatanaka, 1993; Kataoka et al., 2000; Steffen & Pawliszyn, 1996). The method is based on the assay of the terminal species of the "lipoxygenase pathway" which are present in the volatile fraction of the sampled compounds (Hatanaka, 1993).

2. Materials and methods

2.1 Extraction of olive oil and storage

The olive oils investigated (60 Italian and 60 Tunisian) were single cultivar virgin olive oils (SCVOOs) produced in different regions of Tunisia (Chamlali Cv.) and Italy (Coratina Cv.). Olives were handpicked at the optimal olive ripening degree. Immediately after harvest, olive fruits were transported and cleaned, each fruit sample was divided into three portions of 20 Kg. One portion was extracted using pressure system (see paragraph 2.1.1), the second and the third were extracted by centrifugation systems, three and two phases, respectively (see paragraph 2.1.2 and 2.1.3). The oils obtained were stored in three types of packaging (opaque glass, transparent glass and polyethylene terephtalate PET) and monitored for six months.

2.1.1 Pressure system (PS)

Olives are ground into an olive paste using large millstones. In general, the olive paste stays under the stones for 45–50 minutes. After grinding, the olive paste is spread onto fibre disks, that are easier to clean and maintain, stacked on top of each other and then placed into the press. Afterwards, this pile of disks are put on a hydraulic piston where a pressure of about 400 atm is applied. By the action of this pressure, a olive paste and a liquid phase is produced.

Finally, the liquid phase containing oil and vegetation water is separated by a standard process of decantation.

2.1.2 Two-phase centrifugation (2P)

This system does not need water addition and produces a liquid phase (oil) and a solid waste-water-dampened phase (pomace). The olive paste is kneaded for 60 minutes at 27°C and the oil is extracted with a horizontal centrifugation decanter and separated by means of an automated discharge vertical centrifuge.

2.1.3 Three-phase centrifugation (3P)

This system allows the crushing of olives into a fine paste. This paste is then malaxed for 60 minutes in order to achieve the coalescence of small oil droplets. The aromas are created during these two steps through the action of enzymes. Then, the paste is pumped into an industrial decanter where the phases are separated. Water (500 liters per ton) is added to facilitate the extraction process with the paste. The high centrifugal force created into the decanter separates the phases readily according to their different densities (solid phase pomace, vegetation water, oil). The solid materials is pushed out of the system by the action of a conical drum that rotates with a lower speed. The separated oil and vegetation water are then rerun through a vertical centrifuge, which separates the small quantity of vegetation water still contained in the oil.

2.2 Analytical methods

The physic-chemical and organoleptic analysis of VOO were carried out according to the methods described by the European Union Regulations (UE 61/2011).

In particular, analysis of fatty acid methyl esters, total phenols, free acidity, peroxide number, conjugated dienes and trienes, sensory analysis and volatile compounds were conducted as described in the following paragraphs.

2.2.1 Fatty acid methyl ester analysis (FAMEs)

FAMEs analysis were carried out after performing alkaline treatment obtained by dissolving the oil (0.05 g) in n-hexane (1 mL) and adding a solution of potassium hydroxide (1 mL; 2 N) in methanol (Christie, 1998). FAMEs were analyzed by gas chromatography by mean of a Shimadzu 17A chromatograph equipped with detector flame ionization and a capillary column. Peaks were identified by comparing their retention times with those of authentic reference compounds.

The fatty acid composition was expressed as relative percentages of each fatty acid calculated considering the internal normalization of the chromatographic peak area.

2.2.2 Total phenols analysis

Total phenols content was determined according to the method developed by Gutfinger (1981). Briefly, an amount of olive oil (2.5 g) was dissolved with hexane (5 mL) and extracted with a solution of methanol and water (5 mL; 60/40). The mixture was then vigorously agitated for 2 minutes. Folin-Ciocalteu reagent (0.5 mL) and bi-distilled water (4.8 mL) were added to the phenolic fraction. The absorbance of the mixture was measured at 725 nm and results were given as mg of caffeic acid per Kg of oil.

2.2.3 Free fatty acids, peroxides, ultra-violet light absorption

Acidity value, peroxide value (PV) and ultra-violet light absorption, conjugated diene (K232) and conjugated trienes (K270), were determined according to the Regulation EEC/ 2568/91 of the European Union Commission (EEC, 1991).

2.2.4 Sensory analysis

Olive oils were evaluated by a panel according to the official method for the Organoleptic assessment of virgin olive oil referenced COI/T.20/Doc. No 15/Rev. 2.

2.2.5 SPME-GC/MS analysis

Aroma components of products of plant origin are dependent on genetic, agronomic and environmental factors (Benincasa et al., 2003). The complexity of the mass-chromatograms in terms of number of components might represent a drawback when different samples are to be matched. Therefore, in order to consider the minimum set of components that mostly reflect the biogenesis of an oil (Aparicio & Morales, 1998), hexanal (1), 1-hexanol (2), (E)-2-hexenal (3), (E)-2-hexen-1-ol (4) and (Z)-3-hexenyl acetate (5) were chosen as markers of linoleic and linolenic acids specific lipoxygenase oxidation [(path A and B), Fig. 1].

2.2.5.1 Preparation of samples and standard solutions

A solution (200 mg/Kg) was prepared by dissolving 0.04 g of each analytes (see paragraph 2.2.5) in 200 g of commercial seeds oil. In the same manner a solution containing the internal standard (ethyl isobutyrate) was prepared.

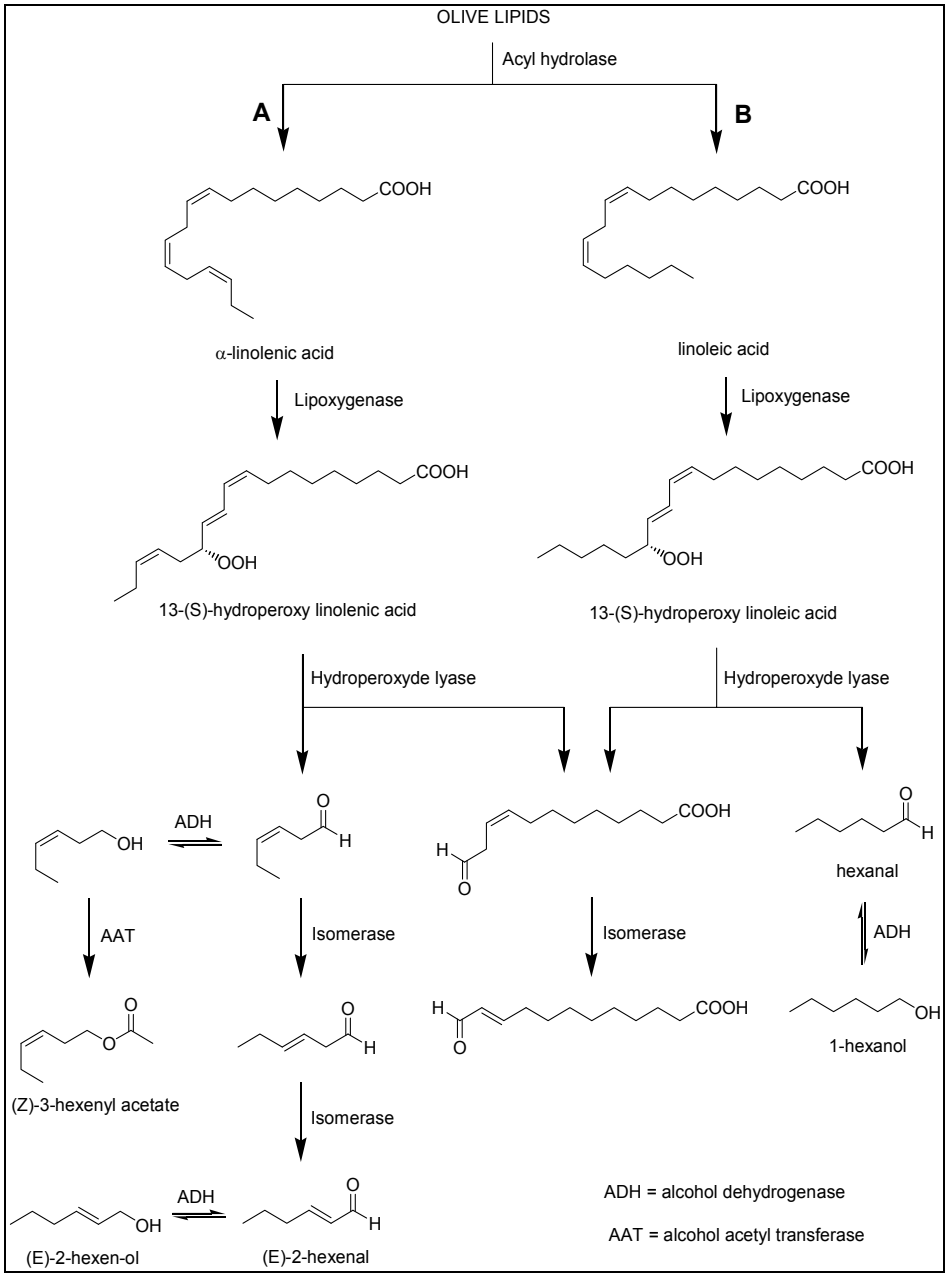

Fig. 1. Linoleic and linolenic acids specific lipoxygenase oxidation.

2.2.5.2 Experimental procedure and instrumentation

The assay of secoiridoid glycosides, such as oleuropein, in virgin olive oil has been proposed as a marker of quality (De Nino et al., 1999, 2005; Perri et al., 1999). With reference to the works previously mentioned, the chromatogram of volatile compounds was considered a useful target. Only the peaks with a certain threshold value (S/N equal to five)

were taken into account and integrated. Identification of analytes was made by comparison of their mass spectra and retention times with those of authentic reference compounds.

The experimental work was carried out using a Varian 4000 Ion Trap GC/MS system (Varian, Inc. Corporate Headquarters, U.S.A.) equipped with a CP 3800 GC. Volatile components were adsorbed by means of a divilbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber and separation was obtained by means of a capillary column FactorFOUR (Varian VF-5ms). The ion trap temperature was set at 210 °C with an ionization time of 2 ms, reaction time at 50 ms and scan rate at 1000 ms. The transfer line temperature was set at 230 °C. The column was a 30 m Chrompack CP-Sil 8 CB low bleed/MS (0.25 mm i.d., 0.25 μm film thickness). The GC oven temperature was initially held at 40 °C for 3 min, then ramped at 1 °C/min to 70 °C and finally ramped at 20 °C/min to 250 °C and held for 8 min. The carrier gas was helium at 1 mL/min. Analyses were performed in splitless mode. Mass spectra were collected in EI in positive mode.

2.2.5.3 Quantitative analysis

The calibration curves were obtained by covering two concentration range: 0.4-4 mg/Kg with six steps at 0.4, 0.8, 1.5, 3, 4 mg/Kg for each analyte, with 1.5 mg/Kg of internal standard and 5-150 mg/Kg with six steps at 5, 10, 25, 50, 100, 150 mg/Kg for each analyte, with 40 mg/kg of internal standard. Each experimental value corresponds to the average of three replicates.

The quantitative assay was performed by selecting the area of the ionic species as follows: m/z 41, 56, 67, 72, 82 for hexanal; m/z 55, 56, 69 for 1-hexanol; m/z 55, 69, 83, 97 for (E)-2-hexenal; m/z 57, 67, 82 for (E)-2-hexen-1-ol; m/z 67, 82 for (Z)-3-hexenyl acetate, respectively and m/z 71, 88, 116 for the internal standard.

2.2.5.4 Statistical analysis

The data obtained for each compound were subjected to statistical analysis. Statistical treatment was performed by STATGRAPHICS Plus Version 5.1 (Statistical Graphics Corporation , Professional Edition - Copyrigth 1994-2001). The approach chosen to analyse the set of data obtained was Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA). Also, in order to check possible differences between the oils, two-way analysis of variance (ANOVA) was performed considering, as main factors, the nationality of the sample and the type of storage container. Moreover, to evaluate significant differences between averages, Tukey test was performed on the oil quality parameters. Differences were considered statistically significant for $P \geq 0.01$ and $P \geq 0.05$. The values obtained for free acidity and FAMEs were analyzed after arcsine transformation in order to meet assumptions for ANOVA.

3. Results and discussions

3.1 FAMEs analysis

VOOs under investigation showed the typical profile of fatty acids of the areas of production. In general, the oils were dominated by palmitic acid (C16: 0), stearic acid (C18: 0), oleic acid (C18: 1) and linoleic acid (C18: 2). The observed values do not show a particular pattern that can indicate the mode of extraction and the type of packing. It is well known, in fact, that fatty acids are dependent on genetic factors, soil and climate (Christie, 1998; Dabbou, et al., 2010; Gharsallaoui, et al., 2011; Manai, et al., 2007).

3.2 Quality parameters

The extraction system has a significant effect on the physical and chemical parameters of the oil: the pressure system can preserve well the colour and the antioxidants of the olive oil, but may affect negatively the sensory profile. From the results obtained, olive oils were characterised by significant differences in free acidity and phenol content (Figures 2 and 3).

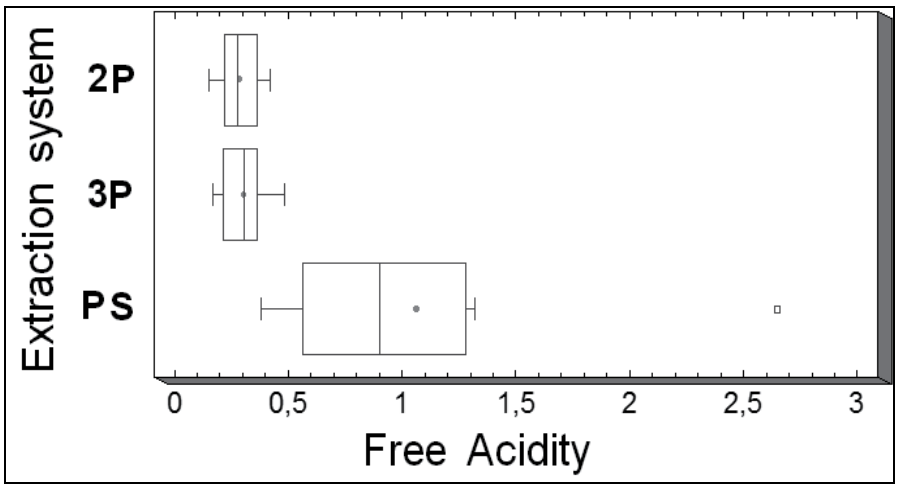

Fig. 2. Box plot of Tunisian VOOs. Free acidity is plotted vs the extraction system.

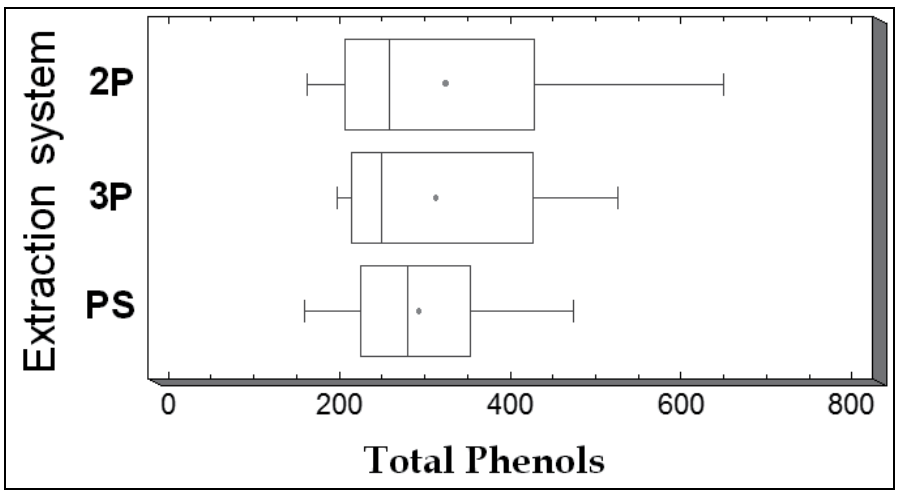

Fig. 3. Box plot of Tunisian VOOs. Total phenols are plotted vs the extraction system.

In general, oils produced with the pressure system have higher free acidity levels than the same oils produced by centrifugation (2P and 3P) and sometime cannot be classified as Extra Virgin Olive Oil (Fig. 2). Moreover, they are often characterised by a lower content of phenols (Fig. 3). In a similar way, the peroxide and K232 and K270 extintion coefficient values were higher than the same oils produced by centrifugation methods.

3.3 SPME-GC/MS and sensory analysis

Cultivar and extraction systems have a considerable effect on sensory attributes of virgin olive oil. A typical mass chromatogram of the volatile component of one of the analyzed sample is reported in Fig. 4, while the bar chart of Fig. 5 shows the distribution of volatile compounds at five and six carbon atoms that mostly contribute to the olive oil aroma.

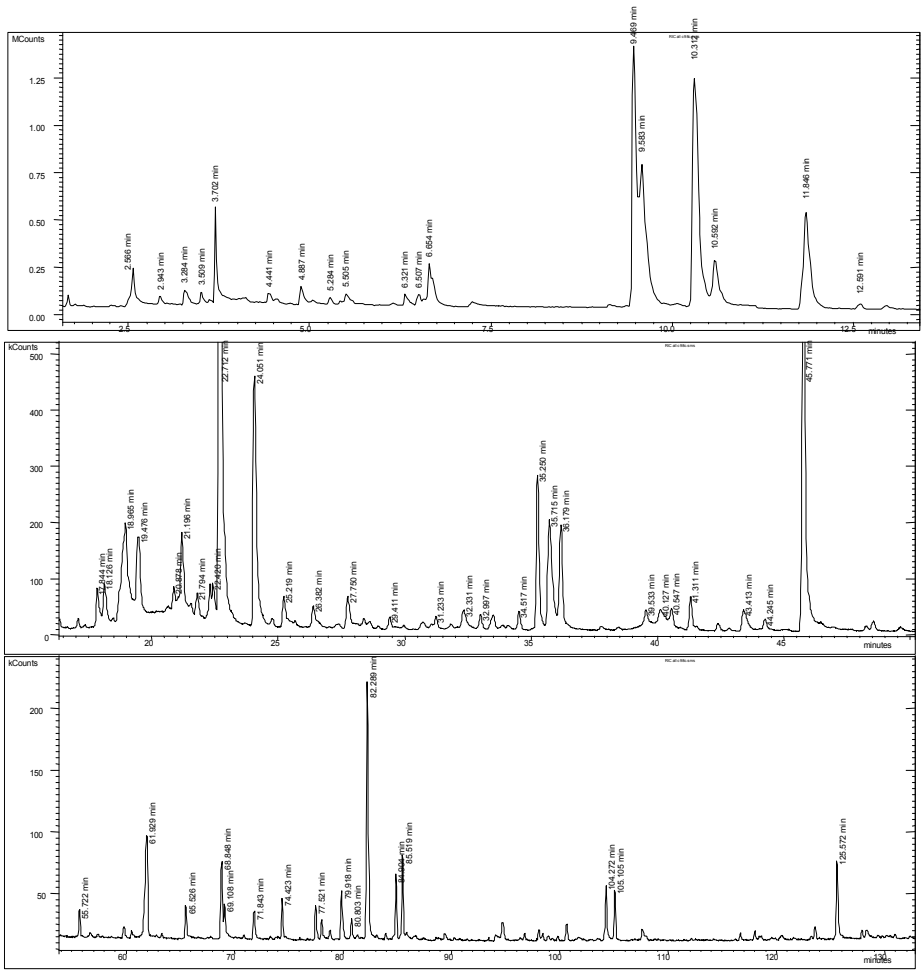

Fig. 4. A typical chromatogram of volatile compounds of one of the analysed samples.

According to the five markers selected as active components of the SPME-GS/MS chromatograms (see paragraph 2.2.5), the distinction of the VOOs under investigation was allowed. Even if both Italian and Tunisian oils were fruity with bitter and pungent characteristics, VOOs of Coratina Cv showed an higher values of fruitiness and bitterness intensity with a clear pungency mainly when they were extracted in centrifugation systems. In fact, these systems can produce olive oils with better organoleptic profiles.

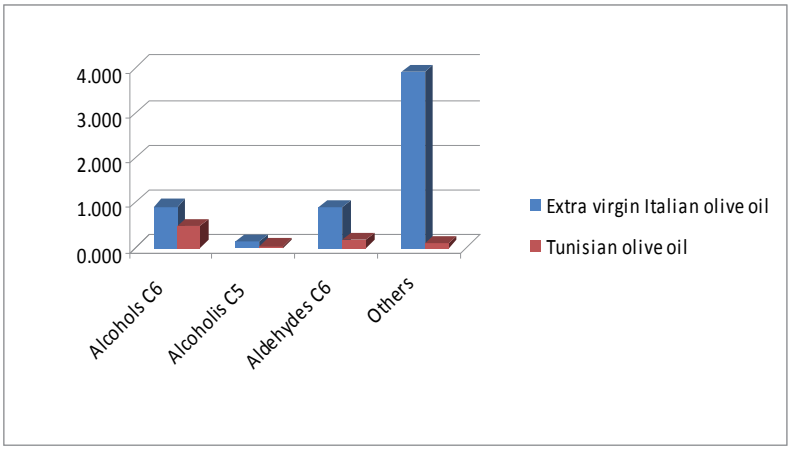

Fig. 5. Bar chart of volatile compounds analysed by SPME-GC/MS. The Cvs under investigation are Coratina and Chamlali from Italy and Tunisia respectively.

Volatile compounds are distributed in a very different concentration in Italian and Tunisian olive oil samples. The flavour of Coratina VOOs was stronger than Chamlali VOOs. In particular, statistical evaluation showed that hexenal (Fig. 6), 1-hexanol (Fig. 7), produced by the lipoxygenase pathway, could discriminate the two VOOs.

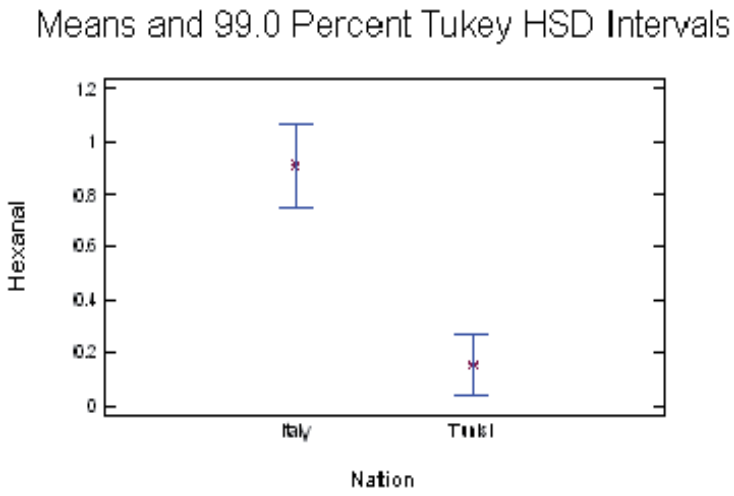

Fig. 6. Biplot of hexenal at 99% confidence level. The genotype was the main factor considered. The Cvs under investigation are Coratina and Chamlali from Italy and Tunisia respectively.

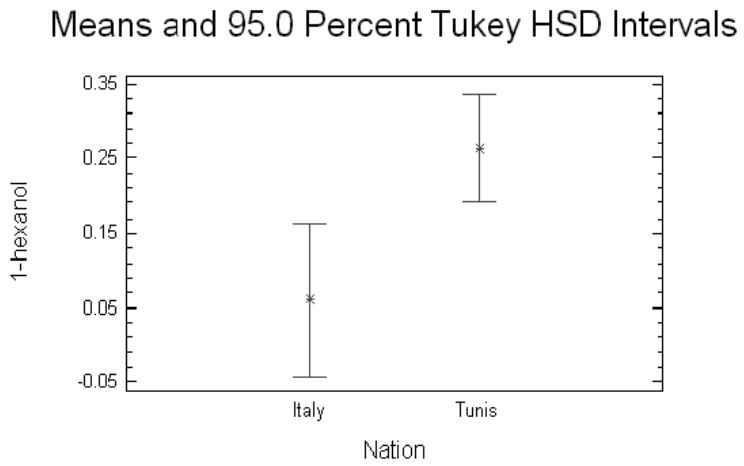

Fig. 7. Biplot of 1.hexanol at 95% confidence level. The genotype was the main factor considered. The Cvs under investigation are Coratina and Chamlali from Italy and Tunisia, respectively.

The VOOs tested by the panelists produced the aromagrams of Figures 8 and 9. According to the panel jury, Coratina olive oils extracted by centrifugation (2P and 3P) were very fruity with a good level of bitterness and astringency. These latter attributes seem to disappear when a pressure system is employed.

Chamlali olive oils extracted by a pressure system were found slightly defected while olive oils extracted by centrifugation systems were fruity with same level of bitterness and astringency. All these results matched those obtained by SPME-GC/MS (see paragraph 3.3).

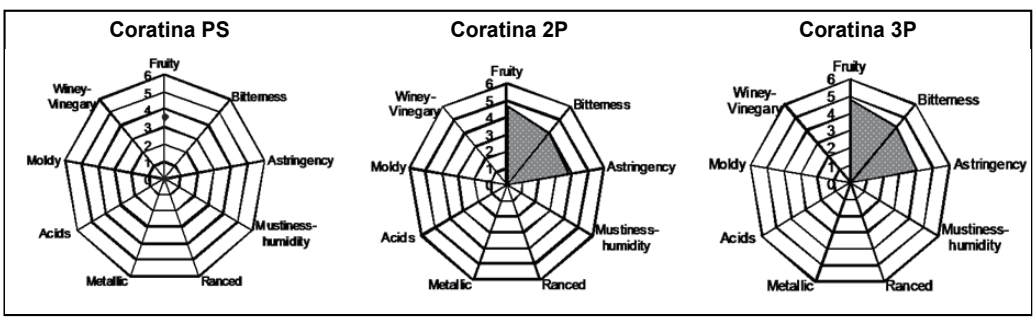

Fig. 8. Sensorial wheels of Italian olive oils of Coratina Cv. extracted by pressure system (PS) and centrifugation two phase and three phase systems (2P and 3P, respectively).

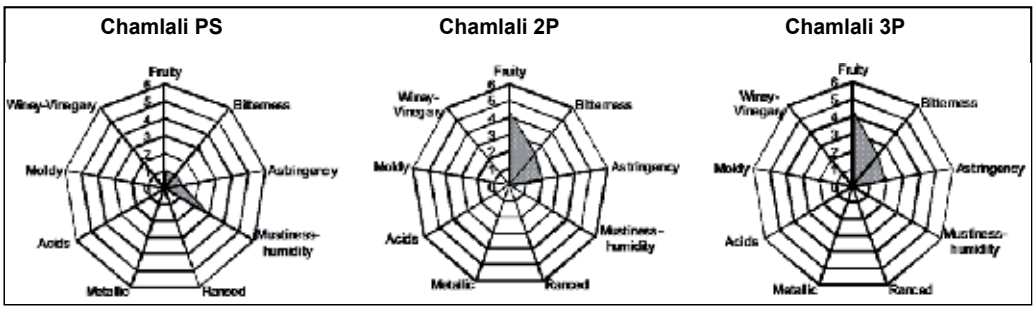

Fig. 9. Sensorial wheels of Tunisian olive oils of Chamlali Cv. extracted by pressure system (PS) and centrifugation two phase and three phase systems (2P and 3P, respectively).

Finally, the organoleptic analysis conducted on custemers demonstrated that consumers prefer olive oils extracted by centrifugation systems rather than olive oils obtained by pressure systems.

3.4 Olive oil storage

Soon after extraction, samples of the sixty Italian and sixty Tunisian VOOs were divided into three groups of twenty and stored in opaque glass, transparent glass and polyethylene terephtalate (PET) bottles. The storage of the oils in opaque glass bottles seemed to be better as it reduced oxidative changes and prolonged shelf life, while polyethylene terephtalate (PET) bottles were the package system that inhibits deterioration to a lesser extent. In fact, free acidity, over the period of six months, became higher when the oils were stored in PET bottles (Fig. 10 and Fig. 11).

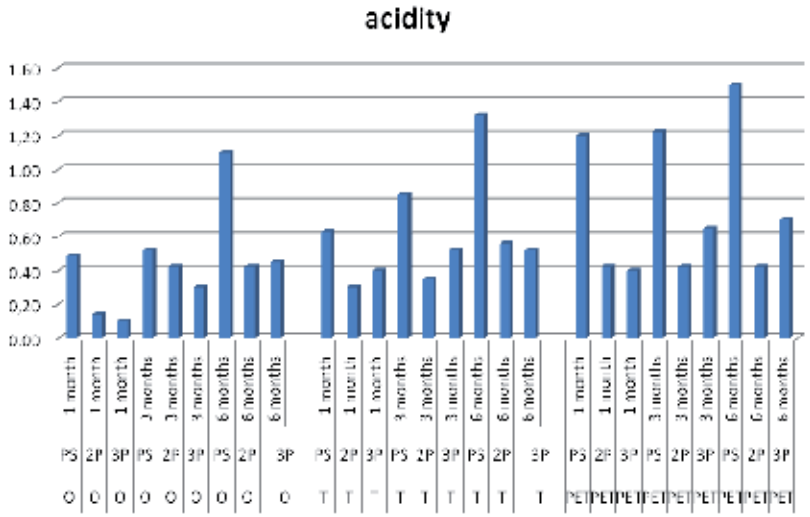

Fig. 10. Bar chart of free acidity of Coratina VOOs during a period of experimentation of six months and depending on the type of packaging utilized. The letters stand for: O opaque glass bottle, T transparent glass bottle, PET polyethylene terephtalate bottle and the extraction system employed: SP pressure system, 2P and 3P centrifugation system at two and three phases respectively.

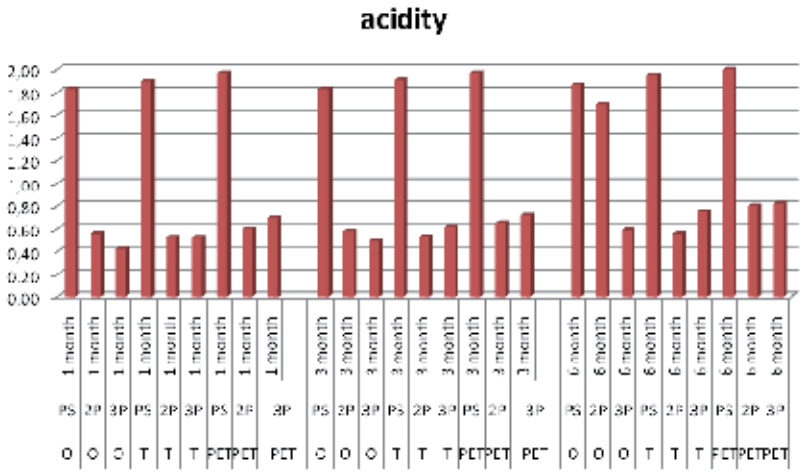

Fig. 11. Bar chart of free acidity of Chamlali VOOs during a period of experimentation of six months and depending on the type of packaging utilized. The letters stand for: O opaque glass bottle, T transparent glass bottle, PET polyethylene terephtalate bottle and the extraction system employed: SP pressure system, 2P and 3P centrifugation system at two and three phases respectively.

Chamlali VOOs were the samples that showed the higher indices of deterioration all over the period. The extraction system plays a key role on the value of the free acidity of an oil. In fact, oils extracted by pressure system have higher free acidity values which increase within the first month (Fig. 12).

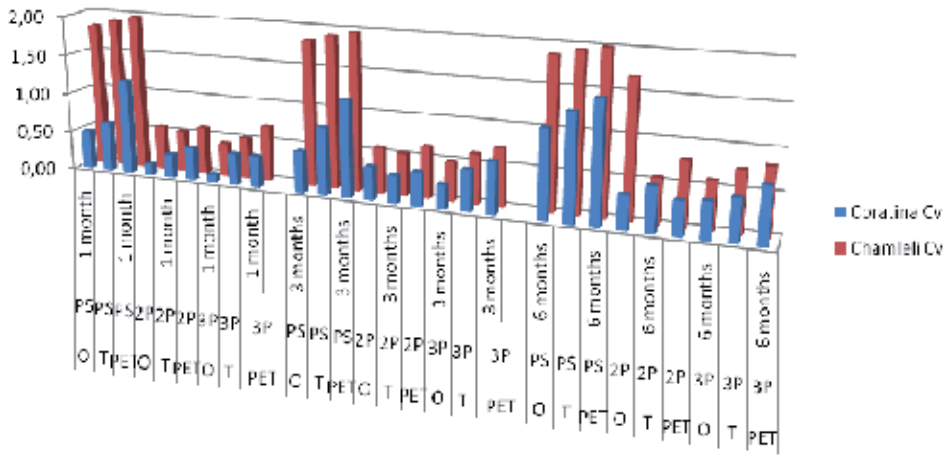

Fig. 12. 3D bar chart of free acidity of Coratina and Chamlali VOOs during a period of experimentation of six months and depending on the type of packaging utilized. The letters stand for: O opaque glass bottle, T transparent glass bottle, PET polyethylene terephtalate bottle and the extraction system employed: SP pressure system, 2P and 3P centrifugation system at two and three phases respectively.

4. Conclusions

The results obtained in this work and discussed in this chapter have shown how important is the method of extraction and the storage of an olive oil (Ben Hassine, et al., 2011; Romano, et al., 2008). A high-quality olive oil can be obtained preferring two phases extraction systems to the classical extraction ones where hydraulic pistons with a pressure of about 400 atm are applied and, storing it in dark glass bottles to better preserve its aroma and phenolic compounds.

5. Acknowledgment

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2

Olive Oil Composition: Volatile Compounds

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1. Introduction

In general olive oil is defined on the basis of its sensory characteristics. European Union (EU) regulations establish the organoleptic quality of virgin olive oil by means of a panel test, evaluating positive and negative descriptors (EU regulations). For the organoleptic assessment, several volatile compounds are considered as the main responsible for negative and positive attributes. Volatile compounds, either major or minor, are crucial to olive oil quality; even when present below their olfactory threshold, they can still be important to understand their formation and degradation pathways and provide useful quality marker information.

Volatile composition of olive oils can be influenced by a number of factors, from agronomic and climatic aspects to technological ones. Cultivar, geographic region, ripeness, harvest and processing methods can affect the volatile composition of olive oil. Storage time is also critical for quality. In order to evaluate the volatile profile of olive oil, sensitive analytical techniques as well as extraction procedures were developed. The big issues on aroma analysis are, the loss of compounds during sample preparation steps, and the knowledge that some of the so-called "compounds of interest" (with higher aroma threshold) are, probably, present only in trace amounts. Due to its nature, olive oil is a difficult matrix; for these reasons several methods have been, so far, proposed. The advantages and drawbacks of these methods will be further discussed. One dimension-Gas Chromatography (1D-GC) analysis was, until recently, the most used method to analyze volatiles in different matrices. The increased development of 2D-GC, allowing higher sensitivity and enhanced separation power, is changing the 1D-GC approach. The type of 2D and/or 3D qualitative and quantitative information, provided by 2D-GC systems, promoted the development of powerful chemometrics tools allowing a useful, and potentially easy, way for data interpretation. Fingerprint comparison can be used on a routine basis, providing important and quick information concerning differences among the olive oils produced and, probably most important, also allowing frauds detection.

This work will be divided in four main parts: 1) a brief summary of the composition and biosynthesis of the volatile fraction of olive oil; 2) the role of volatile compounds in olive oil quality: nutritional and sensorial quality; 3) the effect of agronomic and technological practices on olive oil aroma; 4) analytical methodologies for quantification and identification of volatiles compounds: new analytical methods.

2. Composition and biosynthesis of the volatile fraction of olive oil

The wide variety of volatile compounds found in high quality virgin olive oil are produced through biogenic pathways of the olive fruit, namely the lipoxygenase (LOX) pathways (Hatanaka, 1993), and fatty acid or aminoacid metabolism, as depicted in fig.1 (Angerosa et al., 2004; Angerosa et al., 2002). Besides the contribution of several volatile compounds, related with the mentioned pathways, the role of other compounds, especially aldehydes derived from auto-oxidation processes, should also be considered to the final aroma of the olive oils (Angerosa, 2002). Other metabolized products, originated from possible fermentations, conversion of some aminoacids, enzymatic activities of moulds or oxidative processes, are closely related with off-flavour of virgin olive oil. As illustrated in fig. 1, several compounds namely carbonyl compounds, alcohols, esters and hydrocarbons contribute to the aroma profile of olive oil (Angerosa et al., 2004).

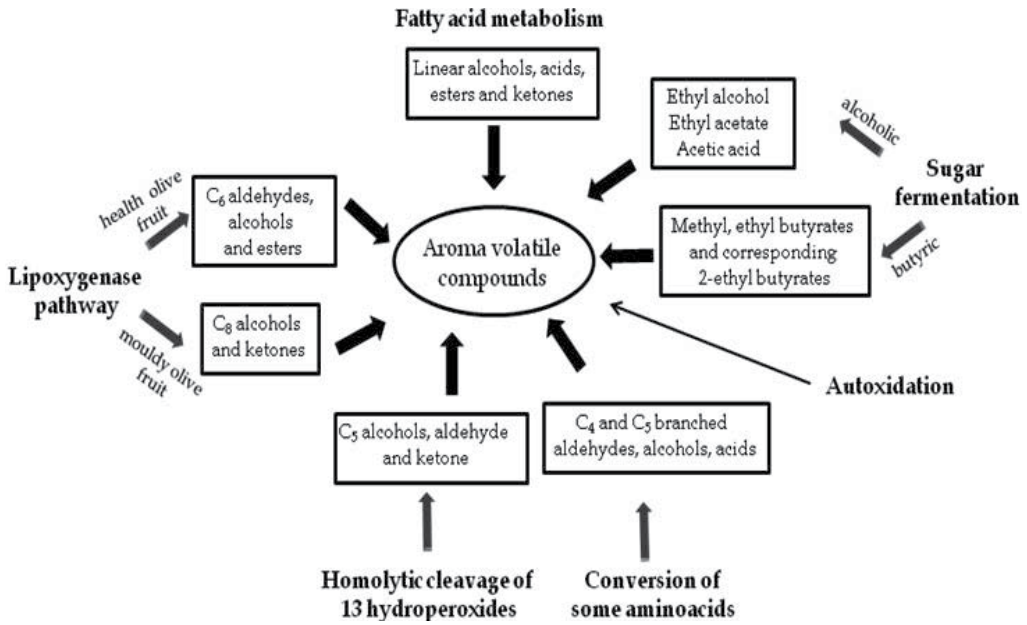

Fig. 1. The main pathways involved in the formation of the volatile profile of high quality virgin olive oils. Adapted from (Angerosa et al. 2004; Angerosa 2002).

The volatile compounds, responsible for virgin olive oil aroma, are usually: low molecular weight (<300 Da); high volatility, sufficient hydrosolubility, fair liposolubility and chemical features to bond with specific proteins (Angerosa et al., 2002).

During crushing and malaxation steps, considerable changes, in olive oil chemical composition occurs accomplished by the activation of olive fruit enzymes due to the

inherent disruption of cellular tissues. Consequently, the LOX pathway is initiated by the hydrolysis of triglycerides and phospholipids, mediated by acyl hydrolase (AH), leading to the release of fatty acids. Lypoxygenases, after their release, become immediately active and transform the unsaturated fatty acids, produced by the action of AH, linolenic (LnA) and linoleic (LA) acids, into their corresponding 9- and 13-hydroperoxides, as shown in figure 2. The subsequent cleavage of fatty acids 13-hydroperoxides is catalysed by specific hydroperoxide lyases (HPL) leading to the formation of C_6 aldehydes (*Z*)-hex-3-enal and hexanal from linolenic and linoleic acids, respectively) and oxoacids. The unsaturated form of C_6 aldehyde ((*Z*)-hex-3-enal) undergo rapid isomerisation to the more stable (*E*)-hex-2-enal. The action of alcohol dehydrogenase (ADH), catalyses the reversible reduction of aliphatic C_6 aldehydes to the corresponding volatile alcohols (Benicasa et al., 2003; Angerosa et al., 1998a). Alcohol species are further transformed into esters due to the catalytic activity of alcohol acetyl transferase (AAT), producing acetates (Kalua et al., 2007) (figure 2). Several factors, such as cultivar and extraction process, including operating temperature, seem to play a relevant role on the improvement of AAT activity (Salas, 2004). When the substrate is LnA, LOX catalyses, besides the hydroperoxide formation, also its cleavage, via an alkoxy radical, increasing the formation of stabilized pent-1,3-diene radicals. These compounds can suffer dimerization leading to the production of C_{10} hydrocarbons (pentene dimmers) or react with a hydroxyl radical present in the medium, leading to C_5 carbonyl compounds (Angerosa et al. 1998b, Pizarro et al., 2011). The most important fraction of volatile compounds, of high quality virgin olive oils, comprises C_6 and C_5 compounds, especially C_6 linear unsaturated and saturated aldehydes. The presence of other volatile compounds, namely C_7-C_{11} monounsaturated aldehydes, C_6-C_{10} dienals, C_5 branched aldehydes and alcohols and some C_8 ketones, in relatively high concentrations, in the aroma of virgin olive oil, is associated with unpleasant notes. The presence, or lack of defects, in the aroma of olive oils is related with the contribution of the various pathways involved on volatiles formation.

When the most active pathway is the LOX cascade the olive oil aroma will not be defective. LOX pathway is predominant in oils of high quality.

3. The role of volatile compounds in olive oil quality: Nutritional and sensorial quality

The International Olive Oil Council (IOOC), European Commission (EC) and Codex Alimentarius have defined the quality of olive oil based on several parameters, such as free fatty acid content, peroxide value, spectrophotometric absorvances in the UV region, halogenated solvents and sensory attributes (Boskou 2006; Kalua et al., 2007; Lopez-Feria et al., 2007). In order to evaluate olive oil quality, the Codex Alimentarius and IOOC include also the insoluble impurities, some metals and unsaponifiable matter determinations (Boskou 2006).

The nutritional value of olive oil arises from high levels of oleic acid and minor components, such as phenolic compounds. It is well recognized that the consumption of some natural antioxidant phenolic compounds produce beneficial health effects. These substances possess strong radical scavenging capacities and can play a relevant role in protecting against oxidative damages and cellular aging. Together with their bioactivity, olive oil phenols have a significant role on the flavour and the bitter taste of olive oil (Boskou 2006; Servili et al. 2002). Sensory quality plays a crucial role in the acceptability of foodstuffs and

some characteristics such as colour and flavour are the main sensations which contribute to their acceptability among consumers. Hence, the evaluation of the sensory quality of olive oils involves perception of both favourable and unfavourable sensory attributes.

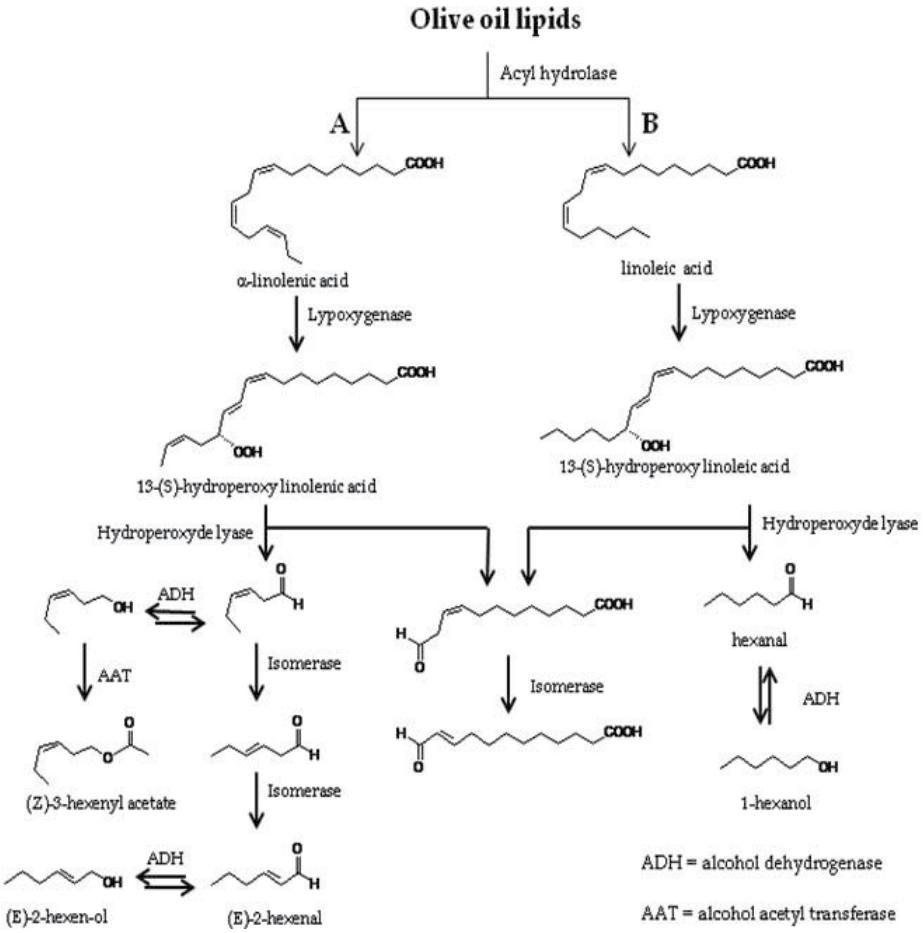

Fig. 2. Lypoxygenase pathway for the formation of major volatile compounds. (Source: Benincasa et al., 2003).

Olive oil possesses a highly distinctive taste and flavor due to specific volatile organic compounds, belonging to several chemical classes, namely aliphatic and aromatic hydrocarbons, aliphatic and triterpenic alcohols, aldehydes, ketones, ethers, esters and furan and thiophene derivatives (Kiritsakis et al. 1998). These compounds, retained by olive oil during the extraction process, stimulate human gustative and olfactive receptors giving rise to olive oil balanced flavour of green and fruity attributes. Such compounds stimulate the free endings of the terminal nerve located in all the palate and in the gustative buds promoting the chemesthetic perceptions of pungency, astringency and metallic attributes. During olive oil tasting, the stimulation of the olfactory epithelium, by a large number of volatile compounds can also occur explaining all other sensations perceived by consumers (Angerosa, 2002). The major volatile compounds of olive oil which contribute for the positive attributes of olive oil

aroma (fruity, pungent and bitter) include hexanal, (*E)*- hex-2-enal, hexan-1-ol and 3-methylbutan-1-ol. Their concentrations, except for (*E*)-hex-2-enal, varying widely, are generally very low reaching minimum levels of ppb. Thus, volatile compounds, which are responsible for most sensory properties of olive oils, play a significant role on the evaluation of the overall oil quality having a decisive influence on acceptability. The sensory defects are also associated with the volatile composition of the olive oil and are, usually, related with chemical oxidation and exogenous enzymes involved in microbial activity. Chemical oxidation is responsible for the formation of off- flavour compounds, such as pent-2-enal and hept-2-enal The off- flavour compounds associated with unpleasant sensory notes can be assembled in five classes- fusty, moistness- humidity, winey- vinegary, metallic and rancid (Morales et al. 1997; Morales et al., 2005; Escuderos et al., 2007; Faria et al.; Angerosa 2002; Kalua et al. 2007). Moistness-humidity, which possesses the highest sensory significance, is related to the presence of C_8 volatile compounds (*e.g*. oct-1-en-3-ol and to a lesser extent oct-1-en-3-one) and short chain fatty acids (Morales et al., 2005). Normally they are a characteristic flavour of oils produced from olives infested with fungi and yeasts as a result of an inappropriate storage. Fusty sensory defect is correlated with the presence of ethyl butanoate, propanoic and butanoic acids, a characteristic flavour of oils from olives stored in piles which have undergone an advanced stage of anaerobic fermentation (Morales et al. 2005). Moreover, the presence of acetic acid, ethanol, 3-methylbutan-1-ol and ethyl acetate contributes to winey-vinegary attributes due to the olives fermentation. The rancid negative attribute is due to oils oxidation, characterized by the absence of C_6 aldehydes and alcohols produced from linolenic acid, the absence of esters and the presence of several aldehydes with low odour threshold (Morales et al, 1997). Metallic flavour is associated to oils that have been in prolonged contact with metallic surfaces, during processing, and is characterized by the presence of pent-1-en-3-one; this ketone has been proposed as a useful marker of metallic off-flavour (Venkateshwarlu et al. 2004). The occurrence of pent-1-en-3-one is also positively correlated with bitter and pungency taste while hexanal is negatively correlated with these characteristics, depending on the final amounts. *Z*-Hex-3-en-1-ol and *E*-hex-2-enal are negatively correlated with bitter and pungent characteristics, respectively. Other common defects of olive oils, such as muddy sediment and cucumber are related with olive oil preservation.

Poor quality olive oils show remarkable modifications on their sensory basic characteristics, namely the decrease or absence of green, bitter and pungent notes. Generally, the intensity of stimuli elicited by volatile substances is related to their amount. Some other chemical factors, such as volatility and hydrophobic character, size, shape and stereochemistry of volatile molecules, type and position of functional groups as well as external factors, such as matrix effects, seem to affect odour intensity, more than their concentration, due to the influence of these chemical features on the interaction with olfactory and gustative receptors (Angerosa et al., 2004). Odour activity value, evaluated by means of the ratio between its concentration and its odour threshold, constitutes a useful tool to identify the main contributors to the olive oil aroma. The thresholds of several of these compounds are already presented in dedicated literature (Bouskou 2006; Angerosa 2002).

4. The effect of agronomic and technological practices on olive oil aroma

Factors affecting volatile composition of olive oils can be classified into four main groups: environmental (soil and climate); agronomic (irrigation, fertilization); cultivation (harvesting,

ripeness) and technological procedures (post-harvest storage and extraction systems) (Aparicio & Luna, 2002). It is generally accepted that volatile profile of virgin olive oils depends on the level and the activity of the enzymes involved in LOX pathway. As previously referred, the major volatile compounds responsible for odour notes of virgin olive oils are the C_6 and the C_5 volatile compounds which emerge from primary or secondary LOX pathway, respectively. The enzymatic levels are determined genetically, so they differ from cultivar to cultivar, but the enzymatic activity is influenced by all factors mentioned above. Apart endogenous plant enzymes, responsible for the positive aroma perception in olive oils, chemical oxidation and microbial activity (associated with sensory defects) should be considered.

4.1 Cultivars

Cultivars and harvest time must be carefully selected in order to correspond to the optimal level of fruit maturity (Esti et al., 1998; Caponio et al., 2001). Olives ripening is quite important for olive oil final composition. The cultivar influence depends on the activity of enzymes and is a genetic characteristic (Tena et al, 2007). The higher LOX activity for linoleic acid than linolenic acid supports the biogenesis of a higher amount of C_6 unsaturated volatile compounds the major constituents of olive oil aroma; usually olive fruits show the highest LOX activity 15 weeks after anthesis; activity decreases during development and ripening periods (Salas et al., 1999). Another enzyme involved is HPL that catalyses the cleavage of fatty acids hydroperoxides producing volatile aldehydes. The highest level of HPL activity is detected in green olive fruits, harvested at the initial development stages. Although there is a slight decrease at maturity, a high activity level is maintained throughout maturation. The decrease in C_6 volatile compounds concentration, in the olive oils of mature olives, is not attributed to HPL activity (Salas & Sanchez, 1999) rather to the availability of substrate. The behaviour of these two enzymes supports the decrease of C_6 volatile compounds content during fruit ripeness. At earlier ripening stages the amount of C_6 aldeydes and alcohols are very similar, and when olive skin colour changes from green to purple most of the C_6 aldehydes reach their maximum concentration (Angerosa & Basti, 2001). With the increase of ripeness a decrease is observed for most of the aldehydes formed from the lipoxygenase pathway, namely *E*-hex-2-enal (the main volatile compound in most European virgin olive oils), being *Z*-hex-3-enal an exception (Aparicio & Morales,1998). Kalua et al (2007), however, state that the decrease in C_6 aldehydes, from the lipoxygenase pathway, might not be characteristic of all cultivars.

The olive cultivar influences also fatty acid composition and, particularly, the ratio of oleic to linoleic acid (C18:1/C18:2), triglyceride profile, and phenolic content of olive oil (Aparicio et al., 2002; Tovar et al., 2002; Beltran et al., 2005). Some differences can be found in the fatty acid content of varietal virgin olive oils (Aparicio 2000); they do not vary so much, however, as to be determinant for the volatile profile. In spite, C_6 volatile compounds (aldeydes, alcohols and acetyl esters) formed from 13-hydroperoxides of linoleic and α-linolenic acids, account for 60 to 80% of the total volatile compounds (Aparicio & Luna 2002). The concentration of C_6 volatile compounds, of 36 monovarietal virgin olive oils produced in countries from Mediterranean basin, show that aldehydes (hexanal, *Z*-hex-3-enal and *E*-hex-2-enal) and pent-1-en-3-one contribute, distinctly, to the sensory profile of these varietal oils, taking into account the odour thresholds of these volatile compounds (75, 3, 1125 and 50 μg Kg^{-1}, respectively) (Aparicio & Luna 2002). These authors found high concentrations of *E*-hex-2-enal in Italian cultivars, in accordance with results previously obtained by Solinas

et al. (1988), and they all suggest that monovarietal virgin olive oils could be distinguished by this compound. According to Solinas et al. (1988) octanal, nonanal and hex-2-enal contents are a cultivar characteristic; the presence of propanol, amyl alcohols, hex-2-enol, hexan-2-ol and heptanol seems also to be related to the olive cultivar. Nevertheless, olive oils from different cultivars, produced under the same exact conditions (extraction system, ripeness stage, pedoclimatic and agronomic conditions), exhibit different amounts of total volatiles, ranging from 9-83 to 35 mg kg^{-1} (Luna et al., 2006). Baccouri et al. (2008), when studying volatile compounds from Tunisian and Sicilian monovarietal virgin olive oils, found that the overall amounts of C_6 aldehydes were clearly higher than the sum of C_6 alcohols in Chemlali and Sicilian samples, whereas, in Chetoui oils, the sum of C_6 alcohols was generally higher than the C_6 aldehydes. The explanation relays again in the differential activity of the enzymes involved. These authors also reported a decrease, in the amounts of C_5 aldehydes and alcohols, during the maturation. Morales et al. (1996) studied the influence of ripeness on the concentration of green aroma compounds; the total content of volatile compounds decreases with ripeness; there are markers for monovarietal virgin olive oils obtained from unripe (*E*-hex-2-enal), normal ripe (hexyl acetate) and overripe olives (*E*-hex-2-enol) regardless of the variety (Aparicio & Morales, 1998)

D'Imperio et al. (2010), when studying the influence of harvest, method and schedule, on olive oil composition, found a remarkable decrease of *E*-hex-2-enal as was previously reported by Aparicio et al. (1998); an increase of hexanal seems to be related to the use of shakers for harvesting. A decrease in unsaturated fatty acids content was also observed relating these findings to the lipoxygenase pathway.

4.2 Environmental factors

Pedoclimatic factors depends on environmental conditions, soil, type and structure, and/or climatic conditions, temperature and rainfall (Beltran et al., 2005). Cultivars do not always grow at the same altitude, but olive grove zones are spread over a wide range of altitudes, where climatic conditions can be quite different. All these have impact on chemical and sensory profiles of olive oils. Monovarietal olive oils, obtained from olives grown at higher altitudes, are in general sweeter and have a stronger herbaceous fragrance, when compared to the ones produced with olives grown at lower altitudes. Lower temperatures, at higher altitudes, may influence the enzymes from lipoxygenase pathway, since hexanal (green-sweet perception) comes from increased levels of linoleic acid, and *E*-hex-2-enal (green odour and astringency taste) from lower levels of *a*-linolenic acids (Aparicio & Luna, 2002). Temime et al. (2006) studied the volatile compounds from Chétoui olive oils, the second variety cultivated in Tunisia, and reported significant differences on volatile compounds when, just, environmental conditions were different. Dabbou et al. (2010) studied the quality and the chemical composition of monovarietal virgin olive oil, from the Sigoise variety, grown in two different locations in Tunisia, a sub-humid zone (Béjaoua, Tunis) and an arid zone (Boughrara, Sfax). Olive oils produced from olives grown at the higher altitude were characterized by higher contents of *E*-hex-2-enal (11.92 mg kg^{-1}) and hexanal (1.24 mg kg^{-1}), whereas the oils, from the lower altitude, were distinguishable by the higher content of *Z*-hex-2-en-1-ol (8.78 mg kg^{-1}) and hexan-1-ol (2.17 mg kg^{-1}). The sum of the products of the lipoxygenase oxidation pathways was higher in oils from Béjaoua (15.92 mg kg^{-1}) than in those from Boughrara (15.20 mg kg^{-1}). Among the LOX oxidation products, the amount of hexanal was higher in Béjaoua oils (1.24 mg kg^{-1}), whereas the content of *Z*-hex-2-en-1-ol was considerably lower.

In a recent study, concerning the behaviour of super-intensive Spanish and Greek olive cultivars grown in northern Tunisia, Allalout et al. (2011) found significant differences between oils; they consider, the majority of the studied analytical parameters, to be deeply influenced by the cultivar-environment interaction.

It seems there is an effect of genotype-environment interaction, responsible for olive oils characteristics.

4.3 Agronomic factors

Irrigation, a practice that has been adequately studied, seems to produce a decrease in the oxidative stability of olive oil volatiles due to a simultaneous reduction in oleic acid and phenolic compounds contents (Tovar et al., 2002).

According to Servili et al. (2007) the olive tree water status has a remarkable effect on the concentration of volatile compounds, such as the C_6-saturated and unsaturated aldehydes, alcohols, and esters. Put simply, deficit irrigation of olive trees appears to be beneficial not only due to its well-known positive effects on water use efficiency, but also by optimizing olive oil volatile quality. Baccouri et al. (2008) reported an enhancement of the whole aroma concentration of Chetouil oils obtained from trees under irrigation conditions when compared to similar ones from non-irrigated trees.

The effect of agronomic practices in oil quality is still controversial: data from Gutierrez et al. (1999) supports the hypothesis that organic olive oils have better intrinsic qualities than conventional ones. These olive oils usually present lower acidity and peroxide index, higher rancimat induction time, higher concentrations of tocopherols, polyphenols, *o*-diphenols and oleic acid. However, this work was carried out during 1 year, with one olive cultivar only, and results can not be generalized. Ninfali et al. (2008) in a 3-year study, comparing organic *versus* conventional practice did not observe any consistent effect on virgin olive oil quality. Genotype and year-to-year climate changes seem to have a proved influence.

4.4 Technogical factors

Volatile compounds are predominantly generated during virgin olive oil extraction, and are important contributors to olive oil sensory quality. Virgin olive oil quality is intimately related to the characteristics and composition of the olive fruit at crushing. Changes in olive fruit quality during post-harvest is considered determinant to the final sensory quality. Kalua et al. (2008) reported that low-temperature storage of fruits can produce poor sensory quality of the final oil. This decrease in quality might be due to lower levels of *E*-hex-2-enal and hexanal, associated with a decrease in enzyme activity, and a concurrent increase in *E*-hex-2-enol, which might indicate a possible enzymatic reduction by alcohol dehydrogenase (Olias et al., 1993,Salas et al. 2000) and reduced chemical oxidation (Morales et al. 1997). Inarejos-Garcia et al. (2010) studied the olive oils from Cornicabra olives stored at different conditions (from monolayer up to 60 cm thicknesses at 10 ºC (20 days) and 20 ºC (15 days)). *E*-hex-2-enal showed a Gaussian-type curve trend during storage that can be related to the decrease of hydroperoxide lyase activity. C_6 alcohols showed different trends, during storage, with a strongly decrease of the initial content of *Z*-hex-3-en-1-ol after 15 and 8 storage days at 20ºC and 10ºC under the different storage layers, whilst an increase of *E*-hex-2-en-1-ol was observed (except for mono-layer). Differences might be related to the

enhancement of alcohol dehydrogenase activity during storage. Besides the evolution and changes observed in the desirable LOX pathway, C_6 fraction, storage may give rise to undesirable volatile compounds, from metabolic action of yeasts, which was more evident when olive were stored at 20 ^{o}C. The effect of the extraction process on olive oil quality is also well documented (Ranalli et al., 1996; Montedoro et al., 1992; Di Giovacchino, 1996; Koutsaftakis et al., 1999; Servili et al., 2004).

Technological operations include several preliminary steps, leaf and soil removal, washing, followed by crushing malaxation and separation of the oil (and water) from the olive paste. This last step can be achieved by pressing (the oldest system), centrifugation (the most widespread continuous system), or percolation (based on the different surface tensions of the liquid phases in the paste).

Ranalli et al. (2008) studied the effect of adding a natural enzyme extract (*Bioliva*) during processing of four Italian olive cultivars (*Leccino, Caroleo, Dritta* and *Coratina*) carried out with a percolation-centrifugation extraction system. The improved rheological characteristics of the treated olive paste resulted in a reduced extraction cycle with good effects concerning olive oil aroma characteristics. Results have shown that enzyme-treated olive pastes always release higher amounts of total pleasant volatiles (*E*-hex-2-enal, *E*-hex-2-en-1-ol, *Z*-hex-3-enyl acetate, *Z*-hex-3-en-1-ol, pent-1-en-3-one, *Z*-pent-2-enal, *E*-pent-2-enal and others). For the individual C_6 metabolites, from the LOX pathway, a similar trend was generally observed, while for the total unpleasant volatiles, *n*-octane, ethyl acetate, isobutyl alcohol, *n*-amyl alcohol, isoamyl alcohol and ethanol, an opposite behaviour was found.

The fundamental step is, however, olive crushing. The release of oil from olives can be achieved by mechanical methods (granite millstones or metal crushers) or centrifugation systems. These different systems affect the characteristics of the pastes and the final oil (Di Giovacchino et al., 2002). Almirante et al. (2006) reported that the oils obtained from de-stoned pastes had a higher amount of C_5 and C_6 volatile compounds, when compared to oils obtained by stone-mills. This increment is due to stones removal, which possess enzymatic activities, metabolizing 13-hydroperoxides other than hydroperoxide lyase, giving rise to a net decrease in the content of C_6 unsaturated aldehydes during the olive oil extraction process. Servili et al. (2007) demonstrate that the enzymes involved in the LPO pathway have different activity in the pulp or in the stone. Stones seem to have a lower hydroperoxide lyase activity and a higher alcohol dehydrogenase activity when compared to the pulp. These authors also found higher amounts of C_6 unsaturated aldehydes olive oils volatiles (VOOs) obtained with the stoning process; the stone presence in traditional extraction procedure increases the concentration of C_6 alcohols (for Coratina and Frantoio cultivars).

The next step is the malaxation. Malaxation is performed to maximize the amount of oil that is extracted from the paste, by breaking up the oil/water emulsion and forming larger oil droplets. The efficiency of this operation depends upon time and temperature. Pressing, percolation, or centrifugation, are finally used to separate the liquid and solid phases. Temperature and time of exposure of olive pastes to air contact (TEOPAC), during malaxation, affect volatile and phenolic composition of virgin olive oil, and consequently its sensory and healthy qualities. Cultivar still plays a fundamental role for the final composition (Servili et al, 2003). These authors showed that TEOPAC can be used to perform a selective control of deleterious enzymes, such as polyphenol oxidase (PPO) and

peroxidase (POD), preserving the activity of LPO. High malaxation temperature (> 25 ºC) reduces the activity of enzymes, involved in LOP pathway, reducing the formation of C_6 saturated and unsaturated aldehydes. A similar result is described by Tura et al. (2004). These authors found that changes in malaxation time and temperature produces differences in the volatile profile of olive oils. Increasing temperature and decreasing time led to a reduction in the amount of volatiles produced, but they also describe cultivar as the single most important factor in determining volatile profile of olive oils. The decrease of olive oil flavour, produced by high malaxation temperature, is due to the inactivation of hidroperoxide lyase (HPL) rather than lipoxygenase (LOX), as both enzymes have different behaviour regarding temperature (Salas & Sánchez, 1999b). LOX, when assayed with linoleic acid as the substrate, displayed a rather broad optimum temperature around 25 ºC and maintained a high activity at temperatures as high as 35 ºC, but HPL activity peaked at 15 ºC and showed a clear decrease at 35 ºC, in assays using 13-hydroperoxylinoleic acid as substrate. Similar results were obtained by Gomez-Rico et al. (2009) who observed a significant increase in C_6 aldehydes, in the final oil, as malaxation time increased; almost no changes in the content of C_6 alcohols were observed. Opposite results were found for the influence of the kneading temperature, where a drop in the C_6 aldehydes content as malaxation temperature increases is observed, especially for *E*-hex-2-enal and a slight increase in C_6 alcohols, mainly hexan-1-ol and *Z*-hex-3-en-1-ol.

The final step of olive oil production also affects olive oil quality. Separation of oil from water can be achieved using a two-phase or a three phase centrifugation system. Comparing monovarietal virgin oils obtained by both processes, the oils from two-phase decanters have higher content of *E*-hex-2-enal and total aroma substances but lower values of aliphatic and triterpenic alcohols (Ranalli & Angerosa, 1996).

Masella et al. (2009), when studying the influence of vertical centrifugation on olive oil quality, observed significant differences both in the total volatile concentration and in the two volatile classes from the LOX pathway involving LnA conversion. The observed decreased of C_6/LnA and C_5/LnA compounds can be explained by the volatiles partition between oil and water phases during vertical centrifugation.

Storage conditions also affect final quality. Light exposure, temperature and oxygen concentration, storage time and container materials are also determinant. A study by Stefanoudaki et al. (2010) evaluating storage under extreme conditions, showed subtle differences, in the pattern of volatile compounds, in bottled olive oils stored indoors or outdoors. When stored with air exposure the levels of some negative sensory components, such as penten-3-ol and hexanal, increased while other positives, like *E*-hex-2-enal were reduced. Filling the headspace with an inert gas can reduce spoilage.

5. Analytical methodologies for quantitation and identification of volatiles compounds: New analytical methods

5.1 Olive oil volatile compounds

In the volatile fraction of olive oils, approximately three hundred compounds have already been detected and identified by means of gas chromatography/mass spectrometry (GC/MS) methods (Boskou, 2006). Among these compounds, only a small fraction

contributes to the aroma of olive oil (Angerosa et al., 2004). The most common olive oil volatiles have 5 to 20 carbon atoms and include short-chain alcohols, aldehydes, esters, ketones, phenols, lactones, terpenoids and some furan derivatives (Reiners & Grosh, 1998; Delarue & Giampaoli, 2000; Kiritsakis, 1992; Boskou, 2006; Vichi et al., 2003a, 2003b, 2003c; Aparicio et al., 1996; Morales et al., 1994; Flath et al, 1973; Morales et al, 1995; Bortolomeazzi et al., 2001; Bentivenga et al., 2002; Bocci et al., 1992; Servili et al., 1995; Fedeli et al., 1973; Fedeli, 1977; Jiménez et al., 1978; Kao et al., 1998; Guth & Grosch, 1991). As all vegetable oils, olive oil comprises a saponifiable and a non-saponifiable fraction and both contribute for the aroma impact. As a result of oxidative degradation of surface lipids (Reddy & Guerrero, 2004) a blend of saturated and mono-unsaturated six-carbon aldehydes, alcohols, and their esters (Reddy & Guerrero, 2004; Matsui, 2006) are produced. As already mentioned they are formed from linolenic and linoleic acids through the LOX pathway, and are commonly emitted due to defence mechanism developed by the plant in order to survive to mechanical damage, extreme temperature conditions, presence of pathogenic agents, among others (Delarue & Giampaoli, 2000; Noordermeer et al., 2001; Pérez et al., 2003; Angerosa et al., 2000; Angerosa et al., 1998b). Volatile phenols are also reported as aroma contributors for olive oil and can play a significant organoleptical role (Vichi et al., 2008; Kalua et al., 2005).

5.2 Analytical methodologies

5.2.1 Sample preparation procedures

When the analysis of a volatile fraction, of complex matrices, is considered sample preparation cannot be underestimated. In biological samples, a wide chemical diversity, in a wide range of concentrations, must be expected (Salas et al., 2005; Wilkes et al., 2000). The chemical nature, and the amount of the detected compounds, strongly depends on the extraction technique used, to remove and isolate them from their matrices. The choice of a suitable extraction methodology depends on sample original composition and target compounds. However, an ideal sampling method does not exist and no single isolation technique produces an extract that replicates the original sample. In order to have enough quantity of each compound to be detected by chromatography, a concentration step must, usually, be considered. Sample preparation can be responsible for the appearance of artefacts, due to the chemical nature of the compounds extracted, and thus detected and quantified, and to a total or partial loss of compounds; this issues can, very strongly, determine the precision, reproducibility, time and cost of a result and/or analysis (Wilkes et al., 2000; Belitz et al., 2004; Buttery 1988; van Willige et al., 2000). These methods are revised in a recent manuscript (Costa Freitas et al.) where sample preparation procedures for volatile compounds are discussed as well as the advantages and drawbacks of each method.

In olive oil analysis, its oily nature strongly influences the choice of the extraction procedure. There are various techniques that can be used for the preparation of the sample analytes in biological material. From those so far applied, liquid extraction with or without the use of ultrasounds (Kok et al., 1987; Fernandes et al., 2003; Cocito et al., 1995) is probably the most used. Besides liquid extraction, simultaneous distillation extraction (SDE) (Flath et al., 1973) has also been widely used. The drawback of these methods is the use of solvents

and consequently the need of compounds isolation from the solvent which represents an extra preparation step, as well as the dilutions steps during the extraction procedure. To avoid these steps, supercritical fluid extraction (SFE) (Morales et al., 1998) was also used for the isolation of volatile constituents of olive oil.

The methods based on extraction from the headspace are an elegant choice (Swinnerton et al., 1962). The more often used procedures are the so called "purge and trap" techniques (Morales et al., 1998; Servili et al., 1995; Aparicio & Morales, 1994) in which the compounds of interest are trapped in a suitable adsorbent, from which they can be taken either directly (using a special "thermal desorber" injector) or after retro-extraction into a suitable solvent which, once again, includes an extra extraction step. Another choice is direct injection of the headspace into the injection port of a GC chromatograph. This possibility does not include a concentration step, and consequently, the minor compounds are usually missing or not detected (Del Barrio et al., 1983; Gasparoli et al., 1986). A direct thermal desorption technique can also be applied, avoiding the use of any types of adsorbents, by just heating the target olive oil sample to a suitable temperature in order to promote the simultaneous, extraction, isolation and injection of the volatile fraction into the analytical column (Zunin et al. 2004, de Koning et al., 2008). The main advantage of this technique is its simplicity, although a special injection system is mandatory, which can be expensive. When SPME was introduced (Belardi & Pawliszyn, 1989; Arthur & Pawliszyn, 1990) several authors have focused their attention on adapting the technique for aroma compounds analysis (D'Auria et al., 2004; Vichi et al., 2003; Vichi et al., 2005; Ribeiro et al., 2008). The main advantages of this technique are: a) it does not involve sample manipulations; b) it is an easy and clean extraction method able to include, in just one step, all the steps usually needed for aroma extraction. The extraction step, in SPME, can be made either by headspace sampling or liquid sampling. Headspace sampling (HS) is usually the method of choice for olive oil aroma analysis. The fibre chemical composition is of main interest and determines the chemical nature of the compounds extracted and further analyzed. There are several coatings commercially available. Polydimethylsiloxane (PDMS) and polyacrylate (PA) coatings extract the compounds by means of an absorption mechanism (Ribeiro et al., 2008) whereas PDMS is a more apolar coating then PA. Polydimethylsiloxane/divinylbenzene (PDMS/DVB), polydimethylsiloxane/carboxene (PDMS/CAR), carbowax/divinylbenzene (CW/DVB), and divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) extract by an adsorptive mechanism. These second group of fibres have usually a lower mechanic stability but present higher efficiency to extract compounds with low molecular weight (Augusto et al., 2001). In both extraction mechanisms, once the compounds are expelled form the matrix, they will remain in the headspace and a thermodynamic equilibrium is established between these two phases (Zhang & Pawliszyn, 1993). When the fibre is introduced a third phase is present and mass transfer will take place in both interphases (sample matrix/headspace and headspace/fibre). When quantification is a requirement, equilibrium has usually to be achieved. Time and temperature are also very important issues to take in consideration, since they will affect equilibrium (Vas & Vékey, 2004) and thus extraction efficiency. Methods that consider quantification in non-equilibrium have also been developed (Ai, 1997; Ribeiro et al., 2008). In order to optimize the extraction procedures by HS-SPME, the efficiency, accuracy and precision of the extraction is also directly dependent on operational parameters like extraction time, sample agitation, pH adjustment, salting out, sample and/or headspace volume,

temperature of operation, adsorption on container walls and desorption conditions (Pawliszyn, 1997).

5.2.2 Chromatographic methods for the analysis of olive oil volatiles

Capillary gas chromatography (GC) is the most used technique for the separation and analysis of volatile and semivolatile organic compounds (Beesley et al., 2001) in biological samples. GC allows to separate and detect compounds present in a wide range of concentrations in very complex samples, and can be used as a routine basis for qualitative and quantitative analysis (Beesley et al., 2001; Majors, 2003). Enantioselective separations can also be performed when chiral columns are used (Bicchi et al., 1999). The most common detector used is the flame ionization detector (FID), known by its sensitivity and wide linear dynamic range (Scott, 1996; Braithwaite & Smith, 1999). When coupled with Fourier transform infrared spectroscopy (GC/FTIR) or mass spectrometry (GC/MS) (Gomes da Silva & Chaves das Neves, 1997; Gomes da Silva & Chaves das Neves, 1999), compounds tentative identification can be achieved.

The most widely used ionization techniques employed in GC/MS is electron ionization (EI normally at 70 eV) and the more frequently used mass analysers, in olive oil volatile research, are quadrupole filters (qMS), ion traps (ITD) and time of flight instruments (TOFMS). The GC/TOFMS instruments allow the simultaneous acquisition of complete spectra with a constant mass spectral m/z profile for the whole chromatographic peak, while in qMS instruments the skewing effect is unavoidable. This fact enables the application of spectral deconvolution (Smith, 2004), and, potentially, a more accurate use of reference libraries for identification and confirmation of analytes may be possible. Nevertheless, for routine laboratory the development of TOFMS dedicated mass spectral libraries, to complement the libraries now generated by using qMS, should be considered. Spectral matching is usually better when qMS data are compared in some instances (Cardeal et al., 2006; Gomes da Silva et al., 2008).

In an ongoing research in our lab, HS-SPME was performed in order to identify volatile compounds in *Galega Vulgar* variety. Four fibres were used and the HS-SPME-GC/TOFMS system operated with a DB-wax column. In table 1 the complete list of compounds identified (using the four different fibres) is provided as well as fragmentation patterns obtained for those not yet reported in olive oils (table 2). Analysis were performed in two columns: a polar column (DB-WAX), usually recommended for volatiles analysis, and an apolar based column DB-5. The use of these two columns, of different polarity, was also very useful to detect co-elutions, occuring when the polar column was used, and helped the identification task, when associated to mass spectrometric and linear retention indices (LRI) data confrontation. Most identification were performed by comparing retention time and fragmentations patterns, obtained for standards, analysed under the same conditions, or by fragmentation studies, when standards were not available. The differences observed, in the LRI experimentally obtained for the DB-WAX column, compared to the literature were expectable since polar columns are known as being much more unstable, then apolar columns, and cross-over phenomena occur (Mateus et al. 2010). Their retention characteristics varies significantly among different suppliers, which suggest the need of LRI probability regions. This fact explains why few LRI data is available for polar columns. These results aims to fullfill some part of this gap.

Compound name	LRI Experimental [Literature]	SPME Fibres	Compound name	LRI Experimental [Literature]	SPME Fibres
Hexane	n.d. [600]	D-C-P	*E*-Pent-2-enal	1060 [1127-1131]	D-C-P
Heptane	n.d. [700]	PA D-C-P	*p*-Xilene	1067 [1133-1147]	PA D-C-P
Octane	800 [800]	PA D-C-P	Butan-1-ol	1074 [1147]	PA D-C-P
Propanone	808 [820]	PA CDVB D-C-P	*m*-Xilene	1077 [1133-1147]	D-C-P
E-Oct-2-ene	818 [n.f.]	PA	Pent-1-en-3-ol	1093 [1130-1157]	PA D-C-P
Ethyl acetate	832 [892]	D-C-P	2,6-Dimethyl-hepta-1,5-diene (isomer)	1101 [n.f.]	D-C-P
2-Methyl-butanal	850 [915]	D-C-P	*Cis*-hex-3-enal	1113 [1072-1137]	D-C-P
Dichloromethane	859 [n.f.]	PA CDVB	Heptan-2-one	1123 [1170-1181]	PA CDVB D-C-P
Ethanol	883 [900-929]	PA D-C-P	Heptanal	1126 [1174-1186]	PA CDVB D-C-P
1-Methoxy-hexane	889 [941]	D-C-P	*o*-Xilene	1128 [1174-1191]	D-C-P
4-Hydroxy-butan-2-one	892 [n.f.]	PA	Limonene	1139 [1178-1206]	PA D-C-P
Pentanal	896 [935-1002]	PA	3-Methyl-butan-1-ol	1141 [1205-1211]	D-C-P
3-Ethyl-octa-1,5-diene (isomer)	907 [n.f.]	D-C-P	2-Methyl-butan-1-ol	1142 [1208-1211]	PA PDMS CDVB D-C-P
3-Methyl-butanal	912 [910-937]	D-C-P	2,2-Dimethyl-oct-3-ene	1144 [n.f.]	D-C-P
Propan-2-ol	918 [n.f.]	PA CDVB D-C-P	*E*-Hex-2-enal	1160 [1207-1220]	PA CDVB D-C-P
3-Ethyl-octa-1,5-diene (isomer)	930 [1018]	PA D-C-P	Dodecene	1164 [n.f.]	PA D-C-P
Pent-1-en-3-one (isomer)	932 [973-1016]	D-C-P	Ethyl hexanoate	1170 [1223-1224]	PA CDVB D-C-P

Compound name	LRI Experimental [Literature]	SPME Fibres	Compound name	LRI Experimental [Literature]	SPME Fibres
Ethyl butanoate	946 [1023]	PA D-C-P	Pentan-1-ol	1184 [1250-1255]	PA CDVB D-C-P
Toluene	952 [1030-1042]	D-C-P	*β*-Ocimene	1186 [1242-1250]	CDVB D-C-P
Ethyl 2-methyl-butanoate	963 [n.f.]	D-C-P	Tridec-6-ene (isomer)	1187 [n.f.]	D-C-P
Deca-3,7-diene (isomer)	985 [1077]	D-C-P	Styrene	1199 [1265]	PA CDVB D-C-P
Deca-3,7-diene (isomer)	994 [1079]	D-C-P	Hexyl acetate	1209 [1274-1307]	PA CDVB D-C-P
Hexanal	1000 [1024-1084]	PA CDVB D-C-P	1,2,4-Trimethylbenzene	1223 [1274]	PA PDMS CDVB D-C-P
3-Methylbutyl-acetate	1037 [1110-1120]	D-C-P	Octanal	1231 [1278-1288]	PA PDMS CDVB D-C-P
2-Methyl-propan-1-ol	1054 [1089]	PA	*E*-4,8-Dimethyl-nona-1,3,7-triene	1247 [1306]	PA PDMS CDVB D-C-P
Ethylbenzene	1056 [1119]	PA CDVB D-C-P	*E*-Pent-2-en-1-ol	1250 [n.f.]	D-C-P
Z-Hex-3-enyl acetate	1258 [1300-1338]	PA CDVB D-C-P	Hepta-2,4-dienal (isomer)	1453 [1463-1487]	PA CDVB D-C-P
E-Hept-2-enal	1272 [1320]	CDVB D-C-P	Decanal	1456 [1484-1485]	PA CDVB
Z-Pent-2-en-1-ol	1281 [1320]	PA D-C-P	*α*-Humulene	1472 [n.f.]	PA
6-Methyl-hept-5-en-2-one (isomer)	1285 [1335-1337]	PA CDVB D-C-P	Benzaldehyde	1488 [1513]	PA CDVB D-C-P
Hexan-1-ol	1290 [1316-1360]	PA CDVB D-C-P	*α*-Terpineol	1493 [1694]	D-C-P
4-Hidroxy-4-methyl-pentan-2-one	1313 [n.f.]	D-C-P	*E*-Non-2-enal	1494 [1502-1540]	PA D-C-P

Compound name	LRI Experimental [Literature]	SPME Fibres	Compound name	LRI Experimental [Literature]	SPME Fibres
E-Hex-3-en-1-ol	1320 [1356-1366]	PA CDVB D-C-P	Propanoic acid	1495 [1527]	D-C-P
Z-Hex-3-en-1-ol	1322 [1351-1385]	PA D-C-P	Octan-1-ol	1504 [1519-1559]	PA CDVB D-C-P
4-Methyl-pent-1-en-3-ol	1330 [n.f.]	PA D-C-P	2-Diethoxy-ethanol	1565 [n.f.]	PA D-C-P
Methyl Octanoate	1331 [1386]	D-C-P	*E,E*-Nona-2,4-dienal	1574 [n.f.]	PA
Nonan-2-one	1340 [1382]	PA D-C-P	Methyl benzoate	1587 [n.f.]	D-C-P
Nonanal	1344 [1382-1396]	PA CDVB D-C-P	Butanoic acid	1588 [1634]	PA D-C-P
E-Hex-2-en-1-ol	1348 [1368-1408]	CDVB D-C-P	4-Hydroxybutanoic acid	1593 [n.f.]	D-C-P
Z-2-Hex-2-en-1-ol	1348 [1410-1417]	PA D-C-P	*E*-Dec-2-enal	1606 [1590]	PA CDVB D-C-P
Oct-3-en-2-one (isomer)	1349 [1455]	D-C-P	Acetophenone	1617 [1624]	D-C-P
Hexa-2,4-dienal (*E,E*), (*E,Z*) or (*Z,Z*)	1349 [1397-1402]	D-C-P	2-Methyl-butanoic acid	1621 [1675]	D-C-P
Ethyl octanoate	1353 [1428]	D-C-P	Nonan-1-ol	1628 [1658]	PA CDVB D-C-P
Hexa-2,4-dienal (isomer)	1360 [1397-1402]	D-C-P	α-Muurolene	1680 [n.f.]	D-C-P
E-Oct-2-enal	1367 [1425]	PA D-C-P	Aromadendrene	1681 [n.f.]	PA PDMS CDVB D-C-P
1-Ethenyl-3-ethyl-benzene	1378 [n.f.]	D-C-P	1,2-Dimethoxy-benzene	1686 [n.f.]	PA PDMS D-C-P
Oct-1-en-3-ol (isomer)	1392 [1394-1450]	PA CDVB D-C-P	4-Methyl-benzaldehyde	1690 [n.f.]	D-C-P
Heptan-1-ol	1400 [n.f.]	PA CDVB D-C-P	Pentanoic acid	1700 [1746]	PA CDVB C-C-P

Compound name	LRI Experimental [Literature]	SPME Fibres	Compound name	LRI Experimental [Literature]	SPME Fibres
Linalool	1403 [1550]	CDVB	Butyl heptanoate	1717 [n.f.]	D-C-P
Acetic acid	1408 [1434-1450]	CDVB D-C-P	*E*-Undec-2-enal	1726 [n.f.]	PA CDVB D-C-P
Hepta-2,4-dienal (isomer)	1421 [1488-1519]	D-C-P	Methyl salycilate	1758 [1762]	D-C-P
2-Ethyl-hexan-1-ol	1436 [1491]	PA CDVB D-C-P	*E, E*-Deca-2,4-dienal	1780 [1710]	PA CDVB D-C-P
α-Copaene	1440 [1481-1519]	PA CDVB D-C-P	2-Methoxy-phenol (guaicol)	1836 [1855]	PA CDVB D-C-P
α-Cubebene	1442 [n.f.]	D-C-P	2-Methyl-naphthalene	1839 [n.f.]	D-C-P
Benzyl alcohol	1846 [1822-1883]	PA CDVB D-C-P	Octanoic acid	2047 [2069]	PA D-C-P
Phenylethyl alcohol	1890 [1859-1919]	PA CDVB D-C-P	Nonanoic acid	2198 [n.f.]	PA CDVB D-C-P
Heptanoic acid	1900 [1962]	PA D-C-P	4-Ethyl-phenol	2212 [n.f.]	D-C-P

n.d. denote not determined; n.f. denote not found;
LRI denote linear retention indices for DB-Wax column. LRI between brackets represents the data range found in literature: Angerosa, 2002; Contini & Esti 2006; Flath et al., 1973; Kanavouras et al., 2005; Ledauphin et al,. 2004; Morales et al., 1994; Morales et al., 1995; Morales et al., 2005; Reiners & Grosch, 1998; Tabanca et al., 2006; Vichi et al., 2003a., 2003b; Vichi et al., 2005; Zunin et al., 2004.

Table 1. Compounds identified in olive oil samples of *Galega Vulgar* by means of HS-SPME-GC/TOFMS. The fibres used are polydimethylsiloxane (PDMS), polyacrylate (PA), carbowax/divinylbenzene (CDVB), and divinylbenzene/carboxene/polidimethylsiloxane (D-C-P). The extraction and analysis procedure for all fibres was: 15 g of olive oil sample in 22 mL vial immersed into a water bath at 38 ºC. Extraction time was 30 min. Fibre desorption time was 300 seconds into an injection port heated at 260 ºC. Splitless time of 1 min. A GC System 6890N Series from Agilent coupled to a Time of Flight (TOF) mass detector GCT from Micromass using the acquisition software MassLynx 3.5, MassLynx 4.0 and ChromaLynx The system was equipped with a 60 m × 0.32 mm i.d. with 0,5 μm d_f DB-Wax column or a 30 m × 0.32 mm i.d. with 1 μm d_f DB-5 column, both purchased from J&W Scientific (Folsom USA). Acquisition was carried out using a mass range of 40-400 u.; transfer line temperature was set at 230 ºC; ion source 250 ºC. Helium was used as carrier at 100 kPa; Oven temperature was programmed from 50 ºC for three minutes and a temperature increase of 2 ºC/min up to 210 ºC hold for 15 minutes and a rate of 10 ºC/min up to 215 ºC and hold.

Compound name	LRI Experimental [Literature]	m/z –fragmentation pattern	SPME Fibres
Ethyl pentanoate	1050 [1127]	57(66%); 60(36%); 71(5%); 73(31%); **85(100%)**; 88(87%); 101(30%); 115 (2%) **130 (1%) M⁺**	D-C-P
2-Methyl-heptan-4-one	1063 [n.f.]	41(41%); 43(45%); 55(10%); **57(100%)**; 69(18%); 71(63%); 85(79%); 95(2%); 100(3%); 113(10%); **128(23%) M⁺**	PA D-C-P
2,6-Dimethyl-oct-2-ene (isomer)	1181 [n.f.]	41(87%); **55(100%)**; 67(11%); 69(73%); 83(25%); 93(12%); 97(25,74%); 111(16%); 126(9,86%); **140(1%) M⁺**	D-C-P
3-Methyl-pent-3-en-1-ol (isomer)	1306 [n.f.]	**41(100%)**; 42(16%); 55(52%); 56(12%); 67(93%); 69(49%); 70(19%); 82(72%); 83(4%); **100(3%) M⁺**	CDVB D-C-P
2,6-Dimethyl-octa-2,4,6-triene (isomer)	1318 [n.f.]	77(15%); 79(38%); 91(3%); 93(22%); 95(10%); 105(55%); **121(100%)**; 122(10%); **136(43%) M⁺**	D-C-P
1-Methoxy-2-(methoxymethyl)-benzene	1346 [n.f.]	51(15%); 65(18%); 77(33%); 79(20%); **91(100%)**; 21(96%); 137 (17%); 152(6%) **M⁺**	D-C-P
Hex-4-enyl propanoate (isomer)	1350 [n.f.]	41(42%); 55(29%); 57(25%); **67(100%)**; 82(51%)	PDMS D-C-P
Decan-2-one	1428 [n.f.]	41(11%); 42(10%); 43(82%); 55(4%); 57(6%); **58(100%)**; 59(24%); 60 (6%); 71(24%); 85(2%); 98(4%); 113 (2%); 127(2%); **156(2%) M⁺**	PA D-C-P
Nonyl acetate	1526 [n.f.]	**43(100%)**; 56(39%); 61(33%); 70(24%); 83(16%); 98(19%); 126(10%)	PA D-C-P
Z-Dec-2-enal	1608 [n.f.]	41(64%); 43(55%); **55(100%)**; 56(98%); 69(71%); 70(94%); 83(57%); 98(34%); 110(5%); 136(2%)	PA D-C-P
Phenyl acetate	1964 [n.f.]	43(39%); 65(22%); 66(28%); 77(8%); 89(16%); **94(100%)**; 95(6%);103(8%); 117(9%); **136(15%) M⁺**	D-C-P
2-Methyl-phenol	2065 [n.f.]	45(7%); 50(5%); 51(9%); 52(4%); 53(8%); 54(4%); 63(3%); 77(24%); 79(19%); 80(8%); 89(4%); 90(8%); 91(3%); **107(100%)**; **108(98%) M⁺**; 109(5%)$(M+H)^+$	D-C-P
4-Methyl-byphenyl	2091 [n.f.]	51(6%); 63(5%); 82(10%); 83(12%); 84(11%); 115(10%); 152(21%); 153(17%); 65(32%); 167(71%); **168(100%) M⁺ ;** 169(17%)$(M+H)^+$	D-C-P

Table 2. New tentatively identified compound in olive oil samples of *Galela vulgar* by means of HS-SPME-GC/TOFMS. Extraction and analytical conditions according to described in table 1. m/z fragmentation patterns are presented; n.f. denote not found; LRI denotes linear retention indices as in table 1. LRI between brackets represents the data range found in literature, according to table 1.

Co-elutions are often impossible to detect and identify with some GC/MS instruments, in spite of the use of selective single ion monitoring mode (SIM), or complex deconvolution processes. The development of new analytical techniques, that maximize analyte separation, has always been a target. Multidimensional chromatography and comprehensive two-dimensional chromatography (David & Sandra, 1987; Bertsch, 1999) are an example of such achievements. The high complexity of the chromatograms points out new ways of chromatography, such as multidimensional-gas chromatography systems (MD-GC), where the analytes are submitted to two or more independent separation steps, in order to achieve separation. In spite of its efficiency, MD-GC is a time consuming technique, with long analysis times, which does not fit with the demands of routine analysis. Additionally, it is technically difficult to carry out sequential transfers in a narrow window of retention times, since co-elutions are foreseen (Poole, 2003). Nevertheless, MD-GC is a precious tool in peak identification for olive oil analysis when co-elutions occur (Reiners & Grosch, 1998). In 1991, comprehensive two-dimensional gas chromatography (GC × GC) was introduced by Liu & Phillips. The GC × GC system consists of two columns with different selectivities; the first and second dimension columns are serially connected through a suitable interface, usually is a thermal modulator (Phillips & Beens, 1999; Marriott & Shellie, 2002). When performing GC × GC technique the entire sample, separated on the first column, is transferred to the second one, resulting in an enhanced chromatographic resolution into two independent dimensions, where the analytes are separated by two independent mechanisms (orthogonal separation) (Venkatramani et al., 1996; Phillips & Beens, 1999; Marriott & Shellie, 2002; Dallüge et al., 2003). The modulated zones of a peak are thermally focused before the separation on the second column, in a mass conservative process; the resulting segments (peaks), of the modulation, are much narrower with higher S/N ratios, than in conventional GC (Lee et al., 2001; Dallüge et al., 2002), improving the detection of trace analytes and the chromatographic resolution. Fast acquisition TOF spectrometers are the suitable detectors for this technique and have considerably enlarged the application of GC × GC. Few applications are still reported for olive oil analysis, nevertheless, they already showed its potential. GC × GC techniques allowed identification of olive oil key flavour compounds, present in very low concentrations (Adahchour et al. 2005); it has also been used as a flexible technique for the screening of flavours and other classes of (semi-)polar compounds, using the conventional orthogonal approach and the reverse, non-orthogonal approach in order to obtain ordered structures that can simplify the identification task (Adahchour et al. 2004); finally this separation technique can allow easy fingerprint analysis of several olive oil matrices directly, or using image processing statistics (Vaz-Freire et al., 2009).

5.3 Future perspectives for olive oil volatile analysis: Identification tools and fingerprinting

A limitation of electron ionization (EI) in MS analysis is due to the fact that, too often, the molecular ions do not survive fragmentation and, consequently, are not "seen". One way to overcome this problem is to use a complementary technique, that provides "soft" ionization of the molecules, allowing molecular ions detection. Chemical ionization (CI) performs this task (McMaster and McMaster, 1998; Herbert and Johnstone, 2003). The mass spectra obtained by CI are simpler than EI, though most of the interpretable structural information is missing. However the compound´s molecular ions appears as a high intensity fragment

and sometimes is the major fragment of the spectra. Thus, molecular weight determination of an analyte becomes possible. Other soft ionization techniques are field ionization (FI) and field desorption (FD). Both produce abundant molecular ions with minimal fragmentation (Herbert and Johnstone, 2003). FI and FD are appliable to volatile and thermally stable samples (Niessen, 2001; Dass, 2007). If high resolution mass analysers are coupled with these ionization techniques, high capability of identification can be achieved. Together with GC × GC a potentially new tool in olive oil compound identification is reachable and desirable.

The application of a multimolecular marker approach to fingerprint allows, in an easy way, the identification of certain sample characteristics. Chromatographic profiles can be processed as continuous and non-specific signals through multivariate analysis techniques. This allow to select and identify the most discriminant volatile marker compounds (Pizarro et al., 2011). The quantity and variety of information, provided by two-dimensional-GC (2D-GC) systems, promoted the increasingly application of chemometrics in order to achieve data interpretation in a usefull and, potentially, easy way. Linear discriminant analysis (LDA) and artificial neural networks (ANN), among other statistical classification methods, can be applied in order to control economic fraud. These applications have been carefully reviewed recently (Cajka et al., 2010). Together with 2D-GC systems the advantage is clear, since, instead of a time consuming trial to determine which variables should be considered for the statistical classification method, the selection may now become as simple as inspecting the 2D contour plots obtained (Cardeal et al 2008, de Koning et al., 2008). Also the use of statistical image treatment, of 2D-GC generated contour plots, can be applied for fingerprint recognitions, precluding the alignment of the contour plots obtained, which already allowed the identification of varieties as well as extraction technologies used to produce high quality Portuguese olive oils (Vaz Freire et al., 2009).

6. Conclusion

A final word should also be addressed to spectral libraries. Commercial spectral libraries are becoming increasingly more complete and specific, making GC/MS one of the most used techniques for routine identifications. However, several compounds are not yet described in library databases and, in spite of better algorithmic calculations, databases are only reliable for target analysis, or when the compounds under study are known, and already characterized with a known mass spectra. Additionally, the full separation of peaks to ensure clean mass spectra, in order to achieve a reliable peak analyte confirmation, is still a necessary goal.

Until now most of the analytical systems used to analyse olive oil volatile compounds are performed in 1D-GC systems with polar or apolar column phases. Since olive oil volatile fraction is very complex, frequent co-elutions occur. Mass spectra obtained are, consequently, not pure, which should preclude the possibility to compare the spectra obtained with the, claimed pure, spectra in the databases. However, tentative identifications are reported in the literature, and it is not rare that some inconsistencies occur, even when linear retention indices LRIs are presented. Because of their nature, the LRIs obtained in apolar columns are more reliable. Nevertheless, a better separation is obtained in 1D-GC systems when polar stationary phases are used, because of the wide chemical variety

comprised in the volatile fraction of olive oils. Unfortunately, these columns present a high variability, at least, among different purchasers, which do not facilitate LRIs comparison with literature data. Multidimensional techniques, hyphenated with mass-spectrometry, are now fullfiling this gap also in the separation of optical active compounds, when chiral column phases are used. Clean mass spectra together with compound LRIs in polar, apolar and chiral column phases represents an improved tool in compound identification and thus in olive oil matrices characterization. LRIs considering probability regions in the 2D resulting plot of a GC × GC experiment (with different column set combinations, e.g. polar × apolar, polar × chiral, etc.), can enable comparing standard compounds with the sample compounds retention indices and thus a more reliable peak identification can be achieved, if mass spectrometric data are simultaneously recorded. In the future, for 2D systems, more comprehensive mass spectral libraries should include retention index probability regions for different column sets in order to allow correlation of the results obtained in the used systems with spectral matches and literature LRIs.

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3

Optical Absorption Spectroscopy for Quality Assessment of Extra Virgin Olive Oil

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1. Introduction

Light travels through space in the form of electromagnetic waves of different wavelengths. The entire wavelength range represents the electromagnetic spectrum. Spectroscopy studies the interaction between light and matter, in order to draw information about the chemical composition inside (Lee et al., 2011). Figure 1 shows the various bands of the electromagnetic spectrum. This chapter refers to measurements performed in the 200-2500 nm band, which is usually subdivided into three portions: the ultraviolet (UV), the visible (VIS) – perceivable by human eyes – and the near-infrared (NIR). They correspond to the 200-400 nm, 400-780 nm, and 780-2500 nm ranges, respectively.

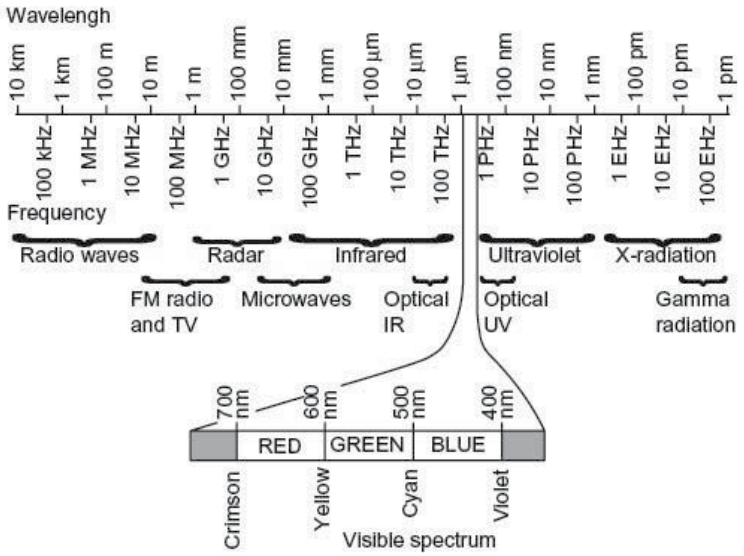

Fig. 1. The electromagnetic spectrum

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A light beam illuminating an olive oil sample gives rise to reflected, transmitted, and scattered intensities. Optical absorption spectroscopy, as shown in Figure 2, makes use of a broadband UV-VIS-NIR source of intensity I_0 to illuminate the olive oil sample. Then, the transmitted light intensity I, as a function of the illumination wavelength is measured. The change in light intensity, providing the transmittance T, is determined by the molar absorptivity ε, the concentration of absorbing species C, and the optical path L, via the Lambert-Beer relationship, expressed by Equations 1 and 2. T is frequently expressed logarithmically as in Equation 3, to give the so called optical absorbance A, which results linearly dependent on concentration.

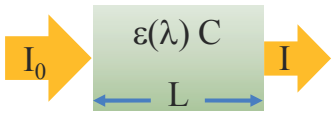

Fig. 2. Optical absorption spectroscopy: the working principle

$$I = I_0 \exp(-\varepsilon C L) \tag{1}$$

$$T = \frac{I}{I_0} = \exp(-\varepsilon C L) \tag{2}$$

$$A = \log \frac{I_0}{I} = \varepsilon C L \tag{3}$$

This chapter focuses on extra virgin olive oil quality evaluation achieved by means of UV-VIS-NIR absorption spectroscopy. The composition of olive oil is about 98% triglycerides and approximately 2% non glycerid constituents.

- The UV spectrum involves the electronic absorption of fatty acids; in particular, the 230-270 nm band shows high absorption when conjugated dienes and trienes of unsaturated fatty acids are present. For this reason, the absorbances measured at 232 nm and 270 nm, namely K_{232} and K_{270}, provide an official method for olive oil quality control, which is capable of detecting product oxidation and adulteration by means of rectified oils. In addition, the 300-400 nm band provides information about polyphenols (Jiménez Márquez, 2003; Cerretani et al., 2005).
- The VIS spectrum reveals the presence of dyes and pigments (Wrolstad et al., 2005). A and B chlorophylls and their derivatives (pheophytins), carotenoids, and flavonoids such as anthocyanins present distinctive absorption bands in the VIS.
- The wide NIR range is informative for the molecular structure of fats, thanks to the presence of overtones and combinations of vibrational modes of C-H and O-H bonds (Osborne et al., 1993; Ozaki et al., 2007).

In practice, the entire UV-VIS-NIR absorption spectrum can be considered an optical signature, a sort of univocal *fingerprint* of the olive oil. The spectroscopic data can be suitably processed for obtaining a correlation to quality indicators, to the geographic origin of production, to product authenticity as well as to adulteration detection.

2. Instrumentation

Absorption spectroscopy in the UV-VIS-NIR range is one of the most popular measuring methods of conventional analytic chemistry (Mellon, 1950; Bauman, 1962). The most relevant advantages offered are:

- direct measurement: little or no sample preparation is necessary; consequently, the analysis is simple, fast, and does not require manual intervention;
- non-destructive analysis by means of a small quantity of sample;
- compatibility for use in an industrial setting by means of compact instruments.

The conventional instrument for absorption spectroscopy is the double-beam spectrophotometer, the working principle of which is depicted in Figure 3. Since quartz-based optical fibers are transparent in the UV-VIS-NIR range, they are used to equip conventional spectrophotometers by flexible means. Indeed, optical fibers offer the unique possibility of localized probing, a particularly attractive feature for online measurements, which can be carried out in real time without any sample drawing. Moreover, the recent availability of bright LEDs and miniaturized spectrometers further enhances the intrinsic optical and mechanical characteristics of optical fibers and makes it possible to implement compact and moderate-cost instruments.

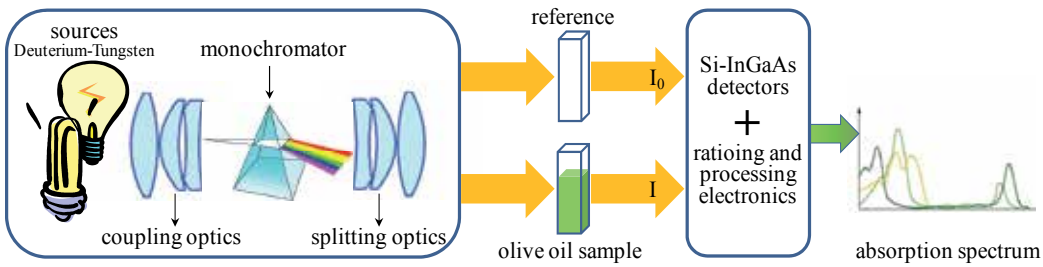

Fig. 3. Working principle of a double-beam spectrophotometer

The conventional spectrophotometer, implemented by means of optical fiber technology, is depicted in Figure 4. In this case, optical fibers are used for both illumination and detection,

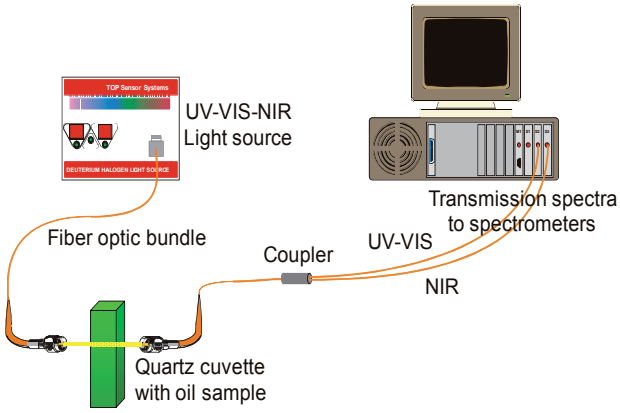

Fig. 4. Fiber optic setup for absorption spectroscopy

and a single cuvette is used. The reference spectrum is measured prior to sample analysis. While the optical fiber strand for illumination can be a single optical fiber or a bundle, the detection is necessarily carried out by means of a bifurcated bundle, or by a coupling device, so as to split the detected light intensity into two spectrometers, for the UV-VIS and the NIR spectroscopic range, respectively.

3. A touch of chemometrics

The chemical information given by an absorption spectrum is contained in the positions and intensities of the absorption bands. Whereas the band positions give information about the appearance and the structure of certain chemical compounds in a mixture, the intensities of the bands are related to the yield of these compounds. Since olive oil contains numerous compounds, the UV-VIS-NIR absorption spectrum shows broad peaks resulting from the convolution of the many overlapping bands, as summarized in Figure 5 (Osborne, 2000).

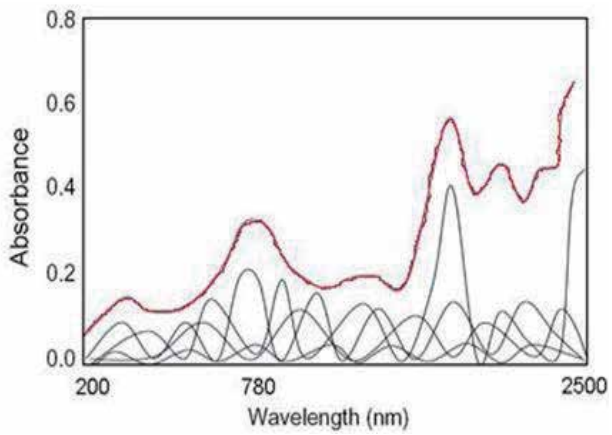

Fig. 5. Absorption spectrum – convolution of absorption spectra of many compounds in a mixture

For qualitative and quantitative analysis, the spectroscopic fingerprinting must be calibrated against reference analytical data from a database of samples representing the best variability in the population. Figure 6 summarizes the steps to follow for achieving a multicomponent analysis from absorption spectroscopy.

- What is needed is a library of representative spectra and relative analytical data to which the spectrum of a test sample may be matched.
- Firstly, a data dimensionality reduction is carried out, which usually leads to a scatter plot where samples are clustered according to the similarity of their spectra. This allows a preliminary inspection of data structure and the detection of what parameters are more likely to be correlated with spectroscopic data.
- Then a more specific analytical tool is chosen according to the type of variable that has to be predicted (quantitative or qualitative). A "Calibration Matrix" is created from which the constituent of interest may be calculated by means of a linear combination of spectroscopic data. The calibration equation has associated statistics which define the closeness of the actual and predicted values. A scatter plot is usually created to detect

any aberrant data. Ideally, the scatter plot should contain data points distributed evenly along a straight line with a narrow confidence limit.

- A validation procedure is then applied for testing the effectiveness of calibration method. While the data dimensionality reduction is usually capable of identifying similarities among products, the correlation to quality indicators always needs the further steps of calibration and validation.

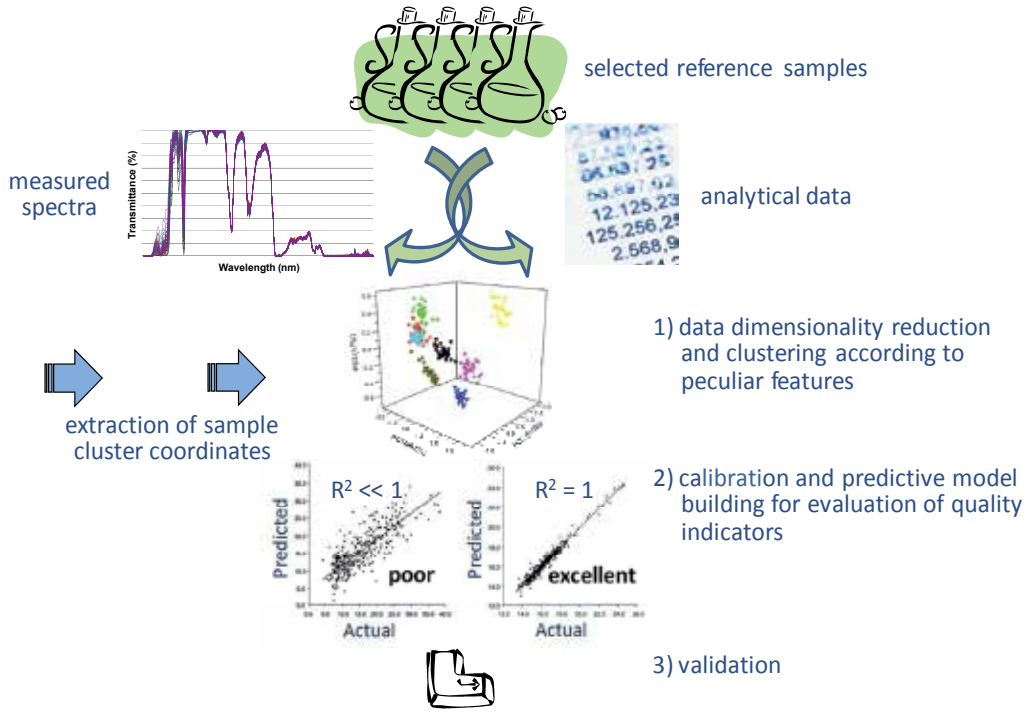

Fig. 6. Steps toward a multicomponent analysis by spectroscopy and chemometrics

Given the nature and complexity of the spectroscopic and analytical data sets involved, many multivariate chemometric techniques have been proposed (Ingle et al., 1988; Mark et al., 2007). The challenge of every multivariate data processing method is to provide excellent classification performance even when few training data are available. Indeed, smart data processing by means of chemometrics makes UV-VIS-NIR optical spectroscopy a rapid and non-destructive method for quality assessment of extra virgin olive oil.

One of the most popular techniques for explorative analysis, data dimensionality reduction, and clustering is the Principal Component Analysis (PCA) that provides the coordinates for identifying the samples in a 2D or 3D map (Jackson, 2003). It linearly combines the spectroscopic data characterizing each oil sample to produce new variables. The coefficients giving the weight of each variable in the linear combination are called *loadings*. The new variables are called Principal Components (PCs), and have the following properties:

- the PCs are mutually uncorrelated (orthogonality);
- the 1st PC (PC1) has the largest variance among all possible linear combination of the starting variables;

- the PCn has the largest variance among all linear combination of the starting variables that are orthogonal to PC1 ... PC(n - 1).

This means that high order PCs have little weight in characterizing the oil samples, and can be disregarded with little loss of information. The loading plots are useful to interpret the score map: they show what variables are important for a given PC. A variable with 0 loading has no importance, a variable with high (positive or negative) loading is important. If a PC has a positive or negative loading at a given wavelength, a sample having high absorbance at that wavelength will tend to have a positive or negative score along that PC.

PCA can also be used for prediction of categorical variables, that is classification, at least in those cases in which they are clearly distinguishable. However, when PCA does not provide satisfactory classification, a more dedicated multivariate data processing tool must be used, such as the Linear Discriminant Analysis (LDA). LDA is a powerful tool that provides both a reduction in dimensionality and automatic object classification. Like PCA, LDA projects a high-dimensional pattern onto a subspace of smaller dimension, but the axes for projection are chosen using a different criterion (Vandeginste et al., 1998).

From the point of view of discriminant analysis, PCA has a drawback: because it weighs the variables in terms of their variance, the features with good discriminating power but limited variance are disregarded. On the contrary, LDA is a tool that is specifically suited for identification, and looks for those variables that show a large spread among different clusters (inter-class variance), but limited variance within each cluster (intra-class variance). Given an N-class problem, the LDA extracts from the data matrix N-1 Discriminating Functions (DFs), which correspond to Principal Components in the PCA, but show a better resolution with regard to poorly-separated clusters. LDA is a *supervised* method. It needs a training set of already classified objects to estimate inter-class and intra-class variances. If all starting variables obey the Gaussian distribution, LDA also provides an easy way to classify unknown patterns. In this case, the points of each cluster in the DF space are distributed following the (N-1)-dimensional Gaussian density function. Thus, classification is achieved simply by evaluating the coordinates of a pattern in the DF space, and then by seeing which of the N density function is higher at that point.

One of the most popular techniques for the prediction of quantitative variables, such as the concentration of an analyte in a multicomponent mixture, is the Partial Least Squares regression (PLS) (Wold et al., 2001). This method is used when the predictor matrix has many collinear variables and the usual Multiple Linear Regression (MLR) cannot be applied. PLS looks for a limited number of PLS factors (PF) which are linear combinations of the original predictors. These new variables are mutually orthogonal (thus uncorrelated) and have the maximum possible covariance with the target variable, among all possible combinations of the original predictors. The idea is that each PF should be linked to a different source of data variance, with the first PF being the most linked to the target variable. The estimation of the optimal number of factors needed to fit the data is a critical issue of PLS. The optimal number of factors is usually assessed by testing each PLS model on the validation set and by minimizing the RMSEP (Root Mean Square Error of Prediction). There are two other fundamental parameters for assessing the goodness of the fit: the RMSEC (Root Mean Square Error of Calibration) and the determination coefficient (R^2), respectively. RMSEC is, like RMSEP, an estimation of the expected prediction error, but is

evaluated on the calibration set. The closeness of RMSEC and RMSEP provides an estimation of robustness of the predictive model. R^2 is, instead, the squared correlation coefficients between predicted and reference values, for the calibration set; thus the fit is as better as this value is closer to 1 ($R^2 \leq 1$).

4. Scattered colorimetry – Looking at olive oil as it is

The extra virgin olive oil is a blend of *cultivars* – different varieties of fruit species – which determine the blend's organoleptic properties. In addition to a distinctive taste, each oil blend has a distinctive color and turbidity. Color is mainly determined by the pigment content of the olives, and by the stage of ripening when they are harvested, whereas turbidity is mainly related to the mill type. Moreover, some commercial oils are filtered before bottling, while other oils are bottled unfiltered to provide a natural appearance. Anyway, both color and turbidity provide a means for oil assessment, and standard techniques are used for their independent measurement.

- Color: the intensity of a white-light source crossing the liquid is measured by a spectrometer, giving the transmission spectrum. The chromaticity coordinates L^*, a^*, and b^* of the CIE1976 Chromaticity Diagram, are then computed (Figure 7, top-left) (Billmeyer et al., 1981; Hunt, 1987).
- Turbidity: the intensities of the monochromatic light crossing the liquid along its axis and scattered at 90° are measured. The ratio between the two is the turbidity in nephelometric turbidity units (Figure 7, top-right) (ISO 7027, 1999).

These color and turbidity measurement methods are popular because of their generality, simplicity, and applicability. In the case of color, since the definition of color is independent of the substance, the method can be applied to any liquid. In the case of turbidity, the independence of the test material is attained by reference to an ISO standard turbid material.

However, both methods view the characteristics separately, i.e., the color method never considers the liquid's turbidity, while the turbidity method never considers the color. Usually, in order to avoid mutual interferences, the olive oil is filtered prior to color measurements, while turbidity measurements are performed at a color-independent wavelength – typically at 830 nm. In order to evaluate the olive oil sample as it is, a new technique combining color and turbidity measurements was proposed, which was called scattered colorimetry or spectral nephelometry, since it extended the color and turbidity standards by adding light sources and observation angles, as shown in Figure 7-bottom (Mignani et al., 2003).

Scattered colorimetry makes use of four white light sources, which span the 450-630 nm spectroscopic range, and a miniaturized optical fiber spectrometer as detector. The sources, positioned at different angles with respect to the detector, are sequentially switched on to measure, in addition to the transmitted spectrum, the scattered spectra at the given angles. The transmitted spectrum mainly provides information regarding color, which is also dependent on the turbidity, while the scattered spectra mainly provide information on turbidity, which is also dependent on the color. Measurements on a vial filled by distilled water are carried out prior to sample analysis, so as to obtain reference signals. Then, the spectroscopic data are processed by means of chemometrics, thus providing few coordinates that summarize the combined effects of color and turbidity.

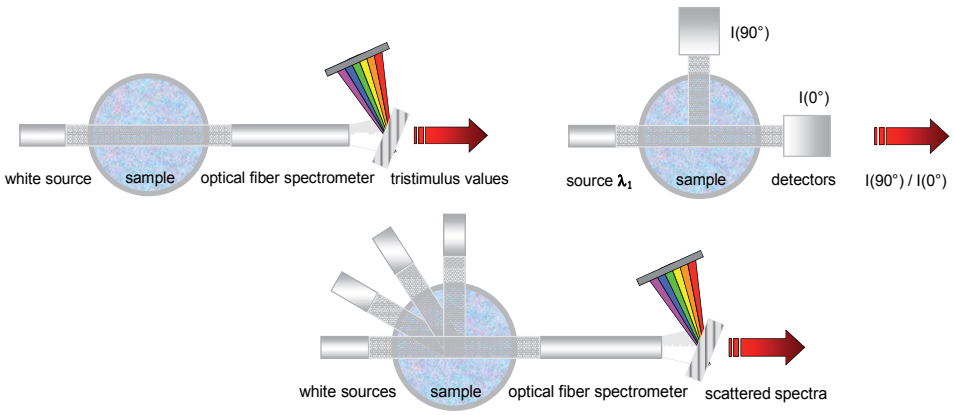

Fig. 7. The working principle of scattered colorimetry (bottom), compared to colorimetry (top-left) and turbidimetry (top-rignt)

Scattered colorimetry showed effectiveness in discriminating the geographic regions of production (Mignani et al., 2005). Figure 8 shows the clustering maps of a couple of experiments carried out by analyzing collections of extra virgin olive oils produced in different geographic regions of the Mediterranean area. Figure 8-left shows the 3D map obtained by LDA processing the spectra of a collection of 236 oils. The collection comprises 115 Tuscan and 53 Calabrian extra virgin olive oils produced by traditional methods, and 68 oils (58 extra virgin and 10 non extra virgin) purchased from retailers. The Tuscan oils are clearly distinguishable from the other extra virgin oils as are the Calabrian oils. As expected, the cluster of non extra virgin olive oils is distinctly separated. Figure 8-right shows another 2D map, again achieved by means of LDA processing of spectroscopic data of a collection of 270 extra virgin olive oils artesanally produced. This collection comprises 213 Italian (90 Tuscan and 123 Sicilian) and 57 Spanish samples. The olive oils of the two countries have intrinsic differences influenced by the diverse cultivars, weather conditions, harvest times, and production methods. An evident clustering according to geographic regions of origin is achieved.

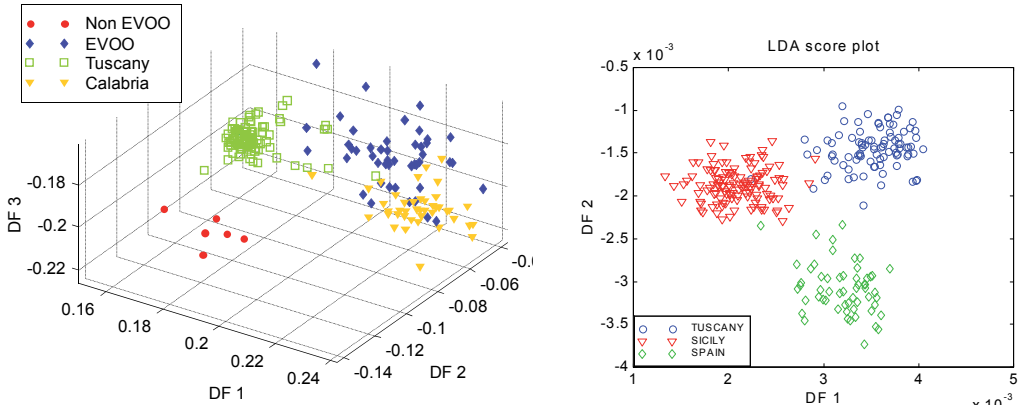

Fig. 8. Effectiveness of scattered colorimetry for discriminating the geographic area of production: Italian extra virgin olive oils from different regions and oils from retailers (left); Spanish and Italian oils (right) (with kind permission of Elsevier and SPIE)

5. UV-VIS-NIR absorption spectroscopy

The little VIS range used in scattered colorimetry only allows to classify oils according to their geographic region of production. In this paragraph, traditional absorption spectroscopy is discussed, which is applied to filtered olive oil samples in order to avoid the influence of turbidity. Indeed, traditional absorption spectroscopy, with or without optical fibers, is certainly the most frequently used spectroscopic technique for olive oil analysis. Since every spectroscopic range is bringing peculiar information, the combination of UV-VIS, or VIS-NIR bands, or the entire UV-VIS-NIR range makes it possible to achieve wider information. Nutraceutic parameters can be predicted, which allow to recognize olive oils of different qualities. In addition, mixtures of extra virgin with lower quality oils can be detected.

Many types of extra virgin olive oils of the Mediterranean region were classified according to their geographic origin by means of absorption spectroscopy combined to chemometrics. The NIR band was used to classify French oils from several regions holding quality labels as registered designation of origin; squalene and fatty acids were also predicted (Galtier et al., 2007). The NIR was also used to classify Spanish oils (Bertran et al., 2000), to predict acidity and peroxide index (Armenta et al., 2007), and to detect and quantify the adulteration with sunflower and corn oil (Özdemir et al., 2007) and other vegetable oils (Christy et al., 2004; Öztürk et al., 2010). Greek oils from Crete, Peloponnese and Central Greece were classified both by the UV-VIS (Kružlicová et al., 2008) and the VIS-NIR bands (Downey et al., 2003), the latter being effective for detecting the adulteration with sunflower oil (Downey et al., 2002). The UV-VIS band was also used to detect the adulteration of extra virgin olive oils mixed with lower quality olive oils (Torrecilla et al., 2010).

The entire UV-VIS-NIR spectrum was exploited to both classify according to geographic region of production, and to predict quality indicators of Italian extra virgin olive oils. The spectra shown in Figure 9-left refer to a collection of 80 extra virgin olive oils produced in four different regions of Italy: Lombardy, Tuscany, Calabria and Sicily. Lombardy is located in the northern part of Italy, Tuscany in the center, while Calabria and Sicily in the south, being Sicily the southest region. A chemometric data processing of these spectra allowed to achieve the regional clustering shown in Figure 9-right, and to predict quality indicators, as shown in Tables 1 and 2 (Mignani et al., 2008).

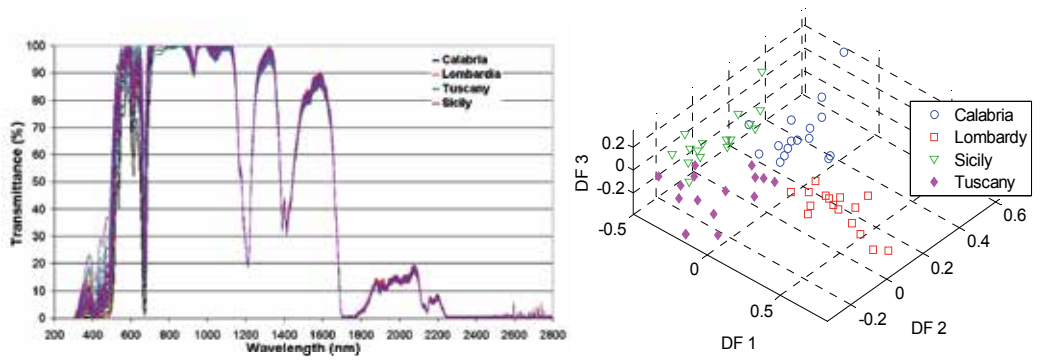

Fig. 9. Transmission spectra in the UV-VIS-VIR ranges of 80 extra virgin olive oil samples from four Italian regions (left), and regional clustering obtained by a chemometric processing of spectroscopic data (right) (with kind permission of SPIE)

Quality parameters	Calibration range	Spectroscopic range (nm)	Number of PLS regressors	R^2
Oleic acidity (% oleic acid)	0.12 - 1.555	780 - 2500	3	0.8407
Peroxide value (meq O / kg. oil)	3.76 - 13.98	1000 – 2333	2	0.9628
K_{232}	0.922 - 1.548	1333 – 2222	3	0.9942
K_{270}	0.062 - 0.1178	1333 – 2222	3	0.9825
ΔK	-0.004 - 0.01	1333 - 2222	2	0.4344

Table 1. Prediction of quality parameters of the extra virgin olive oil collection of Figure 9

Fatty acids	Calibration range (%)	Spectroscopic range (nm)	# PLS regressors	R^2
Oleic	65.847 - 76.334	1333 - 2222	1	0.9986
Palmitic	9.62 - 17.113	300 - 2300	2	0.9847
Linoleic	4.469 - 10.95	1333 - 2222	1	0.9553
Stearic	2.565 - 4.046	780 - 2500	2	0.9942
Palmiticoleic	0.367 - 1.457	1333 - 2222	2	0.9504
Linolenic	0.646 - 1.066	1000 - 2300	1	0.9822
Arachiric	0.382 - 0.642	1000 - 2222	1	0.9896
Eicosenoic	0.212 - 0.431	1000 - 2300	2	0.9821
Behenic	0.042 - 0.411	300 - 2300	2	0.8892
Heptadecenoic	0.053 - 0.356	300 - 2300	2	0.8081
Heptadecanoic	0.025 - 0.29	1000 - 2300	2	0.8337
Lignoceric	0.026 - 0.205	1333 - 2222	1	0.8532

Table 2. Prediction of fatty acids of the Sicilian extra virgin olive oils of Figure 9

6. Diffuse-light absorption spectroscopy

The scattered colorimetry technique allows for assessing the olive oil by considering both color and turbidity. Indeed, although the intrinsic turbidity of the oil can be regarded as a peculiar characteristic, it has an unstable and non-reproducible influence on absorption measurements because of its time dependent nature. In fact, suspended particles created during production of the olive oil usually settle down in a non-reversible way, because they tend to aggregate at the bottom of the container, creating a sort of sludge. Absorption spectroscopy in the UV-VIS-NIR of filtered samples demonstrated effectiveness to achieve wider quality information. However, sample filtering is not only a time-consuming procedure, but is also an action that alters the composition of the sample. In fact, turbidity is also due to the presence of water, and water removal causes a serious loss of water-soluble compounds–such as polyphenols–that are responsible for the unusual character and authenticity of olive oil.

Diffuse-light absorption spectroscopy, that is, spectroscopy carried out by means of an integrating cavity, is an alternative spectroscopic technique which allows to achieve scattering-free absorption spectra, that is, without caring about the intrinsic turbidity of the olive oil. It has been proposed in the literature as an effective method for overcoming scattering problems in process control (Fecht et al., 1999) and biological applications (Merzlyak et al., 2000), as well as for more general quantitative spectrophotometry (Jàvorfi et al., 2006).

Diffuse-light absorption spectroscopy makes use of an integrating sphere that contains the sample under test. The source and the detector are butt-coupled to the sphere, as shown in Figure 10.

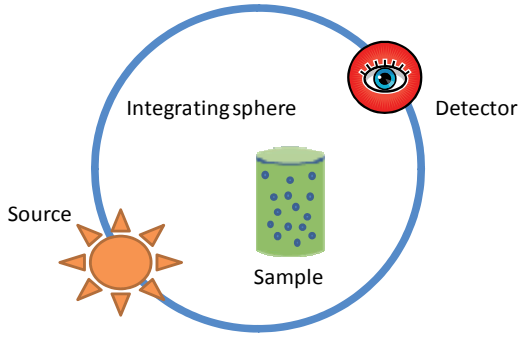

Fig. 10. Setup for diffuse-light absorption spectroscopy by means of an integrating sphere

Almost all the light shining on the sphere surface is diffusely reflected, and the detector can be placed anywhere in the sphere in order to gather the average flux (Elterman, 1970; Fry et al., 1992; Nelson et al., 1993; Kirk, 1995). By inserting an absorbing medium in the cavity, a reduction of the radiance in the sphere occurs. The reduction is related to the absorption of the medium and to its volume, and is independent of non-absorbing objects within it, such as suspended scattering particles. The light intensity detected by means of this measuring setup is described by Equation 4:

$$I = \frac{R\, I_0\, A_d}{S} \frac{1}{1 - \frac{R}{S}\left(S - A_s - \alpha\, V \right)} \tag{4}$$

where I_0: source power; I: detected power; $\alpha=\varepsilon C$: sample absorption coefficient; V: sample volume; A_d: detector area; A_s: source area; R: cavity power reflectivity; S: cavity surface area.

This technique was used to detect the adulteration of high quality extra virgin olive oils produced in Tuscany caused by lower quality olive oils such as olive pomace, refined olive pomace, refined olive, and deodorized olive oils (Mignani et al., 2011). Mixtures of four original extra virgin olive oils and the four types of adulterants were artificially created at different adulterant concentration. Figure 11-top shows the diffuse-light absorption spectra of high quality oils (left) and the others used as adulterants (right). Then, chemometrics was applied for achieving adulterant discrimination and prediction of relative concentration. Figure 11-bottom shows the discriminating maps: PCA was capable of discriminating

samples adulterated by means of deodorized olive oil (left), while a deeper LDA processing was needed for discriminating the other adulterants (right).

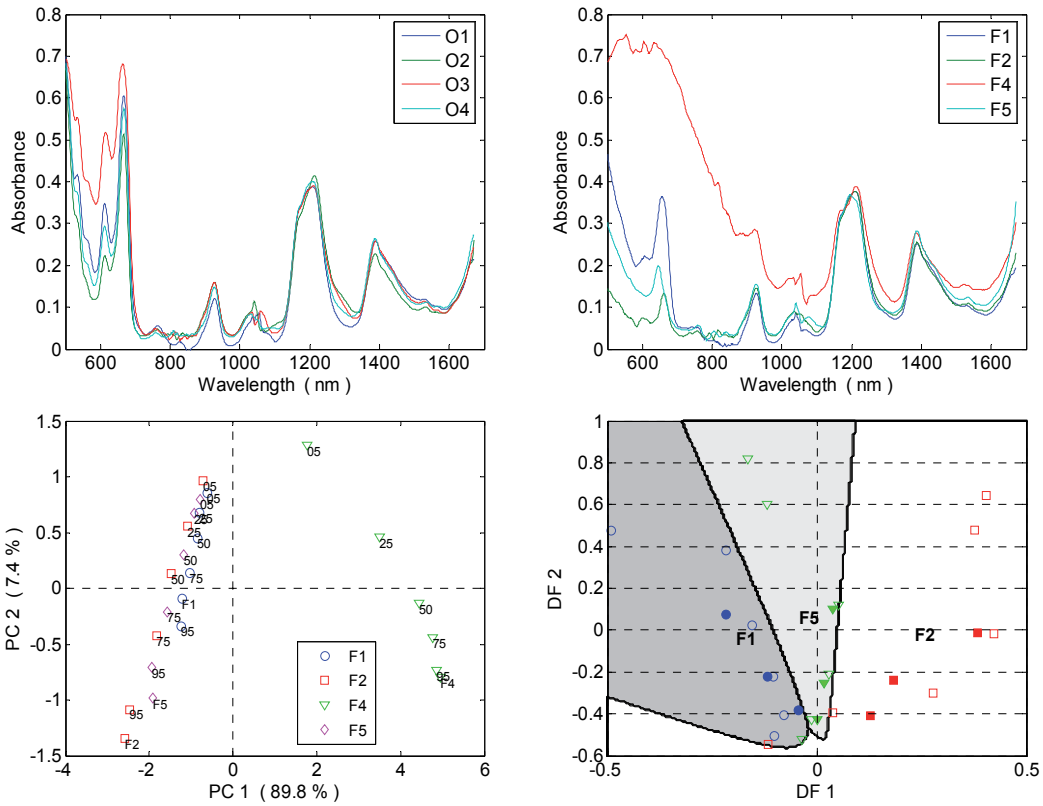

Fig. 11. Top: Diffuse-light absorption spectra of original extra virgin olive oils (left) and other lower quality olive oils commonly used as adulterants (right). Bottom: Discriminating maps obtained by chemometric data processing of absorption spectra (with kind permission of Springer Science+Business Media)

7. Perspectives

Absorption spectroscopy, carried out in the UV-VIS-NIR and combined with chemometrics, can be used for authentication, fraud detection, as well as for quantifying quality indicators. Optical fibers can be used for localized or online analyses.

Absorption spectroscopy for assessing the extra virgin olive oil can be performed also in other spectroscopic bands of the electromagnetic spectrum, especially in the mid-infrared (MIR) and far-infrared (FIR). Recently, NIR and MIR bands were successfully combined and showed effectiveness not only for classifying Italian and French olive oils (Sinelli et al., 2008; Galtier et al., 2011), but also for a classification on the basis of the fruity sensory attribute (Sinelli et al., 2010). The instrumentation for MIR absorption spectroscopy makes use of other types of sources/detectors/optics and measuring schemes. Usually, the attenuated total reflection modality is used, which allows absorption measurements even in high absorption liquids, like olive oil is in the MIR.

Other optical spectroscopic techniques are emerging for the quality assessment of extra virgin olive oils, especially the fluorescence and the Raman spectroscopies. The interested readers can have a look at the most recent bibliography (Ross Kunz et al., 2011; El-Abassy et al., 2010; Paiva-Martins et al., 2010; El-Abassy et al., 2009; Tena et al., 2009; Zou et al., 2009; Sikorska et al., 2008; Poulli et al., 2007).

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4

Analysis of Olive Oils by Fluorescence Spectroscopy: Methods and Applications

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1. Introduction

Fluorescence spectroscopy is a well established and extensively used research and analytical tool in many disciplines. In recent years, a remarkable growth in the use of fluorescence in food analysis has been observed (Christensen et al., 2006; Sadecka & Tothova, 2007; Karoui & Blecker, 2011). Vegetable oils including olive oil constitute an important group of food products for which fluorescence was successfully applied. Fluorescence is a type of photoluminescence, a process in which a molecule, promoted to an electronically excited state by absorption of UV, VIS or NIR radiation, decays back to its ground state by emission of a photon. Fluorescence is emission from an excited state, in which the electronic spin is equal to that in the ground state, and typically equal to zero. Such transitions are spin allowed, and occur at relatively high rates, typically 10^8 s^{-1} (Lakowicz, 2006).

A unique feature of fluorescence, distinguishing it from other spectroscopic techniques, is its inherently multidimensional character (Christensen et al., 2006). Excitation of molecules results from absorption of radiation at the energy corresponding to the energy difference between the ground and excited states of a given fluorophore. Subsequently, radiation at a lower energy characteristic for the specific molecule is emitted during its deactivation. Thus, fluorescence properties of every compound are characterized by two types of spectra: excitation and emission. This feature and the fact that not all of the absorbing molecules are fluorescent both contribute to higher selectivity of fluorescence as opposed to absorption spectra.

Another important advantage of fluorescence is its higher sensitivity. In contrast to absorption measurements, the emitted photons are detected against a low background, making fluorescence spectroscopy a very sensitive method. The sensitivity of fluorescence is 100-1000 times higher than that of the absorption techniques, enabling to measure concentrations down to parts per billion levels (Guilbault, 1999).

The fluorescent analysis of olive oils takes advantage of the presence of natural fluorescent components, including phenolic compounds, tocopherols and pheophytins, and their oxidation products. Oils are complex systems and therefore conventional fluorescent

techniques, relying on recording of single emission or excitation spectra, are often insufficient if directly applied. In such cases, total luminescence or synchronous scanning fluorescence techniques are used, improving the analytic potential of the fluorescence measurements. With contributions from numerous analytes, the autofluorescence of olive oil exhibits numerous overlapping bands. Such complex spectra should be analyzed using multivariate and multiway methods.

Analytical applications of fluorescence to olive oils include discrimination between the different quality grades, adulteration detection, authentication of virgin oils, quantification of fluorescent components, monitoring thermal and photo-oxidation and quality changes during storage.

In this chapter the application of fluorescence spectroscopy to qualitative and quantitative analysis of olive oils is reviewed. Methodological aspects of fluorescence measurements and analysis of fluorescence spectra are also discussed.

2. Fluorescence of olive oils

2.1 Fluorescence characteristics of olive oil and its components

Conventionally, two basic types of spectra characterize the fluorescent properties: excitation and emission spectra. For a system containing a single fluorophore, the shape and location of the excitation and emission spectra are independent of respective chosen emission and excitation wavelengths. However, for a system containing several fluorescent components, the excitation and emission spectra depend on particular emission and excitation wavelength used for measurements. Therefore, in systems containing several fluorophores, single-wavelength spectra are insufficient for a comprehensive description of fluorescent properties, thus multidimensional measurement methods should be used.

The most comprehensive characterization of a multicomponent fluorescent system is obtained by measurement of an excitation-emission matrix, known also as a total luminescence spectrum or fluorescence landscape. This technique was first introduced by Weber (1961). After the first application to edible oils by Wolfbeis & Leiner (1984), it has been intensively used for exploring oil fluorescence. Total luminescence spectra are usually obtained by measurement of emission spectra at several excitation wavelengths. They may be presented as a three dimensional plot, with the fluorescence intensity plotted in function of the excitation and the emission wavelengths (Ndou and Warner, 1991; Guilbault, 1999). Another representation of the total luminescence is obtained using two-dimensional contour maps, in which one axis represents the emission and another – the excitation wavelength, and the contours are plotted by linking points of equal fluorescence intensity, Fig.1. The total luminescence spectrum gives a comprehensive description of the fluorescent components of the mixture and may serve as a unique fingerprint for identification and characterization of the sample studied. The acquisition of contour maps at sufficient resolution (determined by the number of individual emission spectra recorded) on conventional spectrofluorometers is time-consuming, requiring a large number of scans for each sample (Guilbault, 1999).

Alternatively, multicomponent fluorescent systems may be investigated by the synchronous fluorescence techniques, proposed by Lloyd, (1971). This technique involves simultaneous

scanning of both excitation and emission wavelengths, keeping a constant difference between them. Synchronous scanning fluorescence spectroscopy is very useful for the analysis of mixtures of fluorescent compounds, because both excitation and emission characteristics are included into a single spectrum. Although it provides less information than the excitation-emission matrix, it may still present a viable alternative to the total luminescence measurements due to its inherent simplicity and rapidity. A set of synchronous spectra recorded at different wavelength intervals may be concatenated into a total synchronous fluorescence spectrum. In such spectra fluorescence intensity is plotted as a function of the excitation wavelength and the wavelength interval. Both single wavelength interval and total synchronous fluorescence spectra were used for studies of olive oils (Sikorska et al. 2005a; Poulli et al. 2005). The relation between various kinds of fluorescence spectra of a virgin olive oil is presented in Fig. 1.

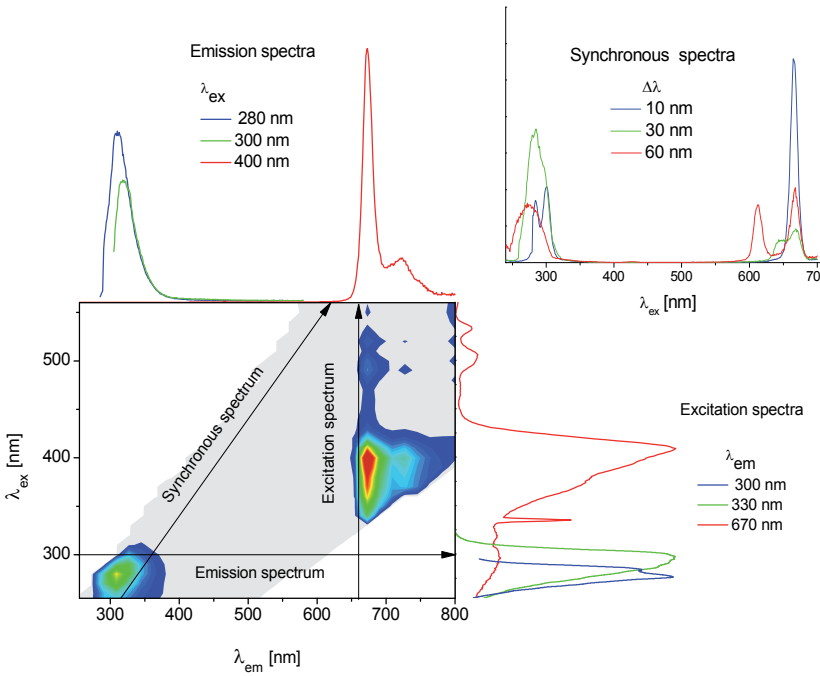

Fig. 1. Different types of fluorescence spectra; fluorescence spectra of a diluted sample of extra virgin olive oil (1%, v/v, in n-hexane) are shown as an example.

Numerous factors affect measured fluorescence intensity and spectral distribution. These factors are related to the nature and the concentration of fluorophores, their molecular environment, and scattering and absorption effects. They may be immeasurably important in complex natural systems, such as oils, and have to be taken into account when measuring and interpreting the fluorescence spectra. Fluorescence intensities are proportional to the concentration over only a limited range of optical densities (Lakowicz, 2006). To obtain proportionality between the fluorescence intensity and the fluorophore concentration, the absorbance at the excitation wavelength should be below 0.05 and close to zero in the emission spectral region. At higher concentrations, the inner filter effects have to be taken into account. These effects may decrease the observed fluorescence intensity by either

reducing the intensity of the excitation or by absorbing the emitted radiation. To avoid the inner filter effects due to the high optical densities or sample turbidity, appropriate geometry of sample illumination should be used. The most common geometry using right-angle observation of the center of a centrally illuminated sample is only appropriate for diluted solutions with low optical densities. For opaque samples, front-face illumination is achieved using either triangular or square cuvettes oriented at 30 or 60° relative to the incident beam.

The effects of concentration and sample geometry on oil spectra were addressed by several authors. Zandomeneghi et al. (2005) in the very detailed studies compared the emission fluorescence spectra of undiluted extra virgin olive oil obtained with the traditional setup (right-angle fluorescence) and using front face fluorescence. The absorption of undiluted olive oil samples was from 2 up to 12 absorbance units, on passing from 325 to 260 nm, and therefore the inner filter phenomena affected the right angle spectra considerably. Excluding the long-wavelength chlorophyll region, significant differences in the number, shape, intensity, and position of the bands in spectra of the same undiluted oil obtained with right angle and front face geometry were observed, Fig. 2. The right angle fluorescence spectra showed considerable distortions, even after the mathematical corrections for the inner filter effects due to the absorption of both the excitation and emission radiation were applied. The front-face fluorescence spectra were less affected by self-absorption and thus provided reliable information about type of fluoropores and their concentration. It was also demonstrated that analysis of spectra affected by inner filter effects may lead to spectral misinterpretation and invalid assignments of origin of some fluorescent bands (M. Zandomeneghi & G. Zandomeneghi, 2005; Zandomeneghi et al., 2006).

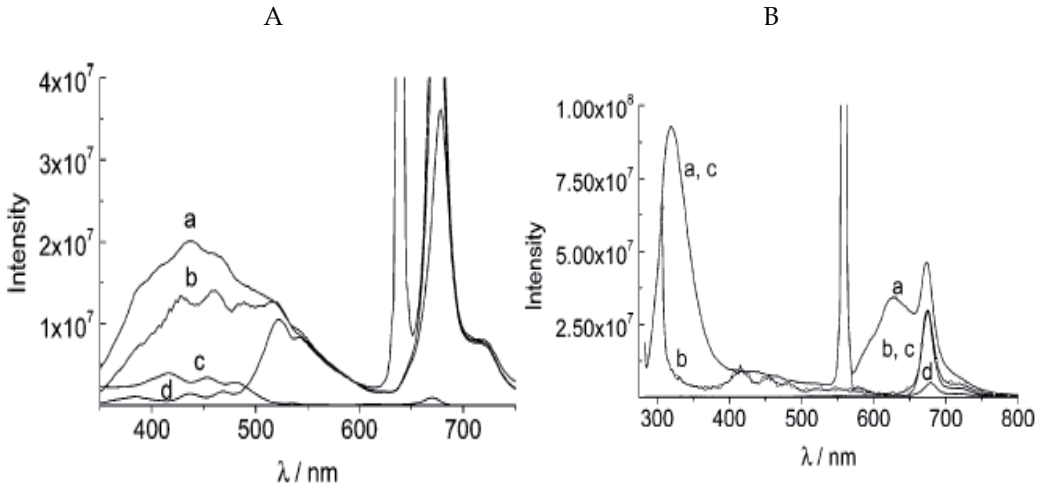

Fig. 2. A. Emission spectra of olive oil with λ_{ex} = 320 nm. (a) front face spectrum; (b) right angle spectrum with the absorption correction; (c) absorption spectrum of the oil multiplied by 10^6; (d) right angle spectrum without the absorption correction. B. Emission spectra of olive oil with λ_{ex} = 280 nm. (a) front face spectrum; (b) right angle spectrum with the absorption correction and multiplied by 3.7; (c) front face spectrum, second-order corrected; (d) right angle spectrum. Reprinted with permission from Zandomeneghi et al. 2005. Copyright 2005 American Chemical Society.

Typical fluorescence spectra of extra virgin and refined olive oils are shown in Fig. 3, (Sikorska et al., 2011). The fluorescence depends on sample concentration; therefore spectra for diluted and intact samples are shown. Both total fluorescence and total synchronous spectra are presented for the same oils, to enable comparison.

Based on the published data, one may conclude that the fluorescence properties depend on the quality grade of olive oils (Kyriakidis&Skarkalis, 2000; Poulli et al., 2006; Guimet et al., 2004a). For a selected quality category, the spectra may show minor differences between samples, however, the general features remain similar, permitting identification and authentication of oil samples.

The total fluorescence spectrum of diluted extra virgin olive oils, measured with the use of right angle geometry, exhibits two intense bands, one with excitation at about 270–330 nm and emission at about 295–360 nm and the second with excitation at about 330–440 nm and emission at about 660–700 nm, Fig. 3. An additional band appears in spectra of refined olive oil, located in the intermediate range, with excitation at 280-330 nm and emission at 372-480 nm. The long-wavelength band has a lower intensity in refined as compared to virgin olive oil (Sikorska et al., 2011).

The spectra of the same undiluted oils measured with the front face geometry method show a clearly different fluorescence pattern. The spectra are not affected by the inner filter effect, because front face geometry was used for measurement of undiluted samples. Additional bands are observed in the spectra of extra virgin olive oil at about 310–390 nm in excitation and 440–580 nm in emission. The ratio of fluorescence intensity of short- and long-wavelength bands is lower as compared to the spectrum of the diluted sample. The spectrum of undiluted refined olive oil exhibits a broad band with emission at 350-600 nm, two maxima at 320/420 nm and 365/450 nm in excitation/emission, and a long wavelength emission at 650-700 nm. Only a trace of the short-wavelength emission is observed with the maximum at 300/331 nm (Sikorska et al., 2011).

The differences in the spectra between diluted and undiluted samples may result from the high fluorophore concentrations in the intact oil samples and a variety of molecular interactions, such as quenching and energy transfer, which alter fluorescence characteristics. The effect of concentration on the total and synchronous fluorescence spectra of vegetable oils including olive oils was reported by Sikorska et al. (2004; 2005b).

The total synchronous fluorescence spectra of undiluted oils showed dependence of spectral shape and intensity on the wavelength interval ($\Delta\lambda$) used in the measurements, with the presence of particular bands dependent on $\Delta\lambda$. At lower values of $\Delta\lambda$ the bandwidths are reduced and the spectrum is simplified as compared to the total fluorescence spectra. Appearance of new bands or splitting of existing bands is typically observed with increasing $\Delta\lambda$. Emission bands are present in the excitation region below 310 nm, 310-350 nm, 350-380 nm, and above 550 nm in spectra of virgin olive oils (Sikorska et al., 2011). Similar spectral characteristics for virgin olive oil were reported by Poulli et al., (2006). The bands in total synchronous fluorescence spectra were observed in the 270–325, 347–365 and 602–685 nm excitation wavelength ranges with the respective wavelength intervals of 20–120, 30–50 and 20–76 nm.

Refined oils are characterized by a relatively weak band between 290-320 nm, a very broad band spreading to about 500 nm, and a band above 550 nm. All of these bands equally appear in the total fluorescence spectra (Sikorska et al., 2011).

The identification of origin of the particular emission bands relies mainly on comparison to the spectra of chemically pure fluorescent components. The fluorescence properties of compounds occurring in oils or suggested to contribute to their emission are listed in Table 1. The short wavelength band in total fluorescence spectra, which covers the region of 270–330 nm in excitation and 295–360 nm in emission, corresponds to the band at 280–310 nm in the total synchronous fluorescence spectra and is assigned to tocopherols and phenols. This assignment has been confirmed by several observations. Firstly, it was shown that a similar band appears in various vegetable oils, either cold-pressed or refined, and not only in olive oils (Sikorska et al., 2004). Olive oils contain considerable amounts of phenolic compounds, with their concentrations significantly reduced in refined oils. This observation seems to confirm that tocopherols also contribute to the emission observed in this wavelength range. In fact, tocopherols are present in most vegetable oils in widely variable amounts, from 70 to 1900 mg/kg (Cert et al., 2000). The vitamin E group includes four natural tocopherols (α-, β-, γ-, δ-) and four tocotrienols (αT3, βT3, γT3, δT3), all — in the R-configuration at the three double bonds in the side-chain of tocotrienols. Due to their structural similarity, all of these compounds exhibit very similar UV-absorption spectra and have similar fluorescence properties, Table 1. Of all tocopherols, α-tocopherol is predominant in olive oils. Indeed, the band in olive oil spectra being discussed is similar to the one in the total luminescence spectrum of α-tocopherol dissolved in *n*-hexane. Moreover, conventional excitation and emission spectra of the olive oils in the wavelength range mentioned are also similar to those of α-tocopherol, and the excitation spectra are in good agreement with the absorption spectrum of α-tocopherol in *n*-hexane (Sikorska et al. 2004). Still, the detailed analysis of excitation and emission spectra suggests contributions from several other fluorophores.

There still remain some inconsistencies concerning the assignment of vitamin E (tocopherol) bands in olive oil spectra. In one of the pioneering papers, where the emission spectra of various oils were reported, it has been suggested that the bands in the emission spectrum (λ_{ex}=365 nm) with the maximum at 525 nm may partly originate from compounds of the vitamin E group, or their derivatives formed upon oxidation (Kyriakidis & Skarkalis, 2000). However, this interpretation is based on spectra of undiluted olive oils measured using right angle geometry, and therefore strongly affected by inner filter effects, and in some cases referring to the spectral region where no emission of tocopherols is present (Zandomeneghi et al. 2005). It should be underlined that the emission of vitamin E in *n*-hexane has its maximum at about 320 nm, with a similar maximum appearing in the spectra of oils. Moreover, it has been stated (Zandomeneghi et al. 2006) that the known products of oxidation of R-α-, β-, γ-, δ-tocopherols, the R-α-, β-, γ-, δ-tocopherolquinones, are all nonfluorescent substances (Pollok & Melchert, 2004).

Note that a considerable number of minor components belonging to different classes of phenolic compounds such as phenolic acids, phenolic alcohols, hydroxyisochromans, secoiridoids, lignans, and flavonoids are present in virgin olive oils (Servili et al., 2004). Most of polyphenols are fluorescent substances, absorbing in the 260-310 nm range and emitting in the near-UV range, with their bands centered at 310-370 nm (M. Zandomeneghi & G. Zandomeneghi, 2005). These phenolic compounds can be detected by fluorescence after separation by HPLC, using excitation/emission wavelengths of 264/354, 310/430 or 280/320 nm (Dupuy et al., 2005). Fluorescence typical for phenolic components of olive oils was reported recently by Tena et al., (2009), using excitation at 270 nm with the fluorescence maxima appearing in the 362-420 nm range, Table 1.

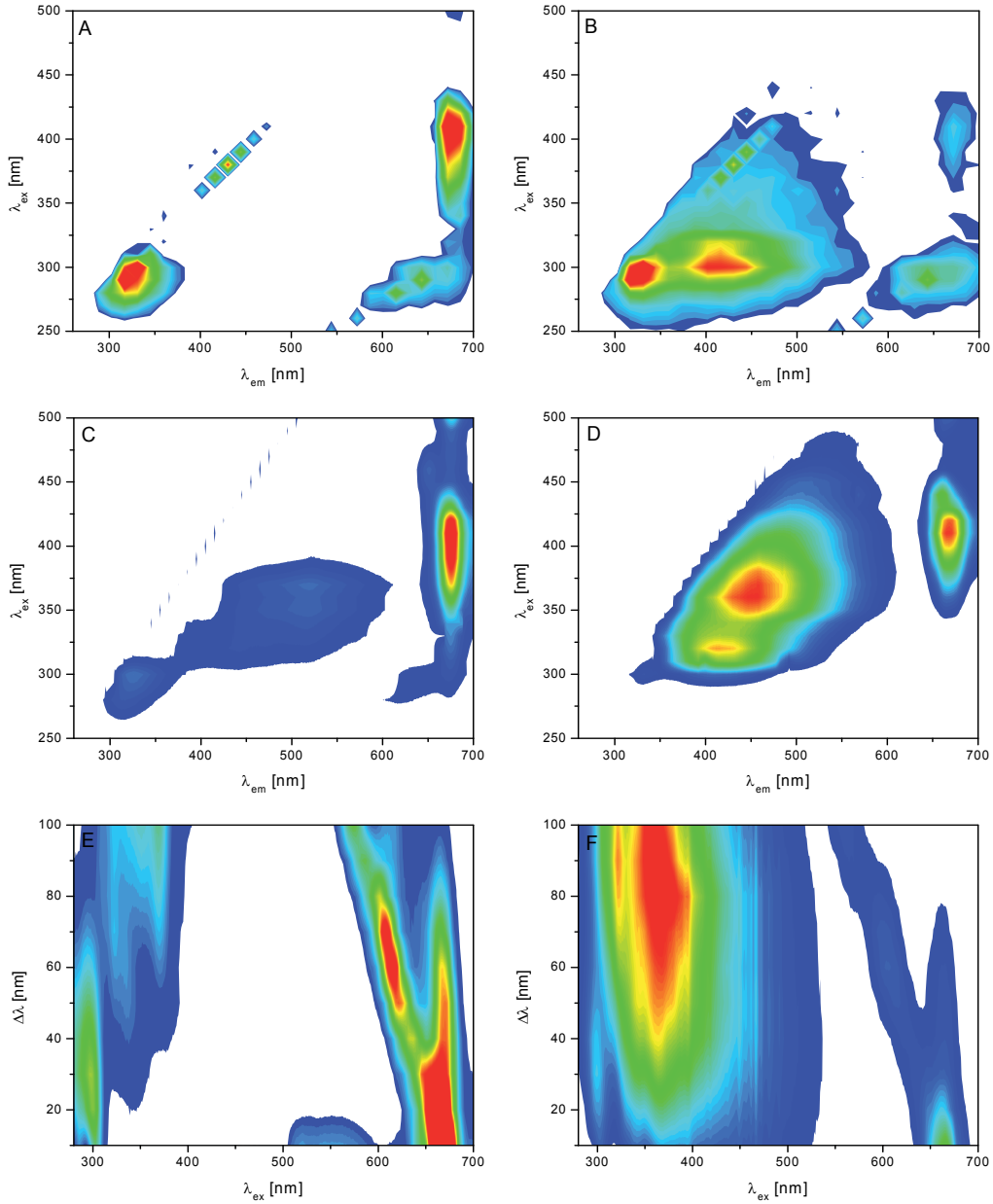

Fig. 3. Fluorescence spectra of extra virgin and refined olive oil: A. Total fluorescence spectrum of diluted olive oil, 1% in n-hexane, B. Total fluorescence spectrum of diluted refined olive oil, 1% in n-hexane, C. Total fluorescence spectrum of undiluted extra virgin olive oil, front face geometry, D. Total fluorescence spectrum of undiluted refined olive oil, front face geometry, E. Total synchronous fluorescence spectrum of undiluted extra virgin olive oil, front face geometry, F. Total synchronous fluorescence spectrum of undiluted refined olive oil, front face geometry (Sikorska et al., 2011).

	Solvent	λ_{abs} [nm]	ε [dm^3 mol^{-1} cm^{-1}]	Solvent	λ_{ex} [nm]	λ_{em} [nm]
Vitamin E						
α-Tocopherol [a]	ethanol	292	3265	n-hexane	295	320
β- Tocopherol [a]	ethanol	296	3725	n-hexane	297	322
δ- Tocopherol [a]	ethanol	298	3515	n-hexane	297	322
γ- Tocopherol [a]	ethanol	298	3809	n-hexane	297	322
α-Tocotrienol [a]	ethanol	292	3652	n-hexane	290	323
β- Tocotrienol [a]	ethanol	292	3540	n-hexane	290	323
δ- Tocotrienol [a]	ethanol	297	3403	n-hexane	292	324
γ- Tocotrienol [a]	ethanol	297	3737	n-hexane	290	324
Chlorophylls						
Chlorophyll *a* [b,c,d]	acetone	430	94700	ether	436	668
		663	75000	acetone	405	669
				9:1 acetone/water	430[e]	669
Chlorophyll *b* [b,c,d]	acetone	455	131000	ether	436	648
		645	47100	acetone	405	652
				9:1 acetone/water	458 [e]	653
Pheophytin *a* [b,c,d]	acetone	409	101800	ether	436	673
		666	44500	9:1 acetone/water	406 [e]	671
Pheophytin *b* [b,c,d]	acetone	434	145000	ether	436	661
		654	27800	9:1 acetone/water	435[e]	658
				-	-	
Pheophorbide *a* [b,c,d]	acetone	409	119200	-	-	-
		667	55200	-	-	-
Phenolic compounds						
Oleuropein	ethanol/n-hexane	282	-	ethanol/n-hexane	270	310
Vanillic acid [e]				methanol	270	349
Syringic acid [e]					270	361
Gallic acid [e]					270	382
p-Coumaric acid [e]						416
o-Coumaric [e]					270	426
Cinnamic acid [e]					270	420
Tyrosol [e]					270	420
Caffeic acid [e]					270	457

λ_{abs}, λ_{exc}, λ_{em} – absorption, excitation and emission maxima, ε - molar absorption coefficient, [a] (Eitenmiller et al., 2008), [b] (Ward et al., 1994), [c] (Undenfriend, 1962), [d](Diaz et al., 2003), [e](Tena et al., 2009).

Table 1. Fluorescence properties of olive oil components.

The fluorescence spectra of a vitamin E standard in hexane and an oil polyphenol extract in methanol–water at the same concentration as found in the extra virgin olive oil were investigated separately (Cheikhousman et al., 2005). The maximums in the fluorescence excitation spectrum measured at the 330 nm emission wavelength of oil polyphenol extract and vitamin E were observed at 284 and 290 nm, respectively. The spectral contribution of both the tocopherols and phenolic compounds to the fluorescence of extra virgin olive oil was confirmed by the similarity between the reconstructed spectrum of the mixture and the spectrum of extra virgin oil (Cheikhousman et al., 2005). Recently the fluorescence intensity at 280/320 nm in excitation/emission was successfully used to determine phenol contents in methanol/water extracts of olive oils (Papoti & Tsimidou, 2009).

Thus, both tocopherols and phenolic compounds contribute to the short-wavelength emission of the olive oils, with the tocopherol contribution dominating in refined oils. The exact positions of the maxima of the short-wavelength emission vary slightly between various oils, which may result from differences in the respective tocopherol composition. Note that in oils obtained by physical methods (cold pressing), including olive, linseed and rapeseed oils, this fluorescence maximum was blue-shifted as compared to refined oils, pointing out the difference between fluorescence of refined and cold-pressed oils in this spectral region (Sikorska et. al., 2004).

Due to the similar fluorescence properties of tocopherols and some phenolic compounds (Table 1), their emission appears as a single broad band, therefore a detailed study of excitation and emission spectra in this region is required to reveal presence of various species (Sikorska et al., 2008a). The excitation and emission spectra of virgin olive oil measured respectively at λ_{em}=330 nm and λ_{ex}=295 nm agree very well with the respective spectra of α-tocopherol, Fig. 4. On the other hand, the excitation and emission spectra measured respectively at λ_{em}=300 nm and λ_{ex}=280 nm are blue shifted as compared to α-tocopherol, being attributed to the phenolic compounds, according to Cheikhousman et al. (2005).

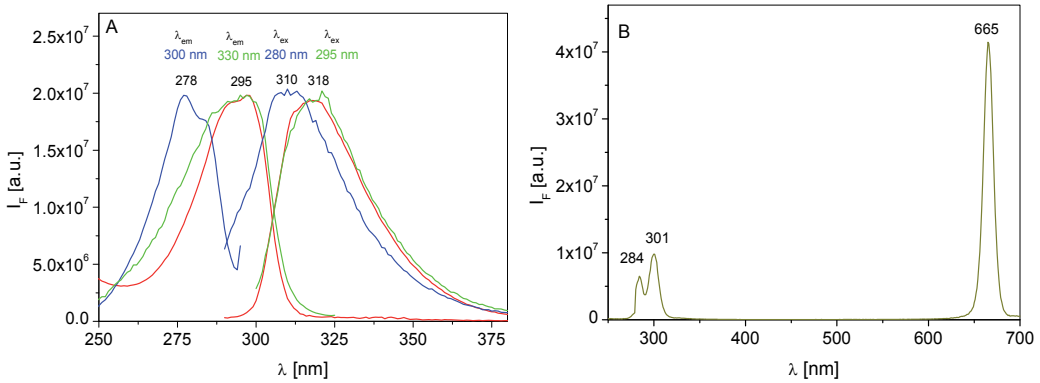

Fig. 4. (A) Excitation spectra (λ_{em}=300 and 330 nm) and emission spectra (λ_{ex}=280 and 295 nm) of extra virgin olive oil (green and blue) and tocopherol (red); the respective excitation and emission wavelengths are shown in the picture; (B) synchronous fluorescence spectrum ($\Delta\lambda$ = 10 nm) of extra virgin olive oil, (Sikorska et al., 2011).

Synchronous fluorescence spectroscopy offers a superior solution to the problem of the overlapping spectral bands, by reducing their spectral widths. The synchronous fluorescence spectrum of virgin olive oil measured at $\Delta\lambda$=10 nm shows two separate bands, with the band at 301 nm attributed to tocopherol. Linear correlation was found between the fluorescence intensity at 301 nm and the total tocopherol concentration as determined chromatographically, for a set of different diluted vegetable oils (1% v/v, in n-hexane) (Sikorska et. al., 2005b).

The band at 284 nm could originate from phenolic compounds such as phenolic aglycons, based on the molecules of tyrosol and hydroxytyrosol, derived from phenolic glycosides present in the olives. Oleuropein aglycons are present in high amounts in virgin olive oil. The synchronous spectrum ($\Delta\lambda$=10 nm) of pure oleuropein exhibits a maximum at about 289 nm and is shifted by about 5 nm as compared to the fluorescence band observed in the virgin olive oil. This shift may result from the solvent, as the phenolic compounds are poorly soluble in nonpolar solvents, the spectrum was recorded in the n-hexane – ethanol mixture. On the other hand, the emission observed in the oil may originate from oleuropein derivatives, with slightly different emission properties (Sikorska et al., 2008).

The band splitting, observed in synchronous spectra only at small $\Delta\lambda$ values, seems to be characteristic for virgin olive oils and has been not observed for refined oils (Sikorska et. al., 2005a). Synchronous fluorescence spectra acquired for virgin olive and sunflower oils at $\Delta\lambda$=20 nm were reported to have one double band at around 275 and 297 nm, and one more band at 660 nm, in contrast to sunflower oil that shows an intense band at around 300 nm and a weak one at 325 nm (Poulli et al., 2006).

The synchronous fluorescence spectra of phenolic compounds (tyrosol, p-coumaric and caffeic acids) present in virgin olive oils were measured at $\Delta\lambda$=30 nm (Dupuy et al., 2005). The spectra present a fluorescence band in the 275-350 nm spectral range, with the maxima for tyrosol and tocopherol observed respectively at 276 and 295 nm. Interestingly, it was shown that the mathematically calculated emission spectrum from a mixture of tyrosol, p-coumaric and caffeic acids, and α-tocopherol was very similar to the experimentally obtained spectrum of olive oil. Therefore, we once more conclude that the fluorescence emission between 275 and 400 nm has contributions from both tocopherols and phenolic compounds (Dupuy et al., 2005).

A long-wavelength band is observed in the olive oil spectra, with excitation at about 350–420 nm and emission at about 660–700 nm, corresponding to the band above 550 nm in total synchronous fluorescence spectra. This band was attributed to pigments of chlorophyll group, based on its excitation and emission characteristics (Zandomeneghi et al., 2005; Diaz et al., 2003). This group includes chlorophylls *a* and *b*, and pheophytins *a* and *b*, derived from chlorophylls by loss of magnesium. The emission spectra of these four chlorophyll derivatives are very similar, with their maxima in the range of 653 to 671 nm in 9:1 acetone/water (Diaz et al., 2003). Their presence is characteristic for virgin olive oils, being reduced to trace amounts in refined oils.

The origin of the emission in the intermediate region for both virgin and refined olive oils is unclear. It seems that various components may be responsible for bands appearing in this region. Wolfbeis & Leiner (1984), suggested that in addition to tocopherols and chlorophylls, parinaric acid, a conjugated 18:4 (n-3) acid, may also contribute to vegetable oil emission.

Recently, fluorescence from conjugated fatty acids including α-eleostearic acid, *cis*-parinaric acid and 8,10,12,14,16-octadecapentaenoic acid, was reported for Borage oil (Smyk et al., 2009). The fluorescence spectrum of methyl ester of the *cis*-parinaric acid has the maximum at 416 nm. It was also suggested that the low intensity emission with the maximum at 524 nm may originate from riboflavin (vitamin B_2) in virgin olive oils. Riboflavin is a polar, water soluble compound and no quantitative data regarding the presence of riboflavin in olive oils are available. This vitamin was found in olive pulp (Zandomeneghi et al., 2005). Additional emission in the intermediate region has been detected as a result of oxidation (Cheikhousman et al., 2005; Poulli et al., 2009a, 2009b; Tena et al., 2009; Sikorska et al., 2008).

2.2 Methods of analysis of fluorescence data

In past decades improvements in both spectroscopic instruments and computers contributed to the extensive application of fluorescence spectroscopy in food analysis, including olive oils. Although even the simple conventional analysis of fluorescence spectra may produce valuable data, most of the successful applications rely on multivariate methods for extracting useful analytical information from the measured fluorescence signals.

According to Christensen et al. (2006), several conditions should be met in an ideal system for fluorescence measurements: 1) the concentration of the fluorophores must be sufficiently low, to be approximately linearly related to the fluorescence intensity, 2) signals from each of the fluorescent components must beindependent of each other, 3) the signal contribution from interfering species must be insignificant compared to the target fluorophore signal. Most of these conditions, however, are routinely violated in intact food samples. Therefore, due to the complex character of the spectra, they are rarely used for direct analysis, being rather used as spectral patterns or fingerprints of particular samples. The vast amount of spectral information contained in such fingerprints could be used in qualitative and quantitative analysis.

Multivariate and multiway methods are specifically suited for treatment of such complex spectral data. The multivariate analysis has several advantages over the univariate approach. It enables analysis of nonselective signals in the presence of spectral interferences, providing diagnostic tools for detection of the outliers. Their application to spectral data has provided important tools for food analysis, where they can be used for exploration, classification and calibration purposes (Christensen et al., 2006).

Traditional multivariate analysis of fluorescence data is performed on a series of emission, excitation or synchronous spectra arranged into a matrix. It starts usually with data exploration that is aimed at discovering structures in the data set, clustering of objects and outlier detection. This analysis does not require any prior knowledge of the explored data, employing unsupervised pattern recognition methods, including principal component analysis (PCA). Other methods used to explore the food fluorescence data include hierarchical cluster analysis, non-negative matrix factorization, common components and specific weights analysis, and canonical correlation analysis (Sadecka & Tothova, 2007).

Three-way models are used for analysis of sets of fluorescence excitation-emission matrices, including parallel factor analysis (PARAFAC) and the Tucker model. The PARAFAC model decomposes the fluorescence data into a number of components. These components correspond to the distinct fluorophores present in the samples. The analysis provides relative concentrations of each of the fluorophores in the mixture, accompanied by the

respective excitation and emission loadings, which correspond to the respective excitation and emission spectra, facilitating identification of the fluorescent constituents. This approach is called mathematical chromatography, enabling qualitative and quantitative analysis of the individual mixture components (Bro, 2003; Christensen et al., 2006).

The analytical problem of the food quality assessment often involves assignment of a particular product to a specific category. To perform such classification, supervised pattern recognition methods are used. In these methods the information about the class membership of the samples in a certain category is used to derive classification rules, which are next applied to classify new samples into correct categories on the basis of patterns present in their measurements (Berrueta et al., 2007). A number of classification techniques were used for analysis of food fluorescence data in the supervised mode: linear discriminate analysis, factorial discriminate analysis, k - nearest neighbors, discriminate partial least squares regression (DPLS), soft independent modeling of class analogy, and artificial neural networks.

Multivariate calibration is the most important and successful combination of chemometrics with spectral data used in analytical chemistry. The calibration consists of building a relationship between a desired chemical, biological or physical property of a sample, and its spectrum. The advantage of such approach is the replacement of the wet chemical measurements of a concentration, which are usually slow and expensive, by the spectral measurements, which are nondestructive and fast, requiring little or no sample preparation and producing no waste chemicals. The multivariate regression methods most frequently used in fluorescence analysis are partial least-squares regression (PLS) and principal component regression (PCR). N-way partial least-squares regression (N-PLS) is used for calibration analysis of fluorescence excitation-emission matrices (Geladi, 2003).

3. Application of fluorescence in olive oil analysis

Application of fluorescence to quality assessment of olive oils was proposed already in the beginning of the 20th century. From 1925, when mercury lamp with the Wood's filter became commercially available, visual observation of oil fluorescence induced by UV light was utilized to detect adulteration of extra virgin olive oils. It was shown that extra virgin olive oils exhibit characteristic yellow fluorescence, due to chlorophylls, while fluorescence of refined oils was blue due to the changes in chlorophyll content during the refining process. This method allowed detecting adulteration of extra virgin olive oils at the level of 5% with refined oils (Sidney & Willoughby, 1929; Glantz, 1930). The use of Wood's lamp was accepted as the U.S. official method for detection of olive oil adulteration. (Kyriakidis & Skarkalis, 2000). The authors of the first papers that reported spectral properties of fluorescence of vegetable oils also point out practical applications of fluorescence spectra as fingerprints in oil analysis (Wolfbeis & Leiner, 1984; Kyriakidis & Skarkalis, 2000).

3.1 Discrimination between quality grades of olive oil

Olive oil is an economically important product and its quality control and detection of possible fraud are of great interest. Olive oils are classified and priced according to acidity. The most expensive is the high-quality extra-virgin olive oil. This oil may be subject of both mislabeling and adulteration. Refined olive oil is obtained from virgin olive oil using

refining methods that do not alter the initial glyceridic structure; pure olive oil (or simply olive oil) consists of a blend of virgin and refined olive oil. The potential of fluorescence to discriminate olive oils of different quality was the subject of several studies. Both total luminescence and synchronous fluorescence spectra combined with various chemometric approaches were successfully used for this purpose.

Scott et al. (2003) used total luminescence spectra of four different types of edible oils: extra virgin olive, non-virgin olive, sunflower and rapeseed oils. The spectra of undiluted oil samples were measured in the excitation range from 350 to 450 nm with 10 nm intervals and in the emission range from 400 to 720 nm with 5 nm interval. Three supervised neural network algorithms were used for sample classification: simplified fuzzy adaptive resonance theory mapping, traditional back propagation and radial basis function. The 100% correct classification was obtained using all methods.

Guimet et al. (2004a) in a series of studies investigated possibility of application of total fluorescence spectra for discrimination between various quality grades of olive oils. The excitation-emission matrices of undiluted oils were measured using right angle geometry. The hierarchical agglomerative clustering method with the Euclidean distance as a similarity measure and the average linkage method were applied to discriminate between three classes of commercial Spanish olive oils (virgin olive oils, pure olive oils, and olive-pomace oils). To optimize the sample grouping into clusters, different preprocessing methods and two spectral ranges were tested, which either included or not the fluorescence peak of chlorophylls. The oils were distinguished using the unfolded excitation–emission fluorescence matrices in the 300-400 nm excitation range and 400-600 nm emission range, thus excluding the chlorophyll band, Fig. 5. The large variations in the chlorophyll band intensity, even between samples of the same type, tend to deteriorate oil discrimination. The optimal preprocessing included normalization of the unfolded spectral excitation–emission fluorescence matrices, followed by column autoscaling. The comparison of the results obtained from the excitation–emission fluorescence matrices to those from a single emission (λ_{ex}=345, 360, 390 nm) and excitation (λ_{em}=410 nm) fluorescence spectrum analysis showed the advantage of the total fluorescence data, which result in a significantly better discrimination.

Other studies used unfold PCA and PARAFAC to explore the excitation–emission fluorescence matrices of virgin and pure olive oils (Guimet, 2004b). The spectral ranges studied were λ_{ex}=300–400 nm, λ_{em}=400–695 nm and λ_{ex}=300–400 nm, λ_{em}=400–600 nm. The first range included chlorophylls, whose peak was much more intense than those of the other components. The second range did not include the chlorophyll peak, being limited to the fluorescence spectra of the oxidation products and vitamin E. The three-component PARAFAC model on the second range (chlorophylls excluded) was found to produce the most useful results. With this model, it was possible to distinguish well between the two groups of oils and to calculate the underlying fluorescent spectra of the three families of compounds. Both unfold PCA and PARAFAC applied to the excitation–emission matrices showed clear differences between fluorescence of the two main groups of olive oils (virgin and pure). Chlorophylls had a strong influence on the models because of their high fluorescence intensity and high variability. Differentiation between the two types of oils was better when the chlorophyll fluorescence region was excluded from the models. The oxidation products are the species that most contribute to the separation between the two

groups. PCA was calculated from the emission spectra of oils between λ_{em}=400 and 695 nm measured at λ_{ex}=365 nm (Guimet, 2004b).

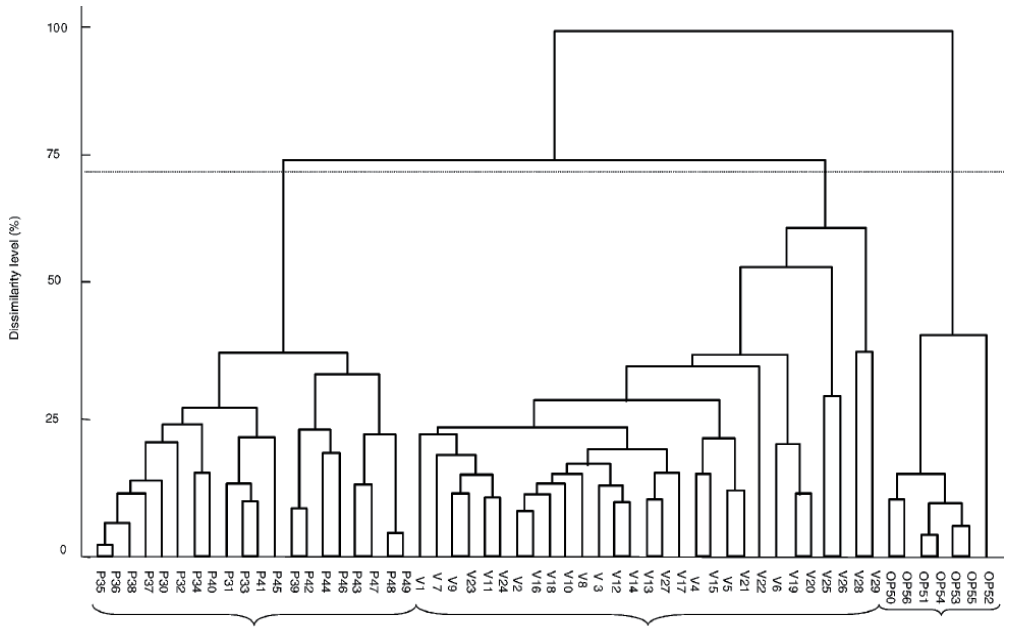

Fig. 5. Dendrogram of the 56 x 861 unfolded matrix (λ_{ex} =300-400 nm, λ_{em} = 400-600 nm) using the Euclidean distance as similarity measure and the average linkage method. The distance is expressed as a percentage of dissimilarity (normalized and autoscaled spectra): virgin V, pure, P; and olive-pomace, OP oils. Reprinted with permission from Guimet et al., 2004a. Copyright 2004, American Chemical Society.

Non-negative matrix factorization with Fisher's linear discriminant analysis were applied for discriminating between different types of olive oils: 1) discrimination between commercial Spanish olive oils of different quality (virgin, pure, and olive-pomace oil); 2) discrimination between virgin oils from two ''Siurana'' (Protected Denomination of Origin) regions; 3) discrimination between the original ''Siurana'' virgin olive oils and oils adulterated with olive-pomace oil at 5% (w/w) level (Guimet et al., 2006). In all cases, classifications at above 90% confidence were achieved. The proposed method was also compared to PARAFAC and discriminant N-PLS regression. The classification results were better with non-negative matrix factorization than PARAFAC for two data sets out of three. Non-negative matrix factorization combined with Fisher's linear discriminant analysis was also comparable with discriminant N-PLS regression, giving better classifications for the second data set, but slightly worse results for the other two. The main advantage of non-negative matrix factorization with respect to discriminant N-PLS regression is that its basis functions are more interpretable than the regression loadings, because they are positive and correspond to parts of the spectra than can be directly related to the fluorescent components of oils.

The fluorescence excitation-emission matrices (λ_{ex}=300-390 nm and λ_{em}=415-600 nm) were used in studies of the Spanish extra virgin, virgin, pure, and olive pomace oils, to investigate the relationship between oil fluorescence and the conventional quality parameters,

including peroxide value, K_{232}, and K_{270} (Guimet et al., 2005c). Multiway methods were applied to the data analysis: PARAFAC with multi-linear regression and N-PLS regression. Better regression fits and lower prediction errors were obtained using N-PLS. The best results were obtained for prediction of K_{270}. The detection of extra virgin olive oils was highly degraded at early stages (with high peroxide value) and little oxidized pure olive oils (with low K_{270}).

Synchronous fluorescence spectroscopy combined with multi-dimensional chemometric techniques was applied to the classification of virgin olive oils according to their quality by Poulli et al. (2005). They studied the fluorescence of virgin olive oils and lampante oils, using total fluorescence, synchronous and total synchronous fluorescence spectra. Total luminescence spectra recorded in the 350–720 nm range while exciting in the 320 to 535 nm range showed different shapes and intensities for the two classes of oils. Lampante olive oil had a broad emission with its maximum at 450-500 nm in addition to the 685-690 nm peak. Total synchronous fluorescence spectra measured at $\Delta\lambda$=20-180 nm had emission peaks between 500-700 nm, depending on $\Delta\lambda$, for both classes of oils. However, lampante oil had additional fluorescence in the of 360–500 nm range, which is not observed for edible virgin olive oil. Classification of virgin olive oils based on their synchronous fluorescence spectra ($\Delta\lambda$=80 nm) was performed by hierarchical cluster analysis and PCA using the 429–545 nm spectral range. The authors conclude that the fluorescence in the 429–545 nm range, which they used for data analysis, originates from oleic acid. PCA provided 100% correct discrimination between the two classes, while hierarchical cluster analysis allowed 97.3% correct classification.

3.2 Adulteration detection of olive oils

A few papers were published in recent years on the use of fluorescence to assess adulteration detection of virgin olive oils. Adulteration of virgin olive oils has been a common fraud practice that involves addition of cheaper oils, including olive oils of lower quality or other plant oils. The most common adulterants found in virgin olive oil are refined olive oil, pomace oil, residue oil, synthetic olive oil–glycerol products, seed oils, and nut oils. The current analytical standards for olive oil enable detection of the presence of almost all of the possible adulterants; however, they require the measurement of several parameters established by the EU Regulations: (EEC) No 2568/91 and (EC) No 796/2002.. Thus, rapid methods to detect olive oil adulteration are important for quality control purposes (Karoui & Blecker, 2011).

Hazelnut oil is chemically similar to virgin olive oil; its presence is difficult to detect at low concentration levels using standard methods. A different approach was tested to detect this type of adulteration using fluorescence (Sayago et al., 2004; Sayago et al., 2007). The emission spectra of undiluted olive oil mixtures with virgin and refined hazelnut oils with excitation at 350 nm were measured (Sayago et al., 2004). The spectra were subjected to mathematical treatment by calculation of the first derivative. One-way analysis of variance was used for the selection of suitable wavelengths to differentiate oil samples. The response to the addition of adulterant, as evaluated by multiple linear regression, was linear for virgin olive and refined hazelnut oil mixtures (R^2=0.99), and for virgin olive and virgin hazelnut oil mixtures (R^2=0.98). Stepwise linear discriminant analysis used to discriminate genuine from adulterated olive oil samples allowed 100% correct classifications for each

mixture separately, and also for the entire set of samples. Another work explored application of the fluorescence spectroscopy to differentiate between refined hazelnut and refined olive oils (Sayago et al., 2007). Classification of these oils based on their excitation (in 300-500 nm spectral range, using λ_{em}=655 nm) and emission spectra (in the 650-900 nm range, using λ_{ex}=350 nm) was performed, using PCA and artificial neural networks. Both methods provided good discrimination between the refined hazelnut and olive oils. Using the artificial neural networks model, the presence of refined hazelnut oils in refined olive oils was robustly detected at levels exceeding 9%.

Several studies devoted to the detection of of adulteration of virgin olive oil with sunflower oil. Poulli et al. (2006) applied total synchronous fluorescence to differentiate virgin olive from sunflower oil and synchronous fluorescence combined with PLS regression for quantitative determination of olive oil adulteration. Total synchronous fluorescence spectra were acquired in the 270–720 nm range, using the wavelength interval variable from $\Delta\lambda$=20 to 120 nm. The emission band at around 660 nm was only observed in virgin olive oil, attributed to pigments of the chlorophyll group. For sunflower, in contrast to virgin olive oil, a fluorescence band in the 325–385 nm excitation range is observed. This band was attributed to linoleic acid, however, there are no published data on fluorescence of this compound. In contrast, virgin olive oil has only small signals in this range if scanned at 30 to 50 nm wavelength interval. Synchronous fluorescence spectra of virgin olive oil recorded at $\Delta\lambda$=20 nm show a double band at 275 and 297 nm and a single band at 660 nm, in stark contrast to sunflower oil that has an intense band at around 300 nm and a weak one at 325 nm. For quantification of the adulteration, the PLS regression model was used for analysis of synchronous fluorescence spectra of mixtures of virgin olive oil and sunflower oil at $\Delta\lambda$=20 and 80 nm, Fig. 6. The detection limits were 3.6% and 3.4% (w/v) when using the 20 and 80 nm wavelength intervals, respectively.

The potential of fluorescence spectroscopy for detecting adulteration of extra virgin olive oil with olive oil has been investigated recently (Dankowska & Małecka, 2009). Synchronous fluorescence spectra were collected in the 240–700 nm range, using $\Delta\lambda$=10, 30, 60 and 80 nm. A narrow band at around 300 nm appeared in the synchronous fluorescence spectrum at $\Delta\lambda$=10 nm, attributed to tocopherols, and an intense band with a peak at around 665 nm, attributed to compounds of the chlorophyll group. The raw spectra were subject to calculation of the first and second derivatives to find the maximum or the intersection point. Five wavelengths at each of the wavelength intervals were chosen for further analysis. Multiple regression analysis was applied separately to the data acquired at each of the wavelength intervals. The ability to detect olive oil in extra virgin olive oil was better at the wavelength interval of 60 or 80 nm, rather than 10 or 30 nm. Using the spectra acquired at 60 and 80 nm wavelength intervals, the lowest detection limits of adulteration were 8.9% and 8.4% at 350 and 302 nm, respectively.

Fluorescence was used to detect adulteration of virgin olive with others oils (Poulli et al., 2007). Synchronous fluorescence spectra of virgin olive, olive-pomace, corn, sunflower, rapeseed, soybean and walnut oils at 20 nm wavelength interval were used for analysis. Virgin olive oil shows a double band in the 275–297 nm range and a single band at 660 nm, in contrast to other oils that show a strong band around 300 nm and a weak to moderate band near 325 nm. Total synchronous fluorescence spectra were acquired for the excitation wavelength in the 250–720 nm range and the wavelength interval $\Delta\lambda$ in the 20 to 120 nm range. Total synchronous

fluorescence spectra for olive oils show a spectral band at around 660 nm, attributable to pigments of chlorophyll group. Moreover, all studied oils save the virgin olive oil show a band at above 315 nm when using $\Delta\lambda$=20 nm. This band could be attributed to linoleic acid. It has been suggested that differentiation of virgin olive oil from low quality oils can be achieved using this wavelength region. The PLS regression model was used to quantify adulteration using 20 nm synchronous fluorescence spectra. This technique enabled detection of olive-pomace, corn, sunflower, soybean, rapeseed and walnut oil in virgin olive oil at levels of 2.6, 3.8, 4.3, 4.2, 3.6, and 13.8% (w/w), respectively (Poulli et al., 2007).

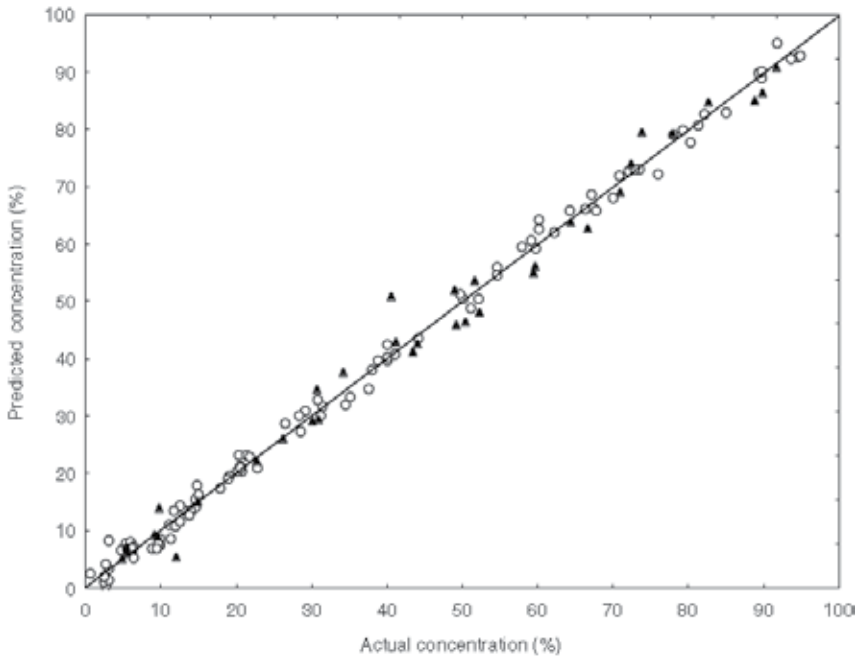

Fig. 6. Predicted versus actual concentrations of sunflower oil in virgin olive oil at a wavelength interval of 80 nm: open circles - calibration samples, filled triangles - validation samples. Reprinted with permission from Poulli et al., 2006. Copyright 2006, Springer.

For adulteration detection of extra virgin olive oil with rapeseed oil a neural network method – a simplified fuzzy adaptive resonance theory mapping - was found to be very efficient, resulting in a total of 99.375% correctly classified oil samples at the 5% v/v adulteration level (Scott et al., 2003). It was shown for extra virgin olive oil adulterated by rapeseed oil that the percentage of adulteration may be described by either a radial basis functional network (2.435% RMSE) or a simple Euclidean distance relationship of the PCA scores (2.977% RMSE).

3.3 Authentication of virgin olive oils geographical origin

Classification of virgin olive oils according to variety and/or geographical origin is of great importance for the producers, importers, and consumers. Dupuy et al. (2005) developed a method to discriminate olive oils according to their geographic origin. Samples of virgin olive oil from five French registered designations of origin (RDOs), namely, Nyons, Vallé des Baux, Aix-en-Provence, Haute-Provence, and Nice, were discriminated by applying multivariate

regression methods to synchronous fluorescence spectra of oils. The synchronous fluorescence spectra were collected in the 250-700 nm range at a constant wavelength difference of 30 nm between the excitation and emission wavelengths. The PLS regression analysis of synchronous fluorescence spectra allowed to determine the origin of the oils with satisfactory results, despite the similarity between two denominations of origin (Baux and Aix) that are composed by some common cultivars (Aglandau and Salonenque). The interpretation of the regression coefficients shows that RDOs are correlated to chlorophylls, pheophytins, tocopherols, and phenolic compounds, present in different amount for each origin (Dupuy et al., 2005).

Guimet et al. (2005a) developed a method based on excitation-emission matrices and three-way methods for detecting adulterations of pure olive oils in extra virgin olive oil from the protected denomination of origin (PDO) "Siurana", which is a prestigious distinction given to the extra virgin olive oils produced in a specific area in the south of Catalonia, Spain. Excitation and emission ranges were λ_{ex}=300–390 nm and λ_{em}=415–600 nm, respectively. Unfold PCA and PARAFAC were used for exploratory analysis. Discrimination between non-adulterated and adulterated samples was performed using Fisher's linear discriminant analysis and discriminant N-PLS regression. Using discriminant N-PLS regression, 100% correct classification was obtained. Adulteration at around 5% level was quantified, with a prediction error of 1.2% (Guimet et al., 2005a). In another study, Guimet et al. (2005b) achieved discrimination between oils from the two PDO "Siurana" regions by means of discriminant unfold PLS regression, giving correct classification for 94% of samples for "Siurana-Camp" and 100% for "Siurana-Montsant" oils.

3.4 Quantification of fluorescent components in olive oils

The olive oil autofluorescence is attributed to minor components, species such as tocopherols, phenols and chlorophylls, thus fluorescence spectroscopy has been used to analyze these compounds in olive oils. Diaz et al. (2003) used fluorescence for determination of chlorophylls *a* and *b* and pheophytins *a* and *b* in olive oil samples. The analysis was accomplished by PLS multivariate calibration using the three types of spectra (excitation, emission, and synchronous spectra of these solutions). The best results were obtained for the excitation spectra. The optimum wavelength range to record the excitation spectra (λ_{em}=662 nm) was selected to minimize the contribution of pheophytin *a* and to maximize the contribution of the other pigments, which are the minor constituents in olive oil. To perform the PLS calibration, a set of samples with final concentration ranges varying from 140 to 560 ng mL^{-1} for pheophytin *a*, from 10 to 40 ng mL^{-1} for chlorophyll *a* and pheophytin *b*, and from 20 to 80 ng mL^{-1} for chlorophyll *b* was used as the calibration matrix. The oil samples were diluted in acetone. Recovery values from olive oil, spiked with chlorophylls *a* and *b* and pheophytins *a* and *b*, were in the ranges of 70-112, 71-111, 76-105, and 82-109%, respectively.

Fluorescence was proposed as an alternative to the Folin-Ciocalteu assay for estimation of the total phenol content in virgin olive oil, olive fruit and leaf polar extracts (Papoti & Tsimidou, 2009). Phenol content in olive oils was determined by measuring the fluorescence intensity of methanol/water extract, with the excitation/emission wavelengths set at 280/320 nm. The method was shown to be more sensitive (limit of detection and limit of quantification values 10-fold lower) and three times faster then the Folin-Ciocalteu assay. Good correlation was found with the results of colorimetric assay ($r = 0.69$, $n = 65$) for virgin olive oil extracts.

Fluorescence combined with PLS regression was used to determine tocopherol homologues (α-, β-, γ-, and δ- tocopherol) in the quaternary mixture and the oils (Diaz et al., 2006). The calibration set that included mixtures of tocopherols dissolved in hexane: diethyl ether (70:30 v/v) was constructed based on the central composite plus a full factorial plus a fractionated factorial design. PLS regression was applied to analyze matrices of fluorescence excitation and emission spectra and with fluorescence excitation, emission, and synchronous spectra. For analysis of synthetic samples, recoveries around 100% were obtained. For the analysis of the oils, the samples were diluted in hexane, cleaned in silica cartridges and then tocopherols were eluted with hexane:diethyl ether (90:10 v/v). The method was applied to different edible oils giving satisfactory results for α-, β-, and γ-, but not for δ- tocopherol.

PLS regression was utilized to develop calibration models between front face and right angle synchronous fluorescence spectroscopy for the characterization of edible oils and total tocopherol content as determined by HPLC (Sikorska et al., 2005b). The studies were performed on commercially available edible oils: olive, grapeseed, rapeseed, soybean, sunflower, peanut, and corn oils were analyzed. The regression models showed a good ability to predict tocopherol content. The best fitting results were obtained for 1% v/v diluted oils and for bulk samples using the entire spectrum, yielding the regression coefficient of 0.991, and the root mean square error of cross-validation of 8%.

3.5 Monitoring thermal and photo-oxidation of olive oils

The studies of thermal deterioration of oils are important because changes during oxidation involve degradation of oil constituents and formation of new products that alter quality attributes and nutritional profile, as the oxidation products are potentially toxic. Fluorophores in olive oils are compounds that can participate in oxidation, thus fluorescence spectroscopy can serve as a tool for better understanding of oil oxidation. The fluorescence was compared to other spectroscopic techniques (NIR/VIS, FT-IR and FT-Raman) and chemical and physical methods in determining the deterioration of frying oils, collected from a commercial Chinese spring roll plant, (Engelsen, 1997). Fluorescence has been measured by using five selected excitation wavelengths varying from 395 to 530 nm. Data analysis was performed using PCA and PLS regression. Overal, fluorescence provided the best models for the anisidine value, oligomers, iodine value, and vitamin E concentration, among the spectroscopic techniques used.

Fluorescence spectrometry and PLS regression were used as a rapid technique for evaluating the quality of heat-treated extra virgin olive (Cheikhousman et al., 2005). Two commercial extra virgin olive oils were heated at 170°C for 3 h. Changes in excitation spectra were correlated with changes in concentrations determined by other methods. The fluorescence excitation band emitting at 330 nm was attributed to vitamin E and some fluorescent polyphenols. This fluorescence decreased during the heating process, with the exponential decay constant similar to that obtained chromatographically. Fluorescence excitation spectra with the emission wavelength at 450 nm were inversely correlated with the hydroperoxide content of oil. Indeed, the degradation products generated during heating, particularly the compounds formed by reaction between amino-phospholipids and aldehydes, fluoresce in this wavelength range.

Thermal deterioration of extra virgin olive oils was studied by Tena et al. (2009). The sample of virgin olive oil was heated at 190°C for 94 h in cycles of 8 h per day. The fluorescence intensity in the spectral region between 290 and 400 nm decreased during the oxidation and

a bathochromic shift of the maximum from 350-360 to around 420-440 nm was observed. The fluorescence observed in the 300-390 nm range was assigned to tocopherols together with polyphenols; the information collected from the spectra was compared to the results of the HPLC analysis of these compounds. The observed changes in the spectral profile were explained by the decrease of the tocopherols and phenols and the increase of the oxidation products of vitamin E homologues correlated to K_{232} and K_{270}, and hydrolysis products. The intensity of the band between 630 and 750 nm, associated with chlorophylls and pheophytins, decreased exponentially with the thermal oxidation time.

The fluorescence intensity recorded at 350 nm and at the wavelength of the spectral maximum occurring in the range of 390-630 nm allowed to explain the increase of the percentage of polar compounds during the experiment. It was stated that the spectra of the undiluted heated oils with maxima at 490 nm or higher correspond to polar compounds exceeding 25%, which is the maximum percentage acceptable for edible oils used in frying.

Poulli et al. (2009b) studied the effect of heating to 100, 150 and 190° C on extra virgin olive, olive pomace, sesame, corn, sunflower, soybean, and a commercial blend of oils. The changes in fluorescence were assessed by measuring total synchronous fluorescence spectra, in the 250-720 nm excitation range, with the wavelength interval, $\Delta\lambda$, from 20 to 120 nm at 20 nm step. The synchronous fluorescence intensities below 315 nm recorded at $\Delta\lambda$=80 nm decreased during heating, presumably due to the consumption of phenolic antioxidants by the lipid radicals generated. The decrease of the fluorescence bands in the 250-350 and 350-400 nm ranges for extra virgin olive and olive pomace oil, respectively, was in accordance with the percentage of trolox equivalent antioxidant capacity reduction. The bands in the total synchronous fluorescence spectra at below 350 nm disappeared during heating, with those at 600-700 nm also decreasing, probably due to the decay of antioxidant compounds and chlorophyll, respectively. The bands in the 400-450 nm range increased, probably due to the formation of secondary oxidation products. PCA of synchronous fluorescence spectra obtained at $\Delta\lambda$=80 nm allowed oil discrimination according to the degree of oxidation. For extra virgin olive, olive pomace, and sesame oil the spectral range of 300-500 nm was used for classification, while the 320-520 nm range was more appropriate for corn, soybean, and sunflower oil, and a commercial blend of oils. Spectroscopic changes are indicative of oxidative deterioration as measured through wet chemistry methods: peroxide value, p-anisidine value, totox value, and radical-scavenging capacity (Poulli, 2009a).

Extra virgin olive oil is very stable in the dark; it is susceptible to oxidation under UV light. An accelerated thermal and photooxidation under UV light was studied by Poulli et al. (2009a, 2009b) on samples of extra-virgin, regular-quality and pomace olive oils. Synchronous fluorescence spectra were collected using the 250–720 nm excitation range at $\Delta\lambda$=80 nm. Extra virgin olive oil bands in the 300–330 nm range decreased during oxidation, while the fluorescence in the 350–550 nm range increased during the initial 8 h and then remained almost constant for up to 12 h. Regular quality olive oil exhibited fluorescence in the 300–550 nm range. The bands in the 300–370 nm range decreased during oxidation, whereas fluorescence bands in the 370–550 nm range increased during the initial period and remained almost constant afterwards. Also, the fluorescence bands of pomace oil in the 350–550 nm range decreased during the initial period of the experiment and then a small additional increase was observed. All olive oils show fluorescence bands in the 550–700 nm range, attributed to chlorophyll pigments, intensive in extra virgin olive oil and with very low intensity in olive-pomace oil. These bands decreased significantly due to deterioration

of the chlorophyll pigments involved in photo-oxidation. Total synchronous fluorescence spectra were obtained by scanning the excitation wavelength in the same spectral range and changing the wavelength interval from 20 to 120 nm at 20 nm steps. These spectra showed considerable changes during oxidation for all of the oils studied, Fig. 7. Fluorescence intensity in the 600–720 nm range, attributed to chlorophylls, decreased significantly. In contrast, the fluorescence bands in the low-wavelength range expanded up to 590 nm. PCA applied to the synchronous fluorescence spectra recorded at $\Delta\lambda$=80 nm in the 300–500 nm range reveals five different classes of oils depending on their oxidation degree.

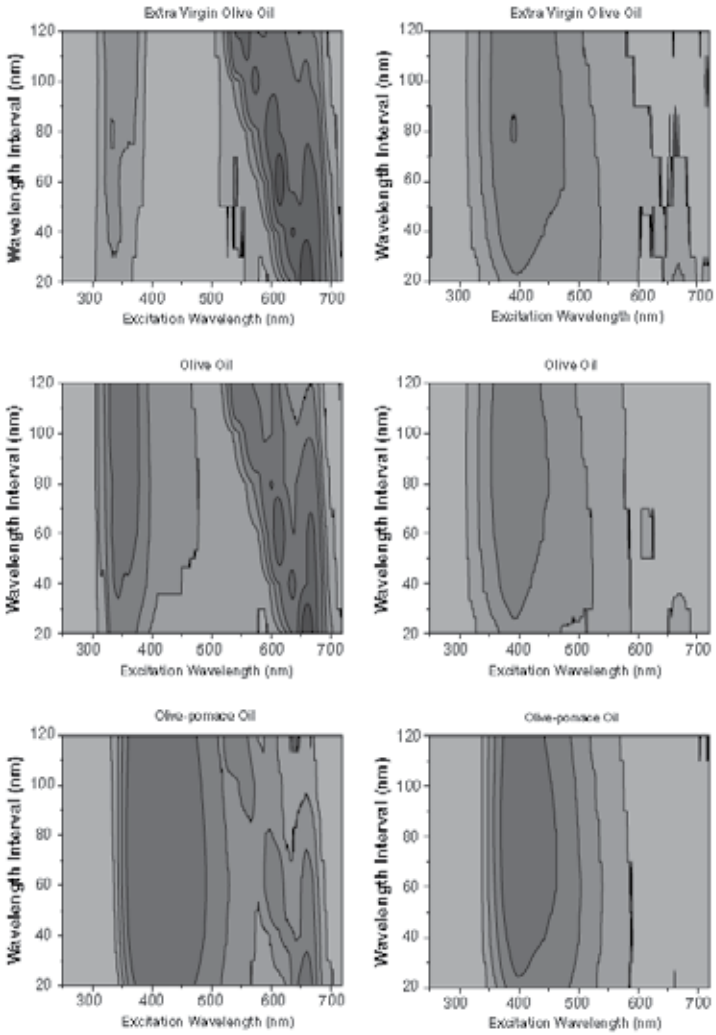

Fig. 7. Total synchronous fluorescence spectra: contour plots of olive oils before (left) and after 12 h (right) exposure to UV light at 80°C. Grayscale indicates fluorescence intensities. Reprinted from *Food Chemistry*, (2009) Vol.117, No.3, Poulli K. I.; Mousdis G. A. & Georgiou C. A., "Monitoring Olive Oil Oxidation Under Thermal and UV Stress Through Synchronous Fluorescence Spectroscopy and Classical Assays", pp. 499-503, Copyright (2009), with permission from Elsevier.

An interesting study on thermal oxidation of extra virgin olive oils has been published recently (Navarra et al., 2011). Among different experimental techniques (including FTIR and rheology) time-resolved luminescence was used to investigate early steps of the thermally induced oxidative process. The oxidation process was followed at three different heating temperatures (30, 60 and 90°C) as a function of time for up to 35 days. The chlorophyll fluorescence lifetime increased from 6.0 ± 0.1 ns, measured before, to 6.3 ± 0.1 ns, measured after 35 days of experiment. These changes were in agreement with the observed viscosity rise, resulting from formation of polar molecules with propensity to form hydrogen bonds. The viscosity increase reduced the frequency of collisions between the chromophore and its environment, consequently lowering the non-radiative contribution to the luminescence decay.

3.6 Assessing quality changes of olive oil during storage

Fluorescence spectroscopy was applied to monitoring changes in virgin olive oil during storage (Sikorska et al. 2008b). The extra virgin olive oil samples were stored for the period of 12 month in different conditions: in clear and green glass bottles exposed to light, and in darkness. Changes occurring in olive oil during storage were assessed by total fluorescence and synchronous scanning fluorescence spectroscopy techniques. In the total fluorescence spectra the intensity of emissions ascribed to tocopherols and chlorophyll pigments decreased during storage, depending on the storage conditions. Additional bands appeared in oils exposed to light in the intermediate range of excitation and emission wavelengths. Bands attributed to tocopherols, chlorophylls and those tentatively ascribed to phenolic compounds were observed in the synchronous scanning fluorescence spectra, allowing monitoring of the storage effects on these constituents. PCA of the synchronous fluorescence spectra revealed systematic changes in the overall emission characteristics dependent on the storage conditions, such as exposure to light, and packaging, Fig. 8.

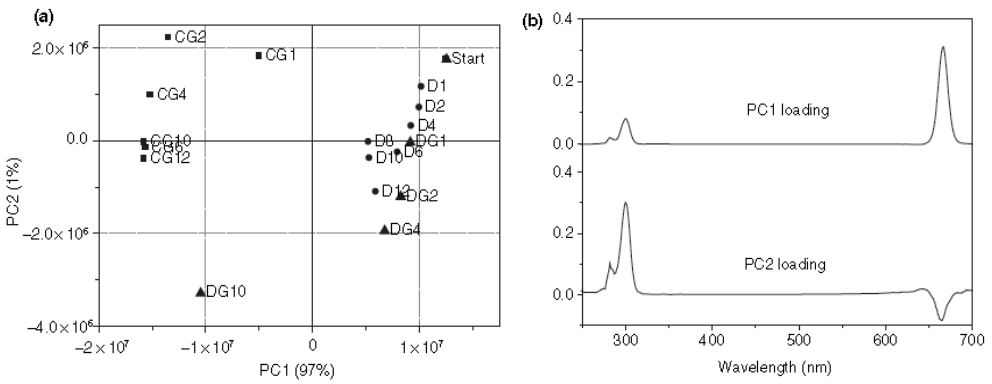

Fig. 8. (a) Scores plot for the two most significant principal components, PC1 vs. PC2, of a PCA of the synchronous scanning fluorescence (SSF) spectra (Δλ = 10 nm) of virgin olive oil samples. A nonstored sample (Start), and samples stored in different conditions: in darkness (D), in green glass bottles (DG) and in clear glass bottles (CG). The samples are numbered according to the months of storage. The values in brackets describe the fraction of the total variation explained by each of the PC. Each point represents an average of the spectra obtained from three replicates . (b) One-vector loading plots for the PC1 and PC2 principal components. Reprinted with permission from Sikorska et al., 2008b. Copyright 2008, John Wiley and Sons.

4. Conclusions

Fluorescence spectra of olive oils contain information about fluorophores (tocopherols, phenolic compounds, and chlorophylls) that are important for oil quality. The spectra may be used to monitor either selected constituents or to determine overall sample characteristics, which may serve as the spectral fingerprint. The analytic potential of fluorescence is enhanced by application of multivariate data analysis methods for the analysis of spectra.

The review of literature data demonstrates that fluorescence measurements conducted directly on olive oil samples with subsequent multivariate data analysis can be efficiently used for qualitative and quantitative analysis as a valid alternative to conventional, chemical methods of quality assessment. These methods can be used for oil discrimination and for quantitative determination of fluorescent components after an appropriate calibration. Further studies are needed to resolve various issues that are important for practical application of the fluorescence techniques, among which are the method verification for specific kinds of oil and identification and quantification of other fluorescent oil constituents.

5. Acknowledgment

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5

Metal Determinations in Olive Oil

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1. Introduction

It is widely known that trace metals have negative effects on the oxidative stability of olive oil. Natural composition of olive fruit, natural contamination from soil, fertilizers, industrial applications or highways near the plantations are the main sources of metals in olive oils. The olive oil may also be contamined with the metals during the production process and contact with storage materials. The level of trace metals in olive oil is one of the quality parameters and also effective on oil oxidation and human health. Oxidation leading to the development of unfavorable odours and taste is one of the major reasons of deterioration of olive oils**.** The factors that most affect the rate of oxidation are the degree of unsaturation, the amount of oxygen, temperature, light and the presence of metals (mainly transition metals such as Fe and Cu) (Meira et al., 2011; Sikwese & Duodu, 2007). The trace metals enhance the rate of oxidation of edible oils by increasing the generation of free radicals from fatty acids or hydroperoxides. Benedet & Shibamoto observed that trace amounts of Fe, Cr, Pb and Cd contribute oxidative effects to lipid peroxidation (Benedet & Shibamoto, 2008).

The determination of metals has been a difficult analytical problem because of the hard organic content of the oil matrix. The analytical techniques used for metal determinations in oils are both emission and absorption spectrophotometry. ICP-OES (Allen et al., 1998; Angioni et al., 2006 ; Anthedimis et al., 2005; Costa et al., 2001; De Souza et al., 2005; Murillo et al., 1999; Zeiner et al., 2005), FAAS (Batı & Cesur, 2002; Carbonell et al., 1991; Köse Baran & Bağdat Yaşar, 2010; Nunes et al., 2011), ETA-AAS (Karadjova et al., 1998; Kowalewska et al., 1999 ; Zeiner et al., 2005), GFAAS (Allen et al.,1998 ; Ansari et al., 2009; Calapaj et al., 1988; Chen et al., 1999; Cindric et al., 2007; De Leonardis et al., 2000; Hendrikse et al., 1988, 1991; Lacoste et al., 1999; Martin-Polvillo et al., 1994 ; Matos Reyes & Campos, 2006; Mendil et al., 2009; Nash et al., 1983; Van Dalen, 1996), and ICP-MS (Benincasa et al., 2007 ; Bettinelli et al., 1995; Llorent-Martinez et al., 2011a, 2011b; Pereira et al., 2010; Wondimu et al., 2000) are the most commonly used techniques for the determination of metal contents in oils (Duyck et al., 2007).

In this chapter, recent determination techniques and sample pretreatment methods have been described and compared with each other. Additionally, a novel metal extraction procedure has been introduced in detail. In recent years, scientists have been interested in defining the bioavailable amount of metals more than total metal concentration. Taking into account this, fractionation and speciation analysis of metals in oil samples have also been discussed in the chapter.

2. Sample pre-treatment

The accurate determination of trace metals in olive oil is an analytical challenge due to their low concentration and the difficulties that arise because of the high organic content. Due to the high organic content, sample pretreatment is a critical step and frequently necessary in olive oil analysis. Sample pretreatment step provides the decomposition of organic matrix or the extraction of metals without matrix decomposition. On the other hand, oil sample can be diluted in a suitable solvent or emulsified with an appropriate emulsifier in a rapid pretreatment for direct determinations. The atomic spectrometers are the most commonly used devices but have some problems such as the reduced stability of the analytes in the solution, requirement of organometallic standards, the use of dangerous organic solvents or sample digestion with an acid or acid mixture (Nunes et al., 2011).

2.1 Acid digestion

Digestion procedures are regularly carried out with either open vessels using acid, acid mixture or basic reagents on hot plates or open- and closed-vessel microwave ovens. The decomposition in open system is hard, time consuming and prone to systematic error sources, i.e. contamination or analyte losses. In case of using microwave radiation, the high cost of instrumentation and dilution of the sample can be considered as disadvantages in the microwave assisted digestion system. Although the amount of sample in vessels is limited due to the generation of gaseous reaction products that can increase of pressure, the use of closed high-pressure vessels is appropriate for efficient sample digestion. On the other hand, in the use of open-focused microwave ovens, the advantages are decreasing the risk to the operator, possible introduction of reagents during procedure, opportunity to digest larger amounts of sample and low cooling time (Sant'Ana et al., 2007).

Microwave-assisted digestion has been performed to dissolve the oil sample for elemental analysis in a large number of papers (Angioni et al., 2006; Ansari et al., 2009; Levine et al., 1999; Llorent-Martinez et al., 2011a, 2011b; Mendil et al., 2009; Sant'Ana et al., 2007), while focused microwave assisted digestion for the same purpose has been employed in a few papers (Sant'Ana et al., 2007). As shown in Table 1, some investigation have been done on microwave digestion for olive oil using various procedures.

2.2 Dry ashing

In general, ashing methods may provide lower analyte recovery and exhibit poorer accuracy compared to acid digestion methods. Although dry ashing procedures are effective, they are time consuming and can often result in loss of analyte species that could occur during the preparation of the sample. Oil is decomposed by high-temperature dry ashing, subsequently the ash is dissolved in an acidic aqueous medium and the metal content of the aqueous phase can be measured by various detection techniques such as AAS, adsorptive stripping voltammetry (AdSV) and derivative potentiometric stripping analysis (dPSA) (Abbasi et al., 2009; Carbonell et al., 1991; Lo Coco et al., 2003). There are limited researches for metal determinations in oils after dry ashing of olive oil (Lo Coco et al., 2003).

Microvawe digestion procedure	Reagent	Metals determined	Reference
130 °C 10 min. 140 psi, 150 °C 10 min. 200 psi, 10 min. cooling, 160 °C 20 min. 200 psi	HNO_3	Mg, Ca, Cr, Fe, Mn, Cu, Ni, Zn, Cd, Pb	Bağdat Yaşar & Güçer, 2004
2 min. for 250 W, 2 min. for 0 W, 6 min. for 250 W, 5 min. for 400 W, 8 min. for 550 W, vent.: 8 min.	HNO_3–H_2O_2	Fe, Mn, Zn, Cu, Pb, Co, Cd, Na, K, Ca, Mg	Mendil et al., 2009
250 W 2 min., 0 W 1 min., 250 W 2 min., 600 W 1 min., 400 W 5 min., vent.: 3min.	HNO_3–H_2O_2	Ca, Fe, K, Mg, Na, Zn, Al, Co, Cu, Mn, Ni, Cr, Pb	Cindric et al., 2007
25_i-90_f °C 5 min. 700 W, 90_i-90_f °C 3 min. 600 W, 90_i-170_f °C 10 min. 600 W, 170_i-170_f °C 7 min. 600 W	HNO_3	Ag, As, Ba, Be, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Sb, Ti, Tl, V	Llorent-Martinez et al., 2011a, 2011b
300 W (83%) for 15 min., 600 W (75%) for 10 min., 1200 W (65%) for 15 min., 300 W (83%) for 5 min.	HNO_3–H_2O_2	Cd, Cu, Pb, Zn	Angioni et al., 2006
750 W 90 °C 6 min., 750 W 90 °C 4 min., 1000 W 180 °C 8 min., 1000 W 180 °C 15 min. (35 bar), vent.: 20 min	HNO_3–H_2O_2	Cu, Fe, Ni, Zn	Nunes et al., 2011
250 W, 4 min., 0 W 4 min., 250 W 5 min., 400 W 7 min., 700 W 6 min., 350 W 5 min.	HNO_3	Be, Mg, Ca, Sc, Cr, Mn, Fe, Co, Ni, As, Se, Sr, Y, Cd, Sb, Sm, Eu, Gd	Benincasa et al., 2007

i: initial ; f: final

Table 1. The summary of microwave digestion procedures for various metals in olive oil.

2.3 Extraction

Sample preparation involves acid extraction (Anwar et al., 2004; De Leonardis et al., 2000; Dugo et al., 2004; Jacob & Klevay, 1975), solid phase extraction (SPE) (Batı & Cesur, 2002) or extraction with special agents (Köse Baran & Bağdat Yaşar, 2010).

After the extraction of metals from oil with nitric acid, hydrochloric acid or acid mixture, the extracts are analyzed. Despite the fact that extraction method has the same advantage both in the separation and preconcentration of metals in oil samples, the recoveries are not satisfactory for many metals in most cases. Batı and Cesur described another method for the preconcentration and separation of copper in edible oils, based on using a solid Pb-piperazine-dithiocarbamate complex for extraction and a potassium cyanide solution for back extraction (Batı & Cesur, 2002).

Anwar et al. reported a simple acid-extraction method for the determination of trace metals in oils and fats. The method has been performed with the use of ultrasonic intensification and successfully applied for accurate determination of iron, copper, nickel and zinc in oils (Anwar et al., 2004). Many extraction procedures are available in literature, the summary of these is given in Table 2.

Extraction method	Metals determined	Detection technique	Notes	Reference
Extraction with 10% HNO_3	Fe, Cu	GF-AAS	[1]Acc.% 94±23 (Cu); 97±12 (Fe)	De Leonardis et al., 2000
Extraction with CCl_4 + 2 N HNO_3 (ultrasonic intensification)	Fe, Cu, Ni, Zn	FAAS	[2]Rec.% 92-98 (Fe); 91-100 (Cu); 92-97 (Ni); 93-101 (Zn)	Anwar et al., 2004
Extraction with 35% H_2O_2 and 36% HCl (30 min., 90 ˚C)	Cd, Cu, Pb, Zn	dPSA	Rec.% 96.5±2.1 (Cd); 97.0±2.7 (Cu); 95.0±1.8 (Pb); 93.5±1.7 (Zn)	Dugo et al., 2004
Extraction with conc. HCl	Cu	Ad-SSWV	[3]LOD: 0.49 ng mL^{-1}	Galeano Diaz et al., 2006
Extraction with conc. HNO_3 and 6% H_2O_2	Cu, Ni	UV-Vis spec.	Rec.% 90-118 (Cu); 96-100 (Ni)	Hussain Reddy et al., 2003
Extraction with 10% HNO_3 (50 Hz, 60 s)	Cu, Fe, Mn, Co, Cr, Pb, Ni, Cd, Zn	ICP-AES	[4]RSD%: < 10 (Cu); 5 (Fe); 15 (Mn); 8 (Co); 10 (Cr); 20 (Pb); 5 (Cd); 16 (Ni); 11 (Zn)	Pehlivan et al., 2008
Extraction with CCl_4 and 2 N HNO_3 after pretreatment with conc. HNO_3 (ultrasonic bath, 30 ˚C)	Fe, Cu, Zn, Ni	FAAS	Rec.% 96.5-97.5 (Fe); 96.5-97.1 (Cu); 95.8-97.5 (Ni); 96.0-97.8 (Zn)	Anwar et al., 2003
Pb-piperazinedithiocarbamate SPE and KCN back-extraction	Cu	FAAS	Rec.% 91-97	Batı & Cesur, 2002
Zn-piperazinedithiocarbamate SPE	Cd	FAAS	Rec.% 93.1-100	Yağan Aşçı et al., 2008
Ultrasonic-Assisted extraction with conc. HNO_3 and H_2O_2 (35 kHz)	Cu, Fe, Ni	FAAS and ETAAS	Rec.% 95.9-98.3 (Cu); 95.7-98.2 (Fe); 95.2-97.5 (Ni)	Ansari et al., 2008
Ultrasonic-Assisted extraction with conc. HCl and 30% H_2O_2	Cu, Pb	SCP	Rec.% 82-107 (Cu); 84-105 (Pb)	Cypriano et al., 2008
Extraction with *N,N'*-bis(salicylidene)-2,2'-dimethyl-1,3-propanediaminato (LDM)	Fe, Cu	FAAS	Rec.% 100.2±5.6 (Fe); 99.4±2.8 (Cu)	Köse Baran & Bağdat Yaşar, 2010

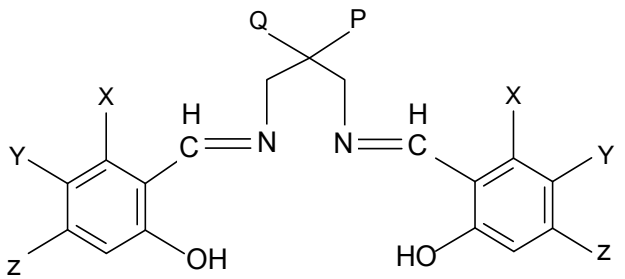
[1]Acc.: Accuracy; [2]Rec.: Recovery; [3]LOD: Limit of detection; [4]RSD: Relative standard deviation

Table 2. The extraction methods used for metal determination in vegetable oils

A Schiff base has been suggested for the extraction of metals from oils as an appropriate chelating agent under the optimum extraction conditions (Köse Baran & Bağdat Yaşar, 2010). In recent analytical applications, Schiff bases have been used in order to form complexes due to their good complexing capacity with metals (Afkhami et al., 2009; Ashkenani et al., 2009; Ghaedi et al., 2009; Khedr et al., 2005; Khorrami et al., 2004; Köse Baran & Bağdat Yaşar, 2010; Kurşunlu et al., 2009; Mashhadizadeh et al., 2008; Shamspur et

al., 2003; Tantaru et al., 2002; Ziyadanoğulları et al., 2008). The chemists have attended to the Schiff bases and their metal complexes because of their widespread applications in biological systems and industrial uses (Issa et al., 2005; İspir, 2009; Kurtaran et al., 2005; Li et al., 2007; Mohamed, 2006; Neelakantan et al., 2008; Prashanthi et al., 2008; Sharaby, 2007).

Although most techniques for metal determinations in edible oils require sample digestion, dilution or emulsification, the improved method can be employed for the same purpose without digestion. The procedure is based on efficient extraction of metals from oil to aqueous solution, and the determination of metals in aqueous phase by FAAS. The proposed approach has been applied for Fe, Cu, Ni and Zn successively. This method includes two main steps. Metal complexes with Schiff bases shown in Fig. 1 were investigated spectrophotometrically as a first step. In this step, the investigation of the complexation reaction as a driving force for the extraction is necessary to decide the appropriate pH and the equilibrium time in terms of complexation efficiency.



X -H ; -OCH_3
Y -H ; -OCH_3 ; -Br
Z -H ; -OCH_3 ; -Br
Q -H ; -CH_3
P -H ; -CH_3 ; -OH

Fig. 1. Chemical structure of Schiff base used in the extractions

As a second step, the experimental conditions affecting the extraction efficiency of metals should be researched. In the procedure of metal extraction with a Schiff base, the optimization of parameters -the ratio of Schiff base solution volume to oil mass, the stirring time and the temperature- for the metal extractions has been achieved by carrying out central composite design (CCD) as an optimization method.

As shown in Table 3, the CCD consisting of a combination of 2^3 full factorial design and a star design was used, in which three independent factors were converted to dimensionless ones (x_1, x_2, x_3) with the coded values at 5 levels: -1.682, -1, 0, +1, +1.682.

Factors		Levels				
		-1.682	-1	0	+1	+1.682
x_1 (1st factor)	V_{LDM} / m_{oil} ratio (mL g^{-1})	0.159	0.5	1	1.5	1.841
x_2 (2nd factor)	Stirring time (minute)	9.56	30	60	90	110.46
x_3 (3rd factor)	Temperature (°C)	13.18	20	30	40	46.82

Table 3. Variables, levels and the values of levels used in CCD (Köse Baran & Bağdat Yaşar, 2010)

Fifteen experiments should be done in a CCD. Additionally, to estimate the experimental error, replications of factor combinations are necessary at the center point (the level, 0). Experiment at the center point has been repeated five times. The total number of experiments in the CCD with three factors then amounts to 20 (Morgan, 1991; Otto, 1999). Accordingly, 20 experiments given in Table 4 were carried out in the extent of the CCD optimization procedure.

	Coded values of levels		
Experiment no.	V_{LDM} / m_{oil} ratio (mL g^{-1}) x_1	Stirring time (min.) x_2	Temperature (˚C) x_3
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	0	0	0
10	-1,682	0	0
11	+1,682	0	0
12	0	-1,682	0
13	0	+1,682	0
14	0	0	-1,682
15	0	0	+1,682
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

Table 4. The coded values of levels for the experiments in the extent of CCD

Organo-metallic standards in oil (Conostan code number; 354770 for iron, 687850 for copper) were used in CCD and metal concentrations of oil standards were fixed to be a certain concentration. The metal concentrations of the extracts gained from each experiment were determined by FAAS. The obtained results were used in order to establish recovery values for the extraction of metals from oil. The response values (y) were calculated from experimentally obtained recovery percentages. The empirical equations were developed by means of response values (Morgan, 1991; Otto, 1999). The following y equations were constructed based on the b values which were calculated by applying to the appropriate matrixes.

$$y = b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (1)$$

New corresponding equations were obtained by equalization of the derivatives of y equation in terms of x_1, x_2, x_3 to zero and solved using software to provide optimum extraction conditions. Optimum conditions are variable depending on the structure of Schiff base and significant metal. The found optimum conditions are given in Table 5 when LDM (Q and P = CH3; X, Y and Z = H) was used as a Schiff base. The recovery values for the extraction of Cu and Fe from oil under the optimum experimental conditions were found to be 99.4(±2.8) and 100.2(±5.6)%, respectively (n=10). To test the applicability of the improved procedure, it was applied on spiked olive, sunflower, corn and canola oils. The recovery percentages were varied between 97.2-102.1 for Cu and 94.5-98.6 for Fe (Köse Baran & Bağdat Yaşar, 2010).

Metal	Optimum Conditions		
	V_{LDM} / m_{oil} ratio (mL g^{-1})	Stirring time (min.)	Temperature (˚C)
Cu	0.76	73	31
Fe	1.19	67	28

Table 5. Optimum extraction conditions for determination of Cu and Fe in edible oils (Köse Baran & Bağdat Yaşar, 2010)

The improved determination strategy after the extraction with Schiff bases has main advantages like independency from hard oil matrix, elimination of explosion risk during decomposition, no requirement for expensive instruments, high accuracy, sensitivity, rapidity and cheapness.

3. Direct determination

The direct determination of metals in oils can be carried out by sample solubilization in an organic solvent, an emulsification procedure in aqueous solutions in the presence of emulsifiers such as Triton X-100 or a solid sampling strategy.

3.1 Dilution with organic solvent

The procedure of the dilution with organic solvents is an easy way to sample pretreatment before detection, but has some requirements: special devices for sample introduction e. g. for FAAS (Bettinelli et al., 1995), the addition of oxygen as an auxiliary gas in ICP-OES or ICP-MS (Costa et al., 2001). The volatile organic solvents have been directly introduced into ICPs for many years, but this can cause plasma instability, less sensitivity, less precision and high cost. Al, Cr, Cd, Cu, Fe, Mn, Ni and Pb contents of olive oil were investigated using diethyl ether, methyl isobutyl ketone (MIBK), xylene, heptane, 1,4-dioxane as solvent and N,N-hexamethylenedithiocarbamic acid, hexamethyleneammonium (HMDC-HMA) salt as a modifier by ETAAS (Karadjova et al., 1998). A transverse heated filter atomizer (THFA) was employed for the direct determination of Cd and Pb in olive oil after sample dilution with n-heptane (Canario & Katskov, 2005). Moreover, Martin-Polvillo et al. (1994) and List et al. (1971) determined trace elements in edible oils based on the direct aspiration of the samples, diluted in MIBK. In another research, the mixture of 2%lecithin-cyclohexane was used to introduce the oil samples to a polarized Zeeman GFAAS (Chen et al., 1999). Van Dalen was

also used lecithin and the organopalladium modifier solutions for the injection of the edible oils (Van Dalen, 1996).

3.2 Emulsification

Taking into account parameters such as economy, safety, environment, time, and low risk of contamination, emulsification appears beneficial over microwave assisted acid digestion. On the other hand, optimization of the particle size effect, slurry concentration and homogeneity are necessary in order to obtain good precision and recoveries with slurry techniques. In spite of optimization, complete destruction of the sample matrix in plasma and then liberation of analyte from the sample matrix are not always succeeded, causes unsatisfactory results. An alternative technique for introduction of oil sample directly into ICP is the on-line emulsification (Anthemidis et al., 2005). Direct introduction of oil samples in the form of emulsion into ICP facilitates the spray chamber and plasma torch owing to no need of extra oxygen or sophisticated desolvation device. In such a case, the use of stable emulsions with proper surfactant concentration is very important (Anthemidis et al., 2005).

Emulsification as sample preparation has been performed for the determination of trace metals in vegetable oils by ICP-OES (De Souza et al., 2005; Murillo et al., 1999), ICP-MS (Castillo et al., 1999; Jimenez et al., 2003), FAAS (List et al., 1971) and GF-AAS (Lendinez et al., 2001). Additionally, the use of microemulsion as sample preparation for vegetable oil analysis by High-Resolution Continuum Source FAAS (HR-CS FAAS) has been described by Nunes et al. (2011). The determination of Zn, Cd and Pb in vegetable oils by electrothermal vaporization in combination with ICP-MS (ETV-ICP-MS) was described in literature (Huang et al., 2001).

3.3 Direct solid sampling

Direct introduction of oil samples into the graphite furnace by solid sampling strategy is rarely used, providing an alternative methodology. Due to technical improvements in spectrophotometer and software capabilities of modern instrumentation, this method has not been entirely accepted (Sardans et al., 2010). Direct solid sampling has some advantages such as no sample dilution, satisfactory LOD levels, calibration probability with aqueous analytical solutions, simple analysis and no sample digestion or extraction. Other advantages of this method are reduced time and cost, required little amount of sample and the achievement of high sensitivity. Additionally, it reduces the risk of contamination due to the nonexistence of sample preparation and use of chemical reagents. Some disputes against the method are the difficulty of introducing small sample masses, faulty measurement of the results due to the heterogeneity of some natural samples and the limiting linear working range of AAS (Sardans et al., 2010). Despite these restrictions, direct solid sampling is a reasonable alternative for the determination of the total content of metals in oils, since it needs almost no sample preparation. A method for the direct determination of Ni and Cu in vegetable oils by GFAAS using the solid sampling strategy has been reported without sample dilution by Matos Reyes et al. (2006).

3.4 Flow injection

Various detection techniques like ETAAS, FAAS, ICP-OES, ICP-MS, voltammetry have been utilized for metal determination in oils. However, all of them have the need for sample

pretreatment procedures in common like: wet digestion, dry ashing, extraction and dilution with organic solvent in order to eliminate hard organic matrix. In the processing large numbers of samples, flow injection analysis (FIA) systems can be preferred for sample pretreatment. The FIA system for oil analysis is frequently based on the on-line preparation of oil-in-water emulsions by using ultrasonic bath with serious drawbacks in efficient preparation of stable emulsions. By this way, more concentrated emulsions (high oil concentration) can be introduced into the plasma and thereby the LODs were improved. A limited number of researches related to metal determination in oils by FIA systems have been presented. Jimenez et al. succeeded multi-element determination in virgin olive oil by flow injection ICP-MS using with HNO_3 and Triton X-100 as emulsifying agents (Jimenez et al., 2003). A magnetic-stirring micro-chamber has been developed for on-line emulsification and has been successfully employed by Anthemidis et al. to detect Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, Mg, Mn, Ni, Pb, Tl and Zn in olive oil using flow injection ICP-OES (Anthemidis et al., 2005). The low concentration of analyte in the sample analysed and difficulty of obtaining stable emulsions with rich oil content were reported as the main problems. On-line emulsion preparation procedure was suggested as simpler, more effective, less time consuming, less labor intensive, less matrix interferences and less contamination risk over the other direct sample introducing procedures. The direct determination of Cu and Fe in edible oils based on the flow injection standard addition method by FAAS was performed without sample dilution in a previous study (Carbonell et al., 1991).

As mentioned above, various pretreatment procedure and detection techniques have been employed for the total determination of metals in olive oil. The researchers have dealt with metallic contents of olive oils during last few decades. As can be seen in Table 6, the concentration range of total amount is given for many metals.

Metal	Concentration ($\mu g\ g^{-1}$) (* $ng\ g^{-1}$)		References
	Minimum	Maximum	
Fe	12.5*	139.0	(Anthemidis et al., 2005; Benincasa et al., 2007; Buldini et al., 1997; Calapaj et al., 1988; Cindric et al., 2007); De Leonardis et al., 2000; Llorent-Martinez et al., 2011a, 2011b; Martin-Polvillo et al., 1994; Mendil et al., 2009; Nunes et al., 2011; Pehlivan et al., 2008; Zeiner et al., 2005)
Cu	1.7*	4.51	(Angioni et al., 2006; Anthemidis et al., 2005; Buldini et al., 1997; Calapaj et al., 1988; Castillo et al., 1999; Cindric et al., 2007; De Leonardis et al., 2000; Galeano Diaz et al., 2006; Jimenez et al., 2003; Karadjova et al., 1998; Llorent-Martinez et al., 2011a, 2001b; Martin-Polvillo et al., 1994; Mendil et al., 2009; Nunes et al., 2011; Pehlivan et al., 2008; Zeiner et al., 2005)
Ni	10.6*	2.26	(Benincasa et al., 2007; Buldini et al., 1997; Calapaj et al., 1988; Castillo et al., 1999; Cindric et al., 2007; Nunes et al., 2011; Zeiner et al., 2005)
Zn	0.6*	4.61	(Angioni et al., 2006; Cindric et al., 2007; Lo Coco et al., 2003; Mendil et al., 2009; Nunes et al., 2011; Zeiner et al., 2005)

Mn	0.7*	0.15	(Anthemidis et al., 2005; Benincasa et al., 2007; Calapaj et al., 1988; Castillo et al., 1999; Cindric et al., 2007; Jimenez et al., 2003; Karadjova et al., 1998; Llorent-Martinez et al., 2011a; Mendil et al., 2009; Pehlivan et al., 2008; Zeiner et al., 2005)
Pb	0.42*	0.032	(Calapaj et al., 1988; Canario & Katskov, 2005; Castillo et al., 1999; Jimenez et al., 2003; Llorent-Martinez et al., 2011a; Mendil et al., 2009; Martin-Polvillo et al., 1994)
Co	0.23*	5.45	(Benincasa et al., 2007; Calapaj et al., 1988; Castillo et al., 1999; Cindric et al., 2007; Mendil et al., 2009; Zeiner et al., 2005)
Cd	0.6*	0.15	(Angioni et al., 2006; Benincasa et al., 2007; Calapaj et al., 1988; Canario & Katskov, 2005; Castillo et al., 1999; Mendil et al., 2009; Yağan Aşçı et al., 2008)
Cr	0.012	2.00	(Anthemidis et al., 2005; Benincasa et al., 2007; Calapaj et al., 1988; Castillo et al., 1999; Llorent-Martinez et al., 2011a)
V	0.005	0.46	(Castillo et al., 1999); (Llorent-Martinez et al., 2011a)
Ge	0.03	0.04	(Castillo et al., 1999)
Zr	0.01	0.04	(Castillo et al., 1999)
Ba	4.9*	0.7	(Castillo et al., 1999; Jimenez et al., 2003; Llorent-Martinez et al., 2011a)
Al	0.030	1.11	(Anthemidis et al., 2005; Cindric et al., 2007; Jimenez et al., 2003; Karadjova et al., 1998; Martin-Polvillo et al., 1994; Zeiner et al., 2005)
Be	0.118*	0.178*	(Benincasa et al., 2007)
Sc	49.94*	747.9*	(Benincasa et al., 2007)
As	1.248*	26.65*	(Benincasa et al., 2007)
Se	1.47*	6.78*	(Benincasa et al., 2007)
Sr	1.52*	48.9*	(Benincasa et al., 2007)
Y	0.082*	0.331*	(Benincasa et al., 2007)
Sb	0.194*	0.411*	(Benincasa et al., 2007)
Sm	0.004*	0.226*	(Benincasa et al., 2007)
Eu	0.004*	0.021*	(Benincasa et al., 2007)
Gd	0.003*	0.094*	(Benincasa et al., 2007)
Sn	0.126	0.159	(Calapaj et al., 1988)
Mg	0.056	4.61	(Bağdat Yaşar & Güçer, 2004; Benincasa et al., 2007; Cindric et al., 2007; Mendil et al., 2009; Zeiner et al., 2005)
Ca	0.63	76.0	(Anthemidis et al., 2005; Benincasa et al., 2007; Cindric et al., 2007; Mendil et al., 2009; Zeiner et al., 2005)
K	0.05	2.14	(Cindric et al., 2007; Mendil et al., 2009; Zeiner et al., 2005)
Na	8.7	38.03	(Cindric et al., 2007; Mendil et al., 2009; Zeiner et al., 2005)

Table 6. The metal levels for olive oils.

4. Speciation and fractionation

Fractionation was defined as "the process of classification of analyte or a group of analytes from a certain sample according to physical (e.g., size, solubility) or chemical (e.g., bonding, reactivity) properties", and speciation of an element was also defined as "distribution of an element amongst defined chemical species in a system" by Templeton et al. (2000). The physicochemical form of an element, i.e. the actual species found in exposure medium and in the different body fractions, is frequently determinant in the evaluation of its bioavailability and toxicity (Flaten, 2002). An element can be found in various species: anionic or cationic inorganic forms, inorganic compounds, complex compounds with protein, peptide etc. Some organometallic compounds are much more toxic than the ions of the corresponding inorganic compounds. Hg, Pb and Sn obey this rule, for example, methyl-Hg and inorganic Hg are both toxic, but methyl-Hg show more toxicity than other (Templeton et al., 2000). In contrast to this, in the case of As and Se, most organo-arsenicals are less toxic than inorganic As species, organic forms of Se are ordinarily less toxic than Se(IV) (Kot & Namiesnik, 2000).

The determination of the total amount of an element in samples cannot give adequate information for understanding its bioavailability or toxicity, that's why the fractionation and speciation of metals in oils are increasingly gaining importance. The fractionation and speciation analysis are more informative than total element determinations for all type of samples.

In general, many works dealing with the total amount of elements in oil samples are reported, but fractionation and/or speciation analysis in vegetable oils are less common in literature. To the best of our knowledge, magnesium fractionation analysis in olive and olive oil was cited firstly in 2004. The improvement of an analytical scheme for fractionation of magnesium in olive products and also the determination of Mg amounts absorbed in stomach and intestine was achieved by Bağdat Yaşar & Güçer (2004). It was reported that 3.37-8.47% of Mg was absorbed in the stomach (ionic and polar groups) and the remaining percentage of Mg was absorbed in the intestine (molecular and complexed structures) in olive oil. As can be seen, the Mg fraction in olive oil is almost absorbable in the intestine. This study can be accepted as a preliminary step for fractionation studies and the fractionation and/or speciation approach for other elements will be described in the future.

5. Detection techniques

Various researchers deal with determination of metals in oils at trace, ultra-trace levels using spectrometric and electrometric techniques. Mentioned detection techniques may be combined with some chromatographic systems. Oils have been analyzed for different metals using atomic absorption spectrometer (FAAS and GFAAS), inductively coupled plasma optical emission spectrometer (ICP-OES), inductively coupled plasma mass spectrometer (ICP-MS). ICP techniques have become more popular since the early 1990s. Although the use of AAS (flame, graphite furnace, hydride generation and cold vapour) has declined during the same period, it is still the most widely used technique (Rose et al., 2001).

Each technique has some special requirements, advantages and disadvantages according to its basic principle. GF-AAS is a sensitive, proper for direct introduction of oil samples in the form of emulsion and does not require a large amount of sample. FAAS and ICP-MS have a requirement of sample pretreatment, but ICP-MS is more sensitive and expensive when compared with FAAS. There are scarce researches dealing with oil samples related to voltammetric and potentiometric techniques such as Ad-SSWV, dPSA (Abbasi et al., 2009; Cypriano et al., 2008; Dugo et al., 2004; Galeano Diaz et al., 2006; Lo Coco et al., 2003).

6. Conclusion

Trace quantities of some metals are naturally present in olive oil. It could be possible to determine the levels of different trace metals with the help of precise and accurate analytical methods. In many cases, a sample pretreatment process is necessary to eliminate the oil matrix prior to the introduction of the sample into the instrument. A direct determination is also possible by sample solubilization in an organic solvent, an emulsification procedure or a solid sampling strategy when ETAAS, GF-AAS or ICP are used for the analysis of edible oils. Microwave-assisted wet digestion sample pretreatment is also employed combined with sensitive detection techniques. An alternative technique can be achieved efficiently and precisely by FAAS after the extraction of metals with a Schiff base ligand.

7. Abbreviations

AAS	Atomic Absorption Spectrometry
FAAS	Flame Atomic Absorption Spectrometry
GF-AAS	Graphite Furnace Atomic Absorption Spectrometry
ETAAS	Electrothermal Atomic Absorption Spectrometry
ICP	Inductively Coupled Plasma
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
Ad-SSWV	Adsorptive Stripping Square Wave Voltammetry
dPSA	Derivative Potentiometric Stripping Analysis
SCP	Stripping Chronopotentiometry

8. References

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6

Sensory Analysis of Virgin Olive Oil

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1. Introduction

Virgin olive oil (VOO) is the supernatant of the fresh juice obtained from olives by crushing, pressure and centrifugation, without additional refining. Its flavour is characteristic and is markedly different from those of other edible fats and oils. The combined effect of odour (directly via the nose or indirectly through a retronasal path, via the mouth), taste and chemical responses (as pungency) gives rise to the sensation generally perceived as "flavour".

Sensory analysis is an essential technique to characterize food and investigate consumer preferences. International cooperative studies, supported by the International Olive Oil Council (IOOC) have provided a sensory codified methodology for VOOs, known as the "COI Panel test". Such an approach is based on the judgments of a panel of assessors, conducted by a panel leader, who has sufficient knowledge and skills to prepare sessions of sensory analysis, motivate judgement, process data, interpret results and draft the report. The panel generally consists of a group of 8 to 12 persons, selected and trained to identify and measure the intensity of the different positive and negative sensations perceived. Sensory assessment is carried out according to codified rules, in a specific tasting room, using controlled conditions to minimize external influences, using a proper tasting glass and adopting both a specific vocabulary and a profile sheet that includes positive and negative sensory attributes (Dec-23/98-V/2010). Collection of the results and statistical elaboration must be standardized (EEC Reg. 2568/91, EC Reg. 640/08). The colour of VOO, which is not significantly related to its quality, may produce expectations and interferences in the flavour perception phase. In order to eliminate any prejudices that may affect the smelling and tasting phases, panelists use a dark-coloured (blue or amber-coloured) tasting glass.

Many chemical parameters and sensory analyses (EEC Reg. 2568/91 and EC Reg. 640/08), with the latter carried out by both olfactory and gustatory assessments, can classify oils in different quality categories (extra virgin, virgin, lampant). Extra virgin olive oil (EVOO) extracted from fresh and healthy olive fruits (Olea europaea L.), properly processed and adequately stored, is characterized by an unique and measurable combination of aroma and taste. Moreover, the category of EVOO should not show any defects (e.g. fusty, musty, winey, metallic, rancid) that can originate from incorrect production or storage procedures.

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Positive or negative sensory descriptors of VOO have been related to volatile and phenol profiles, which are responsible for aroma and taste, respectively.

The characteristic taste of VOO, and in particular some positive attributes such as bitterness and pungency that are related to important health benefits, is not completely understood or appreciated by consumers. In this respect, it is interesting to consider the degree of acceptability of VOO in several countries based on literature data. In this way, it is possible to lay the foundations for correct instruction of the sensory characteristics of EVOO. The main chemical, biochemical and technological processes responsible for the positive and negative (defects) descriptors of VOO are summarized in this chapter. An overview on the sensory methodologies proposed, applied and modified during the last 20 years is also presented.

2. Flavours and off-flavours of virgin olive oil: The molecules responsible for sensory perceptions

VOOs are defined by the European Community as those "…oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil…" (EEC Reg. 2568/91). This production method renders VOO different from other vegetable oils that undergo refining, which leads to loss of most of the minor components such as volatile molecules and "polar" phenolic compounds.

Many authors (Angerosa et al., 2004; Kalua et al., 2007) have clarified that several variables affect the sensory characteristics and chemical composition of an EVOO. These include environmental factors, cultivation and agronomic techniques, genetic factors (cultivar), ripening degree of drupes, harvesting, transport and storage systems of olives, processing techniques, storage and packaging conditions of the oil.

The sensory attributes of EVOO mainly depend on the content of minor components, such as phenolic and volatile compounds. The independent odours and tastes of different volatile and phenolic compounds that contribute to various and typical EVOO flavours have been extensively studied; the sensory and chemical parameters of EVOO have been correlated in a large number of investigations (Bendini et al., 2007; Cerretani et al., 2008).

Each single component can contribute to different sensory perceptions. It is well established that specific phenolic compounds are responsible for bitterness and pungency (Andrewes et al., 2003; Gutiérrez-Rosales et al., 2003; Mateos et al., 2004). Few individuals, except for trained tasters of EVOO, know that the bitterness and pungency perceived are considered positive attributes. These two sensory characteristics, more intense in oils produced from olives at the start of crop year, are strictly related to the quali-quantitative phenolic profile of EVOO.

Even in small quantities, phenols are fundamental for protecting triacylglycerols from oxidation. Several authors (Gallina Toschi et al., 2005, Carrasco-Pancorbo et al., 2005; Bendini et al., 2006; Bendini et al., 2007) have reported their importance as antioxidants as well as nutracetical components. The major phenolic compounds identified and quantified in olive oil belong to five different classes: phenolic acids (especially derivatives of benzoic and cinnamic acids), flavones (luteolin and apigenin), lignans ((+)-pinoresinol and (+)-

acetoxypinoresinol), phenyl-ethyl alcohols (hydroxytyrosol, tyrosol) and secoiridoids (aglycon derivatives of oleuropein and ligstroside). The latter are characteristic of EVOOs.

Several investigations (Gutiérrez-Rosales et al., 2003; Mateos et al., 2004) have demonstrated that some phenols, and in particular secoiridoid derivatives of hydroxytyrosol, are the main contributors to the bitterness of olive oil; other phenolic molecules such as decarboxy-methyl-ligstroside aglycone, which seems to be a key source of the burning sensation, can stimulate the free endings of the trigeminal nerve located in the palate and gustative buds giving rise to the chemesthetic perceptions of pungency and astringency (Andrewes et al., 2003). Using a trained olive oil sensory panel, some investigators (Sinesio et al., 2005) have studied the temporal perception of bitterness and pungency with a time-intensity (TI) evaluation technique. It has been shown that the bitterness curves had a faster rate of increase and decline than the pungency curves. It was also demonstrated that differences in kinetic perception are linked to the slower signal transmission of thermal nociceptors compared to other neurons.

On the other hand, approximately 180 compounds belonging to several chemical classes (aldehydes, alcohols, esters, ketones, hydrocarbons, acids) have been separated from the volatile fractions of EVOOs of different quality. Typical flavours and off-flavour compounds that affect the volatile fraction of an oil obtained from olives originate by different mechanisms: positive odours are due to molecules that are produced enzymatically by the so-called lipoxygenase (LOX) pathway. Specifically both C_6 aldehydes, alcohols and their corresponding esters and minor amounts of C_5 carbonyl compounds, alcohols and pentene dimers are responsible for pleasant notes. In contrast, the main defects or off-flavours are due to sugar fermentation (*winey*), amino acid (leucine, isoleucine, and valine) conversion (*fusty*), enzymatic activities of moulds (*musty*) or anaerobic microorganisms (*muddy*), and to auto-oxidative processes (*rancid*).

Volatile molecules can be perceived in very small amounts (micrograms per kilogram or ppb) and these compounds do not have the same contribution to the global aroma of EVOO; in fact, their influence must be evaluated not only on the basis of concentration, but also on their sensory threshold values (Angerosa et al., 2004; Kalua et al., 2007). In addition, antagonism and/or synergism among different molecules can occur, affecting the global flavour of EVOO. Chemical factors of molecules (volatility, hydrophobic character, size, shape, conformational structure), type and position of functional groups appear to affect the odour and taste intensity more than their concentration due to their importance in establishing bonds with receptor proteins (Angerosa et al., 2004).

In general, it is correct to surmise that from healthy olives, picked at the right degree of ripening and properly processed, it is always possible to obtain an EVOO, independent of the olive variety. However, from unhealthy olives or from those harvested off the ground it is inevitable to produce an olive oil characterized by unpleasant flavours and sensory defects. Thus, both natural (olive variety, environmental conditions, degree of ripening and health status of olives) and extrinsic (technological processing by olive farmer/mill worker) factors may profoundly influence olfactory and gustative notes.

Several agronomic and climatic parameters can affect the volatile and phenolic composition of VOOs. The genetic characteristics of the olive cultivar are some of the most important

aspects that determine the level of enzymes in fruit (Angerosa et al., 1999) that are involved in synthesis of volatile molecules (LOX pathway) and phenol compounds (biosynthetic pathways via PPO and β-glucosidase) present in VOOs.

Even if enzymatic activity depends on the stage of ripeness (Morales et al., 1996; Aparicio & Morales 1998) agronomic (fertilization, irrigation) and climatic (temperature and rainfall) conditions also play an important role.

2.1 Key points in obtaining a high quality VOO

- Processing of healthy olives:

When the common olive fly (*Bactrocera oleae*) attacks olives (from the beginning of summer to the start of harvesting), damage occurs as a result of larval growth: oils from damaged fruits show changes in both volatile and phenolic compounds that influence negatively the sensory properties and oxidative stability of the product, especially during oil storage (polar phenols have a fundamental role as antioxidants during storage). The bad taste due to these changes caused by the olive fly is well known as a *grubby defect* (Angerosa et al., 1992; Gómez-Caravaca et al. 2008).

In order to obtain a high quality olive oil, it is necessary to process olives that are not overripe. The use of fruits that have partially degraded tissues cause an increase in enzymatic and microrganism activities and oxidative reactions; therefore the produced oil probably will be characterized by an higher free acidity and perceivable sensory defects. When olives are accumulated in piles for many days, the high temperature and humidity inside the mass promotes proliferation of bacteria, yeasts and moulds, producing undesirable fermentation and degradation that give rise to specific volatile molecules responsible for unpleasant odours (i.e. winey, fusty and mouldy).

Winey, the typical pungent sensory note perceptible in oils produced by olives stored in piles or in jute sacks for several days, arises from alcoholic fermentation: *Lactobacillus* and *Acetobacter* have been detected in olives inducing fermentative processes. The main microorganism found in olives depends on the length of storage: at the beginning the enterobacteriaceae genera *Aerobacter* and *Escherichia* prevail, while *Pseudomonas*, *Clostridium* and *Serratia* are predominant after longer periods of time. The activity of these microorganisms results in the presence of low concentrations of biosynthetic volatiles and large amounts of compounds such as the branched alcohols due to degradation of amino acids that lead to the typical undesirable sensory note known as fusty (Angerosa, 2002; Morales et al 2005). The most abundant deuteromycetes found in olives stored at high humidity are several species of the genus *Aspergillus* together with ascomycetes *Penicillium*; these organisms oxidize free fatty acids producing mainly methyl ketones, in contrast to yeasts of the genera *Candida*, *Saccharomyces* and *Pichia* which are able to reduce carbonylic compounds. Enzymes from these microorganisms interfere with the LOX pathway to produce volatile C_8 molecules characterized by very low odour thresholds, and reduce some C_6 compounds. This volatile profile is responsible for the musty defect of EVOO.

- Selection of the most suitable milling conditions

The phenolic content is greatly influenced by this technological step. In general, the use of the more violent crushing systems (i. e. with hammers instead of blades) causes an increase

in extraction of phenolic compounds due to more intense tissue breaking; therefore, a more vigorous milling system should be used to process olive varieties that are naturally low in phenolic compounds, and permit enrichment of bitter and pungency intensities. The use of more violent milling systems also produces a significant increase in olive paste temperature and a corresponding decrease of the activity of enzymes that play a key role in the production of volatile compounds responsible for fruity and other green notes (Salas & Sanchez, 1999; Servili et al., 2002).

Concerning the malaxation phase, which consists in a slow kneading of the olive paste, the time-temperature pair should be carefully controlled to obtain a high quality EVOO. The lipoxygenase pathway is triggered by milling of olives and is active during malaxation. The volatile compounds produced are incorporated into the oil phase to confer its characteristic aroma. Specifically, a temperature above 28°C for more than 45 min should be avoided; in fact, these conditions can lead to the deactivation of enzymes that produce both positive volatile compounds and oxidize the phenolic compounds causing changes in oil flavour (Salas & Sanchez 1999; Kalua et al., 2007). The reduced concentration of oxygen in paste, obtained by replacing air with nitrogen in the headspace of malaxer during processing, can inhibit these enzymes and minimize the oxidative degradation of phenolic compounds during processing (Servili et al., 1999; Servili et al., 2003). Malaxation under erroneous conditions is responsible for the unpleasant flavor known as a "*heated defect*" due to the formation of specific volatile compounds (Angerosa et al., 2004).

- The application of different oil separation systems

One of the main disadvantages of discontinuous mill systems is the possible fermentation and/or degradation phenomena of residues of pulp and vegetation waters on filtering diaphragms; these reactions give rise to a defect termed "*pressing mats*", but also promote winey and fusty defects (Angerosa et al., 2004). It is well known that among continuous systems, discontinuous mill systems with a three-phase decanter need lukewarm water to dilute olive paste in contrast to a two-phase decanter, which has two exits producing oil and pomace and separates the oil phase from the olive paste This latter system has advantages in terms of water reduction and major transfer of phenols from the olive paste to the oil, with a consequent increase in oxidative stability, bitterness and pungency.

The amount of water added determines the dilution of the aqueous phase and lowers the concentration of phenolic substances that are more soluble in vegetable waste water. Consequently, a large amount of antioxidants is lost with the wastewater during processing. In addition to phenolic compounds, some volatile compounds accumulate more in oil from a dual-phase decanter than in oils extracted with three-phase decanters. Therefore, the use of a two-phase decanter promotes greater accumulation of volatile and phenolic compounds that are not lost in the additional water as in a three phase decanter. The higher concentrations of these compounds are related to the high intensities of bitter, pungent, green fruity, freshly cut lawn, almond and tomato perceptions (Angerosa et al., 2000; Angerosa et al., 2004; Kalua et al., 2007).

- Storage of oil under suitable conditions

In unfiltered oil, the low amounts of sugars or proteins that remain for extended times in oil can be fermented or degraded by specific anaerobic microrganisms of the *Clostridium* genus,

producing volatile compounds responsible for an unpleasant muddy odour by butyric fermentation. The filtration of newly-produced oil can avoid this phenomenon. It is known (Fregapane et al., 2006; Mendez & Falque, 2007; Lozano-Sanchez et al., 2010) that EVOO has a low amount of water, and for this reason it can be considered as a water-in-oil emulsion (Koidis et al., 2008)

The orientation of phenolic compounds in the oil-water interface and the active surface of water droplets can protect against the oxidation of oil. According to some researchers (Tsimidou et al., 2004; Gómez-Caravaca et al., 2007), the stability of unfiltered samples is significantly higher than that of the corresponding filtered oils. This coincides with a higher total phenolic content in unfiltered oils due to a greater amount of emulsified water. On the other hand, higher water levels are expected to favour enzymatic catalysis, including lipase, lipoxygenase and polyphenol oxidase activities. Thus, a more rapid oxidation of unfiltered oil is expected. Some authors (Montedoro et al., 1993) observed that hydrolytic processes occurr in parallel with oxidation during long term storage.

Lipid oxidation is an inevitable process that begins immediately after oil extraction and leads to a deterioration that becomes increasingly problematic during oil storage. The presence of a rancid defect, typical off-flavour for the fatty matrices, can be avoided or substantially slowed. The most advanced oxidation stages are characterized by the complete disappearance of compounds arising from the LOX cascade and by very high concentrations of saturated and unsaturated aldehydes together with unsaturated hydrocarbons, furans and ketones that contribute mainly to the rancid defect because of their low odour thresholds (Guth & Grosch, 1990; Morales et al., 1997; Bendini et al., 2009). To avoid the rancid perception, it is fundamental to control factors that promote lipid oxidation. These include a decrease in the availability of oxygen, the protection of the oil from light and storage at a temperature of 12-14°C. Before bottling, it is advisable to maintain the oil in stainless steel tanks under an inert gas such as nitrogen equipped with devices that periodically eliminate sediments from the bottom of the tank.

3. Sensory methodology for evaluating the quality of VOO: Basic concepts

A sensory codified methodology for virgin olive oils, known as the "COI Panel test", represents the most valuable approach to evaluate the sensory characteristics of VOO. The use of statistical procedures to analyze data from assessors' evaluation provides results that can be trusted as well as methods usually adopted in scientific fields. The purpose of this international method is to standardize procedures for assessing the organoleptic characteristics of VOO, and to establish the methodology for its classification. This methodology, incorporated into regulations of the European Union since 1991, uses, as an analysis tool, a group of 8-12 persons selected in a controlled manner, who are suitably trained to identify and measure the intensity of positive and negative sensations (EEC Reg. 2568/91).

A collection of methods and standards has been adopted by the International Olive Oil Council (IOOC or COI) for sensory analysis of olive oils. These documents (IOOC/T.20/Doc. 4/rev.1 and IOOC/T.20/Doc.15/rev.2) describe the general and specific terms that tasters use. Part of the vocabulary is common to sensory analysis of all foods (general vocabulary), while a specific vocabulary has been developed *ad hoc* and established by sensory

experts of IOOC. In addition, the official method (IOOC/T.20/Doc.5/rev.1 and IOOC/T.20/Doc.14/rev.2) includes precise recording of the correct tasting temperature, as well as the dimensions and colour of the tasting glass and characteristics of the test room.

The panel leader is the person responsible for selecting, training and monitoring tasters to ascertain their level of aptitude according to (IOOC/T.20/Doc.14/rev.2). The number of candidates is generally greater than that needed in order to select people that have a grater sensitivity and discrimination capability. Screening criteria of candidates are founded on sensory capacity, but also on some personal characteristics of candidates. Given this, the panel leader will personally interview a large number of candidates to become familiar with their personality and understand habits, hobbies, and interest in the food field. He uses this information to screen candidates and rejects those who show little interest, are not readily available or who are incapable of expressing themselves clearly.

The determination of the detection threshold of the group of candidates for characteristic attributes is necessary because the "threshold concentration" is a point of reference common to a “normal group” and may be used to form homogeneous panels on the basis of olfactory-gustatory sensitivity.

A selection of tasters is made by the intensity rating method, as described by Gutiérrez Rosales (Gutiérrez Rosales et al., 1984). A series of 12 samples is prepared by diluting a VOO characterized by a very high intensity of a given attribute in an odourless and tasteless medium (refined oil or paraffin). The panel leader sends out the candidate, removes one of the 12 tasting glasses from the series, and places the remaining together; the candidate is called back in the room and is asked to correctly replace the testing glass withdrawn from the series by comparing the intensity of this last with that of the others. The test is carried out for fusty, rancid, winey and bitter attributes to verify the discriminating capacity of the candidate on the entire scale of intensities.

The stage training of assessors is necessary to familiarize tasters with the specific sensory methodology, to heighten individual skill in recognizing, identifying and quantifying the sensory attributes and to improve sensitivity and retention with regards to the various attributes considered, so that the end result is precise and consistent. In addition, they learn to use a profile sheet.

The maintenance of the panel is made through continuous training over all duration of life of the same panel, the check of the sensory acuity of tasters, and exercises that allow the measurement of the panel performance.

Every year, all panels must assess a number of reference samples in order to verify the reliability of the results obtained and to harmonize the perception criteria; they must also update the Member State on their activity and on composition changes of their group.

3.1 Evolution of sensory methodology: From old to new

A method for the organoleptic evaluation of olive oils was introduced in the Regulation (EEC) No 2568/91, Annex XII, that is inspired by the COl/T.20/Doc. no.15, published in 1987. In the profile sheet of EEC Reg. 2568/91, a number of positive attributes and defects were evaluated, giving each a score from 0 to 5 (Figure 1).

Drawing on experience, the International Olive Oil Council has devised a new method of organoleptic assessment of VOOs (Decision Dec-21/95-V/07) that is simpler and more reliable than that in EEC Reg. 2568/91. In particular, the EC Reg. 796/2002 introduced a reduction of the attributes of the old profile sheet, asking tasters to consider only the defects of the oil (fusty, mustiness/humidity, winey/vinegary, muddy sediment, metallic, rancid and others) and only the three most important positive attributes (fruity, pungent and bitter). The most important innovation of EC Reg. 796/2002 is the use of continuous scales, from 0 to 10 cm, for evaluating the intensity of perception of the different attributes (positive and negative), as reported in Figure 2. In this way, tasters are free to evaluate the intensity of each attribute by ticking the linear-scale, without having a prefixed choice (as with the discrete scale of EEC Reg. 2568/91, see Figure 1).

Sensory analysis and its application to olive and virgin olive oil

VIRGIN OLIVE OIL

PROFILE SHEET

GRADING TABLE

OLFACTORY-GUSTATORY-TACTILE NOTES

	0	1	2	3	4	5
Olive fruity (ripe and green)						
Apple						
Other ripe fruit						
Green (leaves, grass)						
Bitter						
Pungent						
Sweet						
Other allowable attribute(s) (Specify.....)						
Sour/Winey/Vinegary/Acid						
Rough						
Metallic						
Mustiness/humidity						
Muddy sediment						
Fusty ("Atrojado")						
Rancid						
Other unallowable attribute(s) (Specify.....)						

DEFECTS	CHARACTERISTICS	OVERALL MARK: POINTS
None	Olive Fruity Olive fruity and fruitness of other fresh fruit	9 8 7
Barely perceptible	Weak fruitness of any tipe	6
Slight perceptible	Rather imperfect fruitness, anomalous odours and tastes	5
Considerable, on the border of acceptability	Clearly imperfect, unpleasant odours and tastes	4
Great and/or serious, clearly perceptible	Totally inadmissible odours and tastes for consumption	3 2 1

1 Barely perceptible
2 Slight perceptible
3 Average
4 Great
5 Extreme

REMARKS.....

.....

NAME OF ASSESSOR.....
LEGEND OF SAMPLE.....

DATE.....

Fig. 1. Profile sheet for EVOO used for designation of origin (EEC Reg. 2568/91, annex XII).

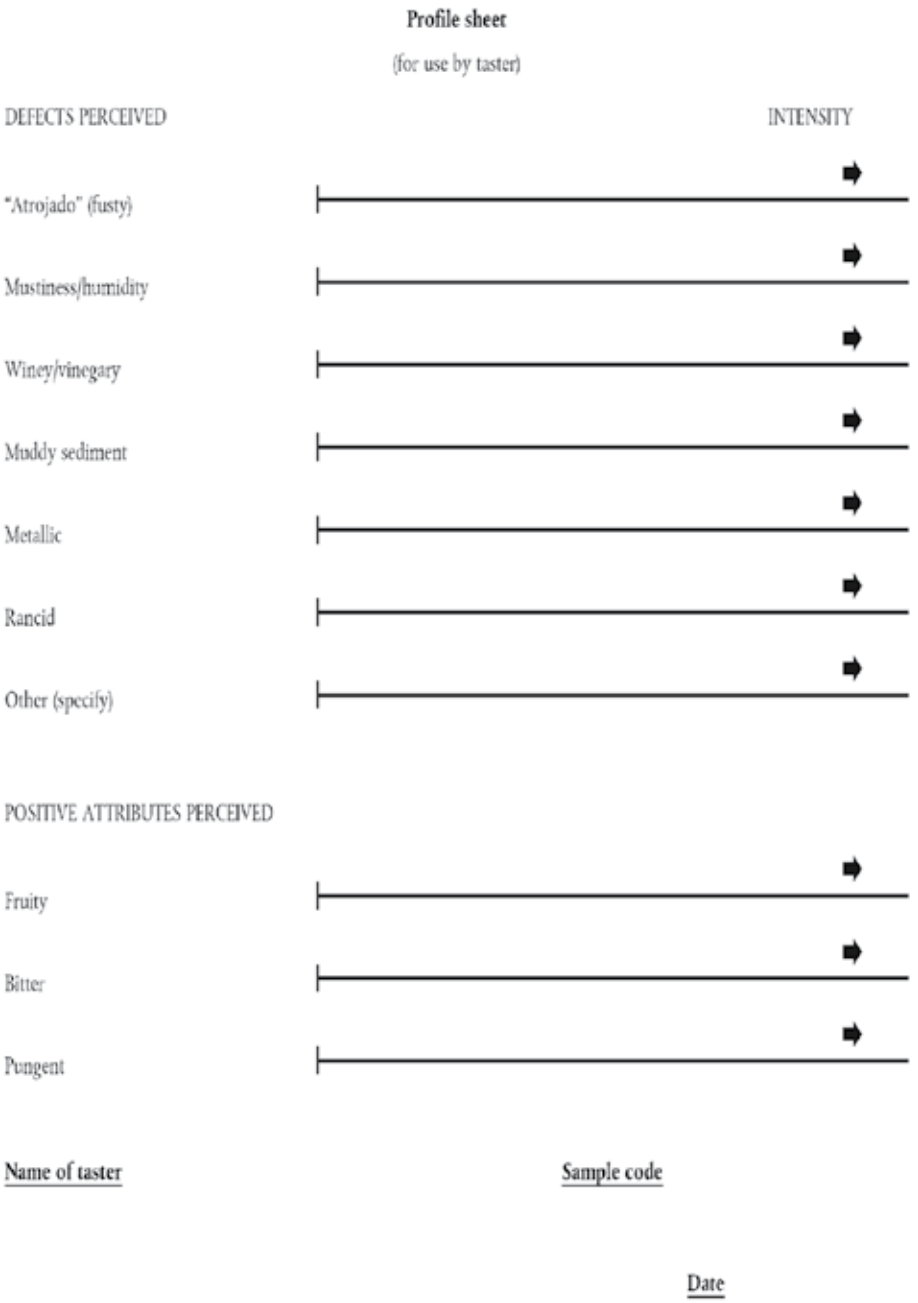

Fig. 2. Profile sheet for VOO assessment currently adopted by the EU (EC Reg. 796/02).

Each attribute is calculated, and the median value of each is used to classify the oil according to the median of the defect perceived with greatest intensity and the median for "fruity". It is important to remember that the value of the robust variation coefficient for this negative attribute must be no greater than 20%.

The classification of olive oils, according to sensory attributes, has also undergone evolution. According to EC Reg. 796/2002, oils are classified as:

a. extra virgin olive oil: the median of the defects is 0, and the median for "fruity" is above 0;
b. virgin olive oil: the median of the defects is above 0, but not above 2.5 and the median for "fruity" is above 0;
c. ordinary virgin olive oil: the median of the defects is above 2.5, but not above 6.0, or the median of the defects is not above 2.5 and the median for "fruity" is 0;
d. lampante virgin olive oil: the median of the defects is above 6.0.

Since November 2003, categories c) and d) have been replaced by (c) "lampante olive oil": the median of defects is above 2.5, or the median of the defects is not above 2.5 and the median for "fruity" is 0.

EC Reg. 640/08 introduced a new upper limit of defect for discriminating between virgin and defective oils: in particular, the evaluation of the median defect ('2.5') was replaced by '3.5'. An important innovation of Reg. 640/08 was also the grouping in only one negative attribute of two different defects: fusty and muddy sediment.

A revised method for the organoleptic assessment of VOO was adopted by the IOOC in November 2007 (Decision No DEC-21/95-V/2007, 16 November 2007) and adopted by the European Community with EC Reg. 640/2008. This revision updated the descriptions of the positive and negative attributes of VOO and the method. It also amended the maximum limit for the perception of defects in VOO. The IOOC's revised method for the organoleptic assessment of VOO also specifies the conditions for the optional use, on labels, of certain terms and expressions relating to the organoleptic characteristics of VOO (optional terminology for labelling purposes).

The most recent change is Decision No Dec-23/98-V/2010 of the IOOC, which defined a new method for assessing the organoleptic properties of VOO and to establish its classification on the basis of those characteristics (IOOC/T.20/Doc. No 15/Rev. 3).

3.2 The method for assigning commercial class: The official profile-sheet and expression of results

The organoleptic assessment of VOO is officially regulated in Europe by a Commission Regulation (EC Reg. 640/2008). This regulation describes the procedures for assessing the organoleptic characteristics of VOOs, the method for classification according to sensory characteristics, the specific vocabulary for sensory analysis of VOOs, including positive and negative attributes, and the optional terminology for labelling purposes. The selection, training and monitoring of skilled VOO tasters, the skills and responsibilities of the panel leader, the specific characteristics of the glass for oil tasting and the test room were also considered, according to previous regulations and IOOC documents (IOOC, 2007 and 2010).

The official profile sheet intended for use by tasters, shown in Figure 3 (EC Reg. 640/08), is quite simple and is formed by an upper section for evaluation of the intensity of defects, and


Profile sheet for virgin olive oil

INTENSITY OF PERCEPTION OF DEFECTS

Fusty/muddy sediment

Musty-humid-earthy

Winey-vinegary — acid-sour

Metallic

Rancid

Other (specify)

INTENSITY OF PERCEPTION OF POSITIVE ATTRIBUTES

Fruity

greenly ☐ ripely ☐

Bitter

Pungent

Name of taster:

Sample code:

Date:

Comments:

Fig. 3. Profile sheet for VOO assessment currently adopted by the EU (EEC Reg. 640/08).

a lower part for the evaluation of the three most important positive sensory attributes (fruity, bitter, pungent). Tasters have to smell the sample, taste the oil (overall retronasal olfactory, gustatory and tactile sensations) and evaluate the intensity with which they perceive each of the negative and positive attributes on the 10-cm scale. If a taster identifies greenly or ripely as fruity attributes, the correct options must be indicated in the profile sheet. Green fruitiness is a characteristic of the oil which is reminiscent of green olives, dependent on the variety of the olive and coming from green, sound, fresh olives. Ripe fruitness is reminiscent of ripe fruit. If any negative attributes not listed in the upper section of the profile are perceived, the taster records them under the "others" heading, using the descriptors among those in the specific vocabulary for the sensory analysis of olive oils (IOOC/T.20/Doc. 4/rev.1).

The panel leader collects the profile sheets and elaborates the results by a statistical approach. In particular, the medians of the greatest perceived defect and fruity attribute are calculated. According to these two parameters, the oil can be graded in different quality categories. Such values are expressed to one decimal place, and the value of the robust coefficient of variation which defines them shall be no greater than 20%. As already mentioned, the classification of the oil is carried out by comparing the medians of the defects and the fruity attribute with the reference ranges established by EC Reg 640/08 for the different categories:

1. Extra virgin olive oil: the median of the defects is 0 and the median of the fruity attribute is above 0;
2. Virgin olive oil: the median of the defects is above 0, but not more than 3.5, and the median of the fruity attribute is above 0;
3. Lampante olive oil: the median of the defects is above 3.5, or the median of the defects is not more than 3.5 and the median of the fruity attribute is 0.

The panel leader can also state that the oil is characterized by greenly or ripely fruity attributes if at least 50% of the panel agrees.

Actually the most important result for sensory analysis of VOO is to identify the presence of defects instead of evaluating the positive attributes, in agreement with the aim of such an analysis, which is essentially to classify the product in different commercial classes.

3.2.1 Optional terminology for labelling purposes

Upon request, the panel head may certify that an oil complies with the definitions and ranges that correspond to the following adjectives, according to the intensity and perception of attributes:

a. for each of the positive attributes mentioned (*fruity* — whether *green* or *ripe* — *pungent* or *bitter*):
   i. the term "intense" may be used when the median of the attribute is greater than 6;
   ii. the term "medium" may be used when the median of the attribute is between 3 and 6;
   iii. the term "light" may be used when the median of the attribute is less than 3;
   iv. the attributes in question may be used without the adjectives given in points (i), (ii) and (iii) when the median of the attribute is 3 or more;

b. the term "well balanced" may be used when the oil does not display a lack of balance, which is defined as the smell, taste and feel that the oil has when the median of the *bitter* and/or *pungent* attributes is two points higher than the median of its *fruitiness*;
c. the term "mild oil" may be used when the medians of the *bitter* and *pungent* attributes are 2 or less.

3.3 Method for organoleptic assessment of EVOO to assign designation of origin: Sensory profile and data processing

In 2005, the IOOC issued a document on methods to be used for the organoleptic assessment of EVOO for granting designation of origin (D.O.) status (IOOC/T.20/Doc. no 22). This document declared that the D.O. authority shall select the characteristic descriptors of the designation of origin (10 at the most) from those defined and reported in Table 1, and shall incorporate them into the profile sheet of the method.

Direct or retronasal aromatic olfactory sensations	
Almond	Olfactory sensation reminiscent of fresh almonds
Apple	Olfactory sensation reminiscent of the odour of fresh apples
Artichoke	Olfactory sensation of artichokes
Camomile	Olfactory sensation reminiscent of that of camomile flowers
Citrus fruit	Olfactory sensation reminiscent of that of citrus fruit (lemon,orange, bergamot, mandarin and grapefruit)
Eucalyptus	Olfactory sensation typical of Eucalyptus leaves
Exotic fruit	Olfactory sensation reminiscent of the characteristic odours of exotic fruit (pineapple, banana, passion fruit, mango,
Fig leaf	Olfactory sensation typical of fig leaves
Flowers	Complex olfactory sensation generally reminiscent of the odour of flours, also known as floral
Grass	Olfactory sensation typical of freshly mown grass
Green pepper	Olfactory sensation of green peppercorns
Green	Complex olfactory sensation reminiscent of the typical odour of fruit before it ripens
Greenly fruity	Olfactory sensation typical of oils obtained from olives that have been harvested before or during colour change
Herbs	Olfactory sensation reminiscent of that of herbs
Olive leaf	Olfactory sensation reminiscent of the odour of fresh olive leaves
Pear	Olfactory sensation typical of fresh pears
Pine kernel	Olfactory sensation reminiscent of the odour of fresh pine kernels
Ripely fruity	Olfactory sensation typical of oils obtained from olives that have been harvested when fully ripe
Soft fruit	Olfactory sensation typical of soft fruit: blackberries,raspberries, bilberries, blackcurrants and redcurrants
Sweet pepper	Olfactory sensation reminiscent of fresh sweet red or green peppers
Tomato	Olfactory sensation typical of tomato leaves
Vanilla	Olfactory sensation of natural dried vanilla powder or pods,different from the sensation of vanillin
Walnut	Olfactory sensation typical of shelled walnuts
Gustatory sensations	
Bitter	Characteristic taste of oil obtained from green olives or olives turning colour; it defines the primary taste associated with aqueous solutions of substances like quinine and caffeine
"Sweet"	Complex gustatory-kinaesthetic sensation characteristic of oil obtained from olives that have reached full maturity
Qualitative retronasal sensation	
Retronasal persistence	Length of time that retronasal sensations persist after the sip of olive oil is no longer in the mouth
Tactile or kinaesthetic sensations	
Fluidity	Kinaesthetic characteristics of the rheological properties of the oil, the set of which are capable of stimulating the mechanical receptors located in the mouth during the test
Pungent	Biting tactile sensation characteristic of oils produced at the start of the crop year, primarily from olives that are still unripe

Table 1. List of descriptors for D.O. of EVOO.

The characteristic descriptors are identified according to the round-table method: the panel supervisor leads a discussion based on a series of samples of known origin that display the most important specific characteristics of the VOO undergoing preparatory analysis. When the descriptor recognition stage is completed, the panel supervisor opens discussions with panel members to establish a list of all descriptors that are considered to be most important and characteristic of the designation that is undergoing preparatory analysis.

Validation should take into account the possible natural variations that may occur in the oil from one crop year to the next. When the profile sheet is completed, tasters shall assess the intensity of perception of the descriptors cited in the profile sheet on the 10-cm scale used for commercial grading of oils. The D.O. authority shall fix the maximum and minimum limits of the median for each descriptor included in the profile sheet and shall establish the limits for the robust coefficient of variation of each descriptor. It shall then enter these values in the *IOOC spreadsheet folder-profile* (software) accompanying this method to define the intervals of the characteristic sensory profile of the designation of origin.

Most of the specifications for the designation of origin of oils before 2005 or those that have not undergone revisions after this date, do not refer to the method IOOC just explained, but to the use of a previous procedure (EEC Reg. 2568/1991) for sensory evaluation of the oils. In Figure 1, the profile sheet according to the old regulation for the commercial grading is shown (EEC Reg. 2568/1991). This method provides a partial description of flavour: tasters are requested to define the fruity type, green or ripe, and recognize the presence of attributes such as grass, leaf, apple and other fruits. For each attribute, a discreet score from 0 to 5 is assigned (0: absence of perception; 1: intensity slightly perceptible; 2: intensity light; 3: average intensity; 4: great intensity; 5: extreme intensity), and there are many positive attributes to evaluate in addition to defects. Tasters rate the overall grading by using a 9-point scale: 9 for oils with exceptional sensory characteristics, and 1 for products with the worst qualities. The mean score identifies the category. An oil could be classified as EVOO if it obtains a final score (expressed as an average of the panel's judgement) of 6.5.

In the case of specifications for the designation of origin of some D.O oils, which have not yet been reviewed according to the new IOOC regulation (IOOC, 2005), it is firstly necessary to verify that the sample has the characteristics provided in the extra virgin category using current methods (EC Reg. 640/08), and to subsequently analyze it according to the old profile sheet (EEC Reg. 2568/1991) to verify the presence of characteristic descriptors. The final score for the D.O must be at least 7, but can be even higher.

4. Consumer acceptability of the sensory characteristics of VOO: An overview of literature data

As previously stated, a virgin oil that is not subjected to any subsequent tecnological refining has a sensory profile standardized by a rich/robust/harmonized regolatory environment (Conte & Koprivnjak, 1997) strongly linked to the quality of the starting olives. Any damage to drupes, which can lead to hydrolysis or fermentation, produces molecules that remain in the product and irreversibly affect its quality. There is no way of correcting

chemical and/or sensory defects in a virgin product. On the other hand, technological refining results in the loss of the superior quality of "extra virgin/virgin" oil, and the transition to a lower category with weaker sensory attributes. The difference in the overall quality between a virgin and a refined oil, the latter adjusted in both quality and the flavour, is not always correctly perceived by the consumer.

Generally, consumers appreciate what is familiar, what is strongly linked to the territory (tradition/origin) or to which they have a precise expectation (brand, other values) (Caporale et al., 2006, Costell et al., 2010). Furthermore, as demonstrated in a recent large study, people do not understand dietary fat, either the importance of the quality or the quantity needed for health and this generally results in consumers adhering to fat choices they are comfortable with (Diekman & Malcolm,2009). In the case of EVOO, for a correct perception of the overall quality the fruity (green or ripe) and bitter and pungent attributes should be perceived by consumers as "healthy" indicators of quality and genuine taste, linked to the raw oil and its richness in pungent and bitter minor components (phenols) (Carluccio et al, 2003). To achieve this purpose, consumers should be made capable, by research dissemination, to appreciate bitterness (primary taste of oil obtained from green olives or olives turning colour) and pungency (biting tactile sensations characteristic of oils produced at the start of the crop year, primarily from olives that are still unripe) (COI/T.20/Doc. no 22) as healthy substances related attributes.

By law, the virgin oil "ideal" sensory profile is quite simple and easy, the fruity attribute is universally recognized as the primary sensory characteristic, and the bitter and pungent aspects are reported as positive attributes (*CODEX STAN 33-1981*). However, due to the superficial knowledge in terms of fat quality, technology (virgin and refined) and sensory characteristics, consumers do not appear to practice an informed/univocal consumption of EVOO. In this regard, research on consumer behaviour has intensified in recent years, and some of the more salient findings are provided below.

A study in Turkey (Pehlivan & Yilmaz, 2010) comparing olive oils originating from different production systems (continuous, organic, stone pressed, refined) declared that, for a sample of 100 consumers, hedonic values of the refined samples were close to the values of the virgin samples. Similar findings were previously reported by Caporale et al (2006), by which consumers are able to differentiate EVOO on their characteristic sensory attributes, but buying intentions (blind test) of the refined samples were as high as the values for the virgin samples. Again, the sensory attributes of EVOO, even if perceived, did not seem to be drivers to purchase it.

In Italy, Caporale et al. (2006) demonstrated that information about origin creates a favourable hedonic expectation, with regards to specific sensory attributes, such as pungency and bitterness. This means that, if familiar with bitter/pungent oils, consumers can have high and positive expectations of bitter and pungency attributes as distinguishing characteristics of typical olive oils (i.e. *Coratina* cv.). To confirm this physiological opportunity to perceive pungent as a positive attribute can be cited an interesting paper on the unusual pungency of EVOO (Peyrot des Gachons et al., 2011), sensed almost exclusively in the throat, suggesting that it is, therefore, perhaps no coincidence if phenols with potent anti-inflammatory properties (oleocanthale, ibuprofen) also elicit such a localized/specific

pungency. In this paper the authors ask what is the functional significance of the pungency to the human upper airways; they suggest that the posterior oral location of toxin and irritant detectors can protect against their intake either by inhalation or ingestion. But if the role of these ion channels, in general, is to protect tissue from harmful compounds, then it is a mystery how one (TRPA1-channel), mediating throat irritation of extra-virgin olive oils, came to be valued as a positive sensory attribute by those who consume them. The authors hypothesize that this pungency, distinguisheing particularly good olive oils in the European Union standards, similarly to other common food irritants (e.g., capsaicin, menthol, and so forth), also important positive components in many cuisines, turns, from a usually negative taste-kinesthetic sensation into positive, because the molecules that elicit it have a body healthy action. This theory requires considerably more investigations to be demonstrated, but is true that many compounds eliciting pungency are also linked to decreased risks of cancer, degenerative and cardiovascular diseases (Boyd et al., 2006; Peng & Li, 2010).

In the case of EVOO, but this is a very general question, the authors suggest that people can transform an inherently unpleasant sensation into a positive one, commonly experienced around the world when consuming pungent EVOO, because it has beneficial health effects (Peyrot des Gachons et al., 2009). If this theory is correct, it means that this kind of pungency colud be easily taught as a positive sensation quality-related, to the unfamiliar consumers.

Infact, it has been reported (Delgado & Guinard, 2011) in the USA, an emergent market, that in a study on 22 samples evaluated in blocks of 5, for the majority of 100 consumers bitterness and pungency were negative drivers of liking.

Descriptive analysis (Delgado & Guinard, 2011) has been proposed as a more effective method to provide a more detailed classification of EVOO; the final method consisted of 22 sensory attributes, some of which were original but infrequent (butter/green tea). But, in the case of EVOO, the challenge for the future does not appear descriptive analysis, which has had the most interesting developments for the characterization/valorization of monovarietal, PDO and PGI (Inarejos-García et al., 2010; Cecchi et al. 2011) with many targeted/robust attributes. Rather it concerns the fact that consumers are actually able to appreciate/perceive its fundamentals of sensory profile (fruity, bitter, pungent) as related to its quality.

Finally, the worldwide problem of two different qualities of EVOO, a high one (expensive) and a "legal" one (less flavour/cheaper), was also highlighted in a means-end chain study (Santosa & Guinard, 2011), explaining that the attributes associated with EVOO generally have high (more flavour, more expensive, smaller size) or, unfortunately, low (cheaper/on sale, big quantity/bulk size, less flavour) levels of product involvement.

5. Conclusion

Sensory analysis of EVOO has been used for classification for more than 20 years. Since 1987, the "COI Panel test" has undergone many revisions, became law in 1991 in Europe and actualy COl/T.20/Doc. no. 15. is the method of analysis accepted by the Codex Alimentarius. Over the years, the profile sheet has undergone simplifications that have

restricted selected specific positive (fruity, bitter, pungent) attributes and defects (fusty/muddy sediment, winey-vinegary-acid-sour, metallic, rancid, others).

On the other hand, in 2005 the IOOC issued document COI/T.20/Doc. no 22 that provides specifics about the methods to be used for sensory assessment of EVOO when granting designation of origin (D.O.) status. The method contains a list of 23 direct or retronasal aromatic olfactory sensations, 2 (bitter, sweet) gustatory sensations, 2 tactile or kinesthetic sensations (fluidity/pungent) and a qualitative retronasal persistence. Even taking into account the recent development of sensory analysis, there is no other food that has such a rich/robust/harmonized regulatory environment regulated by the EU, International Olive Oil Council and, as any food, Codex Alimentarius (FAO-OMS).

At present, origin, tradition and habits, more than sensory profile, are purchase drivers for EVOO and the real challenge for the future is improving consumer education in appreciating the foundamental attributes: fruity, together with taste and tactile sensations of phenols, functional and healthy substances naturally present in EVOO, respectively, bitterness and pungency.

Therefore, nowadays, the key to provide the consumer a truly effective EVOO organoleptic knowledge is the worldwide dissemination of the three basic quality-related and "healthy" sensory attributes.

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7

Quality Evaluation of Olives, Olive Pomace and Olive Oil by Infrared Spectroscopy

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1. Introduction

Olive oil extraction starts by crushing olives and ends by obtaining olive oil, vegetative water and partially de-oiled olive pomace (Petrakis 2006; Di Giovacchino 2000). In the industry it is important to know the amount of oil and water present in both olive fruits and olive pomace. In fact, the amount of oil is the parameter that establishes the price of raw materials and by-products and is critical for the optimization of extraction procedures.

There are publications compiling the various technological aspects of olive oil production, its quality, authenticity, chemical composition and the numerous analytical methodologies used for its characterization (Boskou 2006; Hardwood & Aparicio 1999; Hardwood & Aparicio 2000 Kiritsakis 1998; EC Reg No 2568/1991 and its amendment EC Reg No 1989/2003).

The intrinsic characteristics of the production demand fast decisions based on rapid analytical results. Therefore, conventional analytical determinations of oil, water and acid value should be replaced by short time or real-time/in-line measurements. Rapid characterization of raw material allows the selection of olives according to quality, enabling the production of higher quality oils.

Nowadays, infrared spectroscopy has become widely used as a non-invasive tool for fast analyses with less to no sample pre-preparation. There are numerous publications on the use of infrared spectroscopy for the analysis of oils, some of them will be referred, later in this document.

Baeten et al. (2000) published a paper on infrared and Raman spectroscopies and their potential for olive oil analysis. They described the instrumental techniques, interpretation of the spectra, data treatment and present potential applications.

This chapter reviews various applications of infrared spectroscopy for the analysis of olive oil, presents some results of the authors' work and emphasizes that infrared spectroscopy coupled with proper chemometric tools is an advantageous instrument, to be used in the industry, for olive quality evaluation and olive oil characterization.

2. Overview of the olive oil quality parameters

As mentioned previously the most relevant parameters for olives and olive pomace are fat and water content. Another important parameter is the free fatty acids (FFA) content of the oil of the fruit, which will determine the acid value of the produced olive oil .

Olive oil, after its extraction, classification and quality evaluation should be labeled and priced. Quality evaluation and authenticity are based on organoleptic assessment and chemical characterization according to the European Commission Regulation (EC Reg No 2568/1991 and its later amendments EC Reg No 1989/2003), the Codex Alimentarius Norm (Codex Alimentarius Commission Draft, 2009) and International Olive Oil Council (IOOC) Trade standards (IOC/T.15/NC nº 3/Rev.4, 2009). Usual adulterations of olive oil are the addition of olive residue oil, refined olive oil, low-price vegetable oils, and even mineral oil (Wahrburg et al. 2002; Dobarganes & Marquez-Ruiz 2003).

IOOC standards for olive oil and olive pomace oils contain a set of values and limits for the following parameters: fatty acid composition, *trans* fatty acid content, sterol composition and total sterol content, content of erythrodiol + uvaol, wax content, aliphatic alcohol content, stigmastadiene content, 2-glyceryl monopalmitate, unsaponifiable matter, free acidity, peroxide value, specific absorbance in ultra-violet, moisture and volatile matter, insoluble impurities in light petroleum, flash point, trace metals, α-tocopherol, traces of heavy metals and traces of halogenated solvents (IOC/T.15/NC nº 3/Rev.4, 2009). Olive oil chemical characterization involves complex, time consuming and expensive analytical methodologies, which also destroy the sample.

3. Interpreting and using the infrared spectra

Spectroscopic techniques are neither invasive nor sample destructive and may contribute to rapid quality and authenticity evaluation, with low operating cost. From a physicochemical point of view, infrared spectroscopy is based on the vibrational transitions occurring in the ground electronic state of the molecules. The infrared absorption requires a change of the intrinsic dipole moment with the molecular vibration. The regions of the infrared spectrum which are used for applications in food analysis are: mid-infrared (MID-IR) and near-infrared (NIR).

Mid-infrared spectra present well resolved bands showing absorbances of varying intensity in the range of 4000 to 400 cm^{-1} originating from the fundamental vibrations.

Figure 1 shows a representative olive oil spectrum in the 4000 – 900 cm^{-1} region, where several characteristic bands related to lipid functional groups can be observed. In the 3100 - 2800 cm^{-1} spectral region appear the signals, assigned to C-H stretching mode from methylene and methyl groups of fatty acid and triacylglycerols. The low intensity peak near 3100 cm^{-1} may be explained by the CH=CH elongation and the signals of weak absorption around 2800 cm^{-1} are the result of the presence of secondary oxidation products, such as aldehydes and ketones. At 1800-1700 cm^{-1} the C=O stretching mode is found. The very strong band located at 1743 cm^{-1} can be ascribed to the triacylglycerol n-C=O ester group and a shoulder found around 1710 cm^{-1} is characteristic of the presence of free fatty acids (carboxylic n-C=O). The C-H deformation is detected between 1400 and 900 cm^{-1}, spectroscopic region which is also known as fingerprint region (Tay et al. 2002).

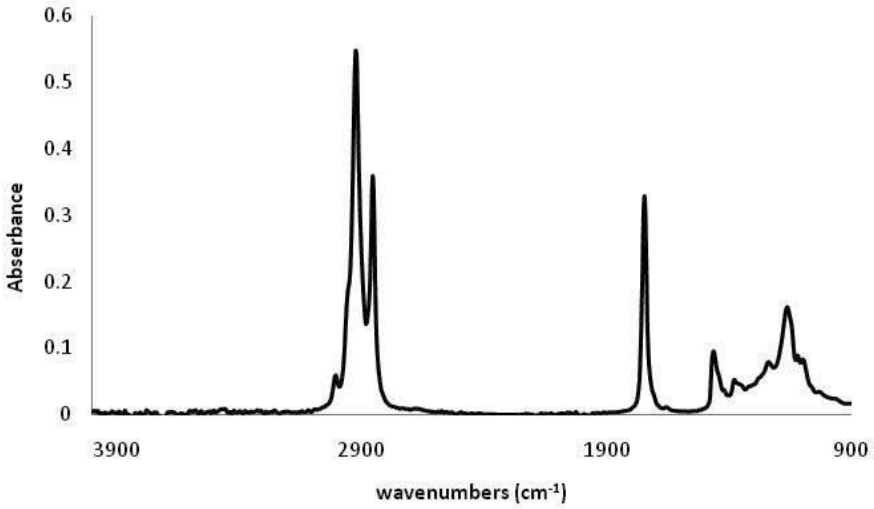
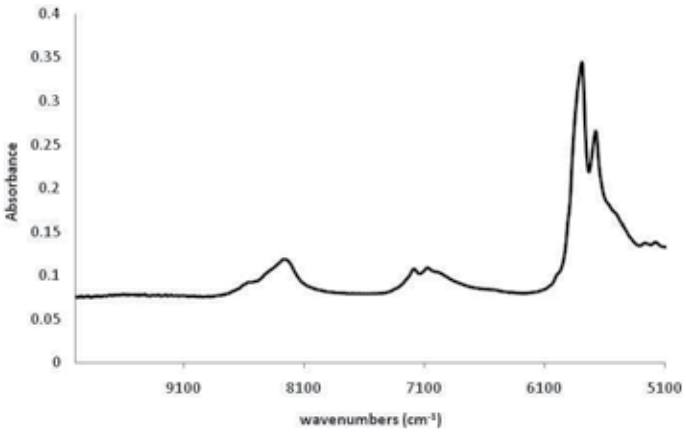


Fig. 1. Typical ATR-Mid-IR spectrum of olive oil.

Near-infrared spectra present less well resolved bands in the range of 14000 to 4000 cm^{-1} corresponding to overtones and combinations of fundamental vibrations.

Figure 2 a) and b) show NIR spectra of olive oil, hammer milled olive and olive pomace. The following main spectroscopic regions can be observed: the region between 9000 – 8000 cm^{-1}, can be ascribed to the second overtone of the C–H stretching vibration of modes of methyl, methylene and ethylene groups of fatty acids and triacylglycerols; the region between 7500 and 6150 cm^{-1} can be attributed to the first overtone of the O-H stretching vibrations; whereas the absorptions located around 6000 – 5700 cm^{-1} correspond to the first overtone of the C-H stretching vibration modes of methyl, methylene and ethylene groups; in next region bands between 5350 and 4550 cm^{-1} result from combinations of fundamentals of the C-H stretching vibration and of bands of water molecules (specially in olives and olive pomace); finally, the 4370 – 4260 cm^{-1} region can be ascribed to the C–H stretching combination of methyl and methylene groups (Galtier et al. 2007; Muick et al. 2004).

Several aspects must be considered when spectroscopic data are used in order to achieve multiple parameter determination, by direct analysis of spectra. A careful calibration framework should be devised, comprising: 1) an adequate sampling strategy, taking in account sampling variability and a suitable physicochemical range set; 2) a robust spectroscopic equipment in order to detect and quantify olive oil parameters in lower amounts, which is particularly important in the industrial in-line process; 3) a proper validation of the results given by infrared spectra and multivariate models; 4) a careful control of the outcome from the instrumental results and chemometric models, by employing control charts to evaluate the performance of the methodology and 5) a plan to address models sustainability through a periodic assessment of models performance, e.g. by performing traditional analysis and comparing to the outcome of the infrared spectra, in order to correct possible deviations. This last aspect is very important due to the nature of the samples (e.g. different harvest periods and samples origin, etc.) and equipment efficacy.



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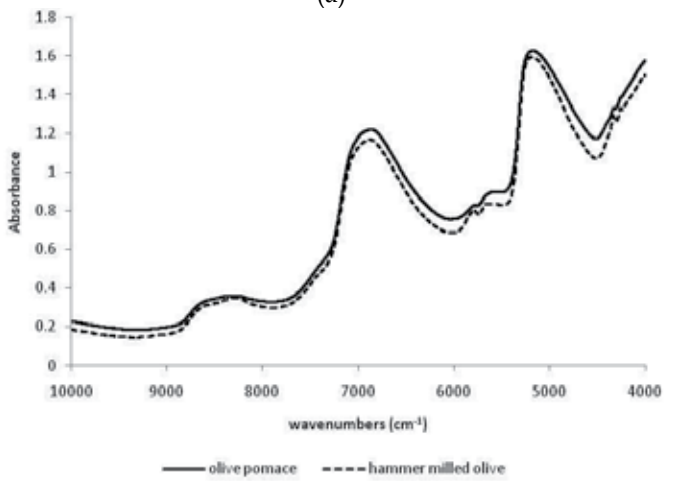


(b)

Fig. 2. Typical NIR spectra of (a) olive oil and (b) hammer milled olive and olive pomace.

4. Quantification of chemical parameters in olive oil

To ensure a more reliable control of every step in the extraction process, a good sampling system is necessary. For industrial in-process analysis of olive oil, spectroscopic techniques (mostly, NIR and MID-IR spectroscopy) in tandem with chemometric methods are the corner-stone for quality control. According to Marquez et al. (2005) these techniques have shown a high potential as an alternative to time-consuming and expensive chromatographic or wet chemistry analysis. In fact the application of optical on-line NIR sensor for olive oil characterization is an appealing approach for real-time chemical evaluation, allowing the estimation of acid value, bitter taste (K_{255}) and fatty acids (Marquez et al. 2005). Near infrared spectroscopy has been valuable for the assessment of physicochemical parameters of vegetable oils (Sato 1994; Hourant et al. 2000; Takamura et al. 1995; Franco et al. 2006). Infrared spectroscopy (NIR and MID), has also been applied successfully for olive oil evaluation and geographical origin determination using chemometric approaches (Tapp et al. 2003; Iñón et al. 2003; Galtier et al. 2007). In addition, NIR technique was employed to detect fraudulent

addition of other vegetable oils and olive pomace oil, to virgin olive oil (Wesley et al. 1995; Yang & Irudayaraj 2001; Doweny et al. 2002; Vlachos et al. 2006); and for olive oil authentication (Bertran et al. 2000; Downey et al. 2003; Woodcock et al. 2008). The chemical characterization of fatty acids and sterols (Ollivier et al. 2006; Ollivier et al. 2003; Aranda et al. 2004; Leardi and Paganuzzi 1987), is useful for assessing quality and authenticity.

The authors attempted to apply NIR for the quantification of fatty acids, sterols and wax in an industrial scenario (for in-line analysis). 40 chemically characterized olive oil samples, from different origins, were used for this study. Partial Least Squares regression (PLS1) was applied in combination with NIR spectra in the 9000 - 4500 cm^{-1} range. Model validations were carried out using Monte-Carlo Cross-Validation (MCCV) (500 runs to evaluate models robustness); the predictive power of each one of the models were assessed through the computation of 1) Root Mean Square Error of Cross Validation (RMSECV); 2) Root Mean Square Error of Prediction (RMSEP); 3) Coefficient of Determination (R^2) and 4) the cross-validated coefficient of determination (Q^2). At the same time several data pre-treatments were tested in order to find the most suitable ones (predictive ability).

A summary of the results obtained is described in Table 1. Two data pre-treatments were selected: 1) Standard Normal Deviate (SNV) and 2) 2nd derivative computed with the Savistzky-Golay procedure, using a 2nd degree polynomial with 11 points (5+5+1). As it can be seen from the table the obtained models have from reasonable to good predictive power.

Parameter	Range	LV	Q^2	R^2	RMSECV (%)	RMSEP (%)	Pre-treatment
Fatty acids							
16:0	9.0 - 12.1 %	4	0.88	0.82	0.270	0.570	2nd derivative
16:1	0.7 - 1.3 %	5	0.88	0.82	0.050	0.101	2nd derivative
18:0	2.8 - 3.8 %	9	0.81	0.98	0.123	0.216	SNV
18:1	73.3 - 78.9 %	9	0.86	0.98	0.521	1.47	SNV
18:2	5.3 - 9.0 %	8	0.91	0.99	0.263	0.32	SNV
18:3	0.7 - 0.8 %	4	0.64	0.81	0.027	0.038	2nd derivative
24:0	0.0 - 0.1 %	7	0.76	0.80	0.021	0.087	SNV
Sterols							
Campesterol	2.97 - 3.64 %	7	0.82	0.98	0.070	0.131	2nd derivative
Cholesterol	0.07 - 0.27 %	6	0.85	0.96	0.020	0.052	2nd derivative
Stigmastenol	0.23 - 0.33 %	9	0.80	0.96	0.012	0.027	SNV
Stigmasterol	0.52 - 1.21 %	8	0.85	0.96	0.088	0.106	SNV
Sitosterol	94.6 - 98.3 %	7	0.63	0.99	0.111	0.175	2nd derivative
Total Sterols	1434 - 1636 (mg/kg)	9	0.70	0.96	23.90	57.0	SNV
Others							
Wax	59.42 - 240.67 (mg/kg)	5	0.71	0.95	29.20	37.7	2nd derivative

Nomenclature: palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), lignoceric acid (24:0).

Table 1. Fatty acids, sterols and wax determination for olive oil by NIR and PLS1 regression in the spectral range of 9000 – 4500 cm^{-1}.

Several factors may reduce the predictive ability of such modeling techniques: 1) low amount of some parameters present in the olive oil; 2) NIR instrument characteristics (selectivity, specificity, signal to noise ratio, etc.) and 3) sampling distribution. At this stage seven fatty acids can be estimated using NIR, but many more could be quantified. It will be necessary to add more samples with wider ranges of parameters in order to enhance the robustness of the models. Galtier et al. (2007) have managed to quantify 14 fatty acids, squalene and triacilglycerols.

The spectral profiles extracted from infrared spectra using chemometric methods could be in many cases a substitute for the traditional analysis, for olive oil overall characterization.

5. Identification and quantification of olive oil adulterants

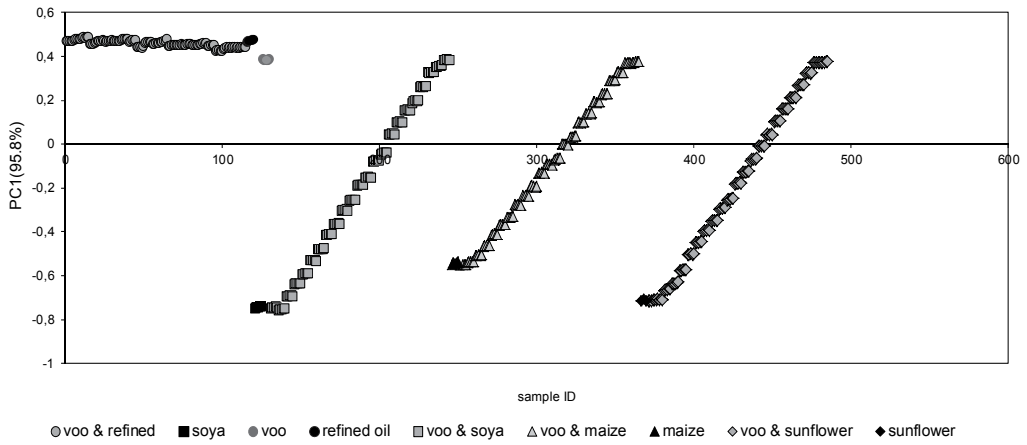
Extra virgin olive oil is adulterated with oils of low quality or price. The natural variation due to geographical origin, weather effect during growth and harvesting makes the task of detecting adulteration difficult. Analytical methods applied in the examination of chemical composition include the determination of fatty acid profiles by gas liquid chromatography (Firestone et al. 1988), high-pressure liquid chromatography (Cortesi 1993; Mariani & Fedeli 1993), pyrolysis mass spectrometry (Goodacre et al. 1993), measurement of iodine values and many other determinations. Rapid, non destructive spectroscopic methods such as Raman (Davies et al. 2000), ultraviolet (Calapaj et al. 1993), MID-IR (Lai et al. 1995; Dupuy et al. 1996; Guillen & Cabo 1999; Küpper et al. 2001) and NIR (Wesley et al. 1995; Bewig et al. 1994; Sato 1994; Wesley et al. 1996; Hourant et al. 2000) have all been applied to quantify adulterants in olive oil.

NIR spectroscopy in tandem with PCA and PLS1 regression, as studied in this laboratory, was found to be suitable for the identification and quantification of adulterants (refined olive oil, sunflower oil, maize oil and soya oil) in virgin olive oil. Binary mixtures were prepared with extra virgin olive oil and each one of the selected potential adulterants. Different amounts of refined olive oil and 3 commercial oil samples (sunflower, soya and maize) were mixed with olive oil giving four different data sets. 25 samples were prepared for each data set (binary mixture), containing additions from 5 to 95 mL of adulterant. Additionally, for each adulterant, an independent prediction set of 8 samples was prepared.

NIR spectra from the samples were obtained with a Perkin Elmer Spectrum One NTS FT-NIR spectrometer. The data were recorded in the spectral range between 10000 – 4500 cm^{-1}, by co-adding 30 scans with a resolution of 8 cm^{-1}. Each sample was acquired five times. PCA allowed the characterization of the sample relationships (scores plans) and the recovery of their sub-spectral profiles (loadings) (Jolliffe 2002). For this analysis, the 6100 - 4500 cm^{-1} region was selected and each spectrum was SNV corrected. A calibration model was built for each adulterant and was validated with the external and independent prediction data sets.

Oil samples are distributed across PC1 axes according to the olive oil content (Figure 3a). The bands located at 4596, 4668 4704, 5880 and 6024 cm^{-1} are related with the samples with larger amount of olive oil (Figure 3b).

Parameters of the best calibration models built for each adulterant are shown in Table 2. The four calibration models were built in the spectroscopic region of 4536-6108 cm^{-1}.



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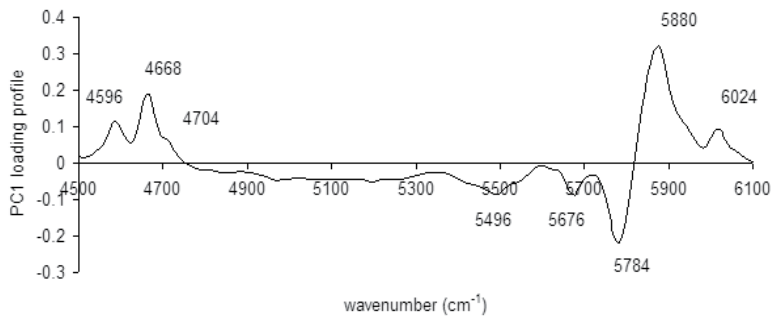


(b)

Fig. 3. (a) Scores plot of the first principal component (PC1), obtained for the set of virgin olive oil (voo) adulterated with refined oil, sunflower oil, maize oil and soya oil. (b) PC1 loading profile.

Spectral region (cm^{-1})	Pre-treatment	LV	RMSEP(%)	R^2	RMSEC(%)
Model 1: quantification of sunflower oil in virgin olive oil					
4536-6108	SNV	6	0.20	0.99	0.29
Model 2: quantification of maize oil in virgin olive oil					
4536-6108	SNV	5	0.23	0.99	0.34
Model 3: quantification of soya oil in virgin olive oil					
4536-6108	SNV	2	0.38	0.99	0.67
Model 4: quantification of refined olive in virgin olive oil					
4536-6108	1st derivative	7	2.81	0.99	3.74

Table 2. Statistical parameters obtained for the calibration models built for each adulterant.

The coefficient of determination higher than 0.99, and the low root mean squared error of prediction (RMSEP) suggest a good predictive power. PLS1 regression based calibration models were used to predict the percentage of adulterant in the independent data sets. Results presented in Table 3 suggest that NIR spectroscopy in tandem with PLS1 regression is suitable to detect and quantify adulteration with other edible oils (sunflower, soya, maize refined olive oil) in extra virgin olive oil up to 2% (w/w).

Prediction Sample nº	Observed Oil Content in Virgin Olive Oil (%)	Predicted Oil Content in Virgin Olive Oil (%)							
		Model 1 (Sunflower)		Model 2 (Maize)		Model 3 (Soya)		Model 4 (Refined Olive Oil)	
P1	0.3	0.1	± 0.1	0.3	± 0.2	0.0	± 0.2	0.4	± 0.4
P2	2.0	1.9	± 0.1	1.8	± 0.3	1.0	± 0.1	1.7	± 1.9
P3	8.0	7.8	± 0.1	7.7	± 0.1	7.5	± 0.1	10.0	± 2.2
P4	16.0	16.1	± 0.1	15.6	± 0.1	15.2	± 0.1	14.9	± 0.7
P5	37.0	36.9	± 0.2	34.5	± 0.1	36.9	± 0.3	36.1	± 2.9
P6	58.0	57.9	± 0.2	56.1	± 0.1	57.7	± 0.1	56.9	± 0.6
P7	71.0	71.1	± 0.1	69.0	± 0.1	70.6	± 0.2	71.2	± 0.8
P8	87.0	86.9	± 0.1	84.9	± 0.2	83.3	± 0.1	82.6	± 1.9

Table 3. Predicted values using the calibration model built for each adulterant.

6. Quality evaluation of the olives at the oil extraction plant

6.1 Determination of oil and water content in olives and olive pomace

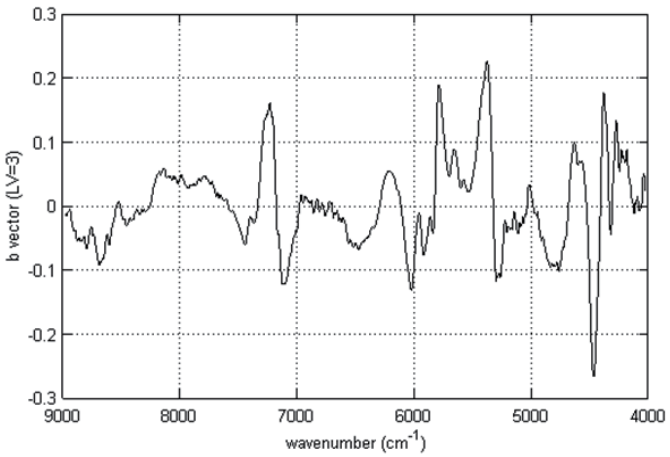
Information about olive quality is very important for the olive and olive oil producers as fruits with larger amounts of oil are highly priced. In addition to water content, fat content is also an important parameter for the optimization of the extraction procedures. Olive pomace can be re-extracted in the same industrial facilities or dried and sold in the form of dried olive pomace (O´Brien 2004).

Conventional oil and water analytical determinations could be replaced by real-time methods that avoid mixtures of high quality with low quality fruits. Muick and coworkers (2004) applied NIR and Raman spectroscopy to the determination of oil and water content in olive pomace. Later on, Bendini et al. (2007) were able to determine fat content, moisture and acid value directly in olives, using a Fourier Transform near-infrared (FT-NIR) instrument located in an industrial mill.

Here, the application of NIR in tandem with a multivariate regression method for the quantification of oil and water directly in fresh hammer milled olive and olive pomace samples is discussed (Barros et al. 2009). A total of 159 olive and olive pomace samples were used to build the calibration set. In order to validate the built models (for oil and water), 108 olive and olive pomace samples were used as independent set. In order to build the calibration models for the quantification of oil and water in hammer milled olive and olive

pomace using NIR and PLS1 regression, a Monte Carlo Cross-Validation (MCCV) (Xu & Liang 2001) framework was used. This approach was needed for building robust calibration models for real-time industrial application.

The model for water content estimation was built by a preliminary spectrum pre-treatment by computing the 1st derivative, in order to minimize the baseline effect, followed by Standard Normal Deviate (SNV). A PLS1 regression model with 3 LVs (Latent Variables) was needed to obtain predictive power with a Q^2 of 0.96 and a relative RMSECV of 1.1%. The **b** vector plot for the water calibration models and the relationship between measured and predicted water values using a PLS1 are presented, respectively, in Figure 4a and Figure 4b.



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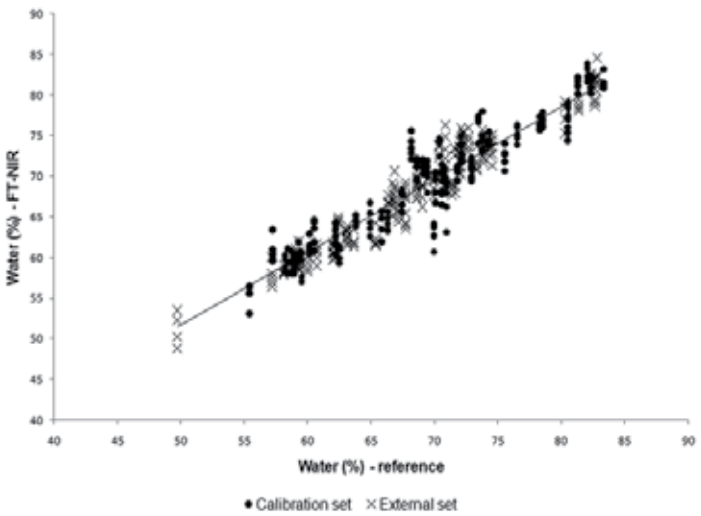
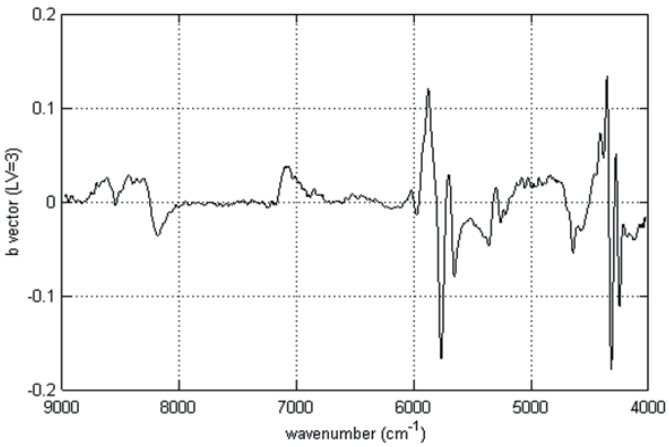


(b)

Fig. 4. (a) b vector plot for the water calibration and (b) relationship between measured and predicted water values using a PLS1 model with 3 Latent Variables (Reproduced with permission from Barros et al. 2009 © Springer 2009).

For oil calibration model the spectra pre-treatment was the same as for the water calibration model and, in this case, 3 LVs were needed to obtain predictive power with a Q^2 of 0.88 and a relative RMSECV of 2.64%. The **b** vector plot for the oil calibration models and the relationship between measured and predicted oil values are shown, respectively, in Figure 5a and Figure 5b.



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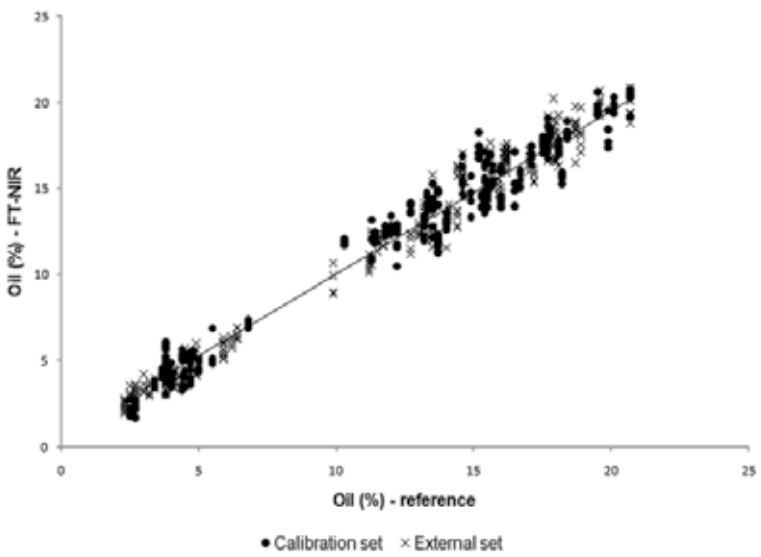


(b)

Fig. 5. (a) b vector plot for the oil calibration and (b) relationship between measured and predicted oil values using a PLS1 model with 3 Latent Variables (Reproduced with permission from Barros et al. 2009 © Springer 2009).

The models showed a good predictive power considering the nature (heterogeneity) of the milled olive fruits and olive pomace samples. NIR technique in tandem with PLS1 regression was found suitable for the quantification of these two important parameters. At industrial scale, the results show that NIR can be used for an extensive screening process of the olive fruits and olive pomace. In fact, when compared to classical approaches of analysis, this methodology is faster, allows larger number of samples in real-time and is environmental sustainable.

6.2 Acid value quantification in olives

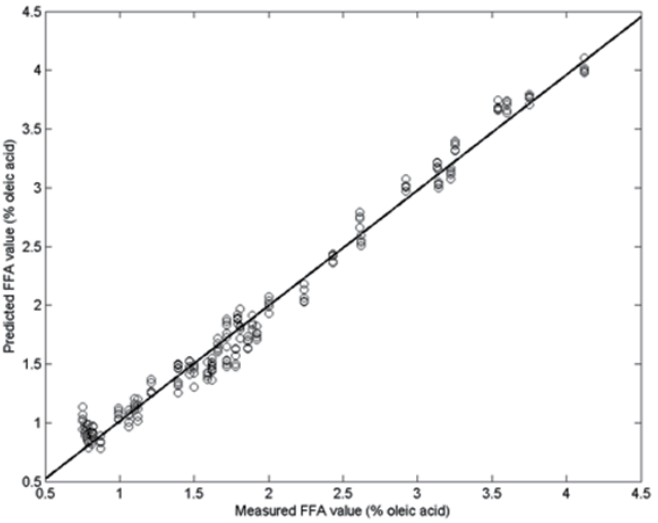
Several factors may affect the olive characteristics and consequently its quality (Muick et al. 2004) specially the increase of free fatty acids (FFA) due to the action of lipases (Morelló et al. 2003). Consequently, the classification of olive oils based on their FFA content prior to processing is an important measure to improve and guarantee the production of good quality olive oil.

Previous works (Muick et al. 2003) report the application of Raman spectroscopy to the direct determination of FFA in milled olives. It is not possible to predict FFA content directly in the milled olive by NIR, probably because of the complexity of the matrix: kernel, pulp and skin.

The authors proposed a method for a rapid determination of free fatty acids in olive (Nunes et al. 2009). This procedure combines Soxhlet extraction for 30 minutes with MID-IR spectroscopy coupled to a multivariate regression method (PLS1). The oil extracted from olives (crushed with a hammer mill) was used for infrared analysis and for free fatty acids determination according to UNE 55030 (AENOR 55030:1961) protocol. MID-IR spectra were acquired by ATR in a Golden Gate accessory (one reflection), in the range of 4000 to 600 cm^{-1}. The data set comprising a total of 210 spectra (42 x 5) was imported into an in-house developed procedure for performing PLS1 (Helland 2001; Martens 2001; Wold et al. 2001).

Figure 6a, shows a linear correlation between the actual olive oil acid values and those estimated by the PLS1 model within the considered values range. The corresponding b vector profile shown in Figure 6b clearly identifies the band located at 1710 cm^{-1} (carboxylic n-C=O) as the most important one, related to the quantified olive oil acid value. Moreover, the band located at 1743 cm^{-1} (assigned to triacylglycerols n-C=O ester group), although weaker than the previous one, also contributes to the modeling of the olive oil acidity.

The PLS1 calibration model with a Monte Carlo Cross-Validation approach was built in the spectroscopic region of 1850-1600 cm^{-1} (with SNV pre-treatment, 4 LVs, a RMSECV(%) of 8.7 and a Q^2 of 0.97). It represents an optimized method for calibration of FFA in extracted olive oil and a proposal for an indirect but quick acid value determination in olives and consequently fruit quality. The short extraction time and the spectroscopic determination of FFA from the MID-IR spectra instead of the titration step (with the consequent decreasing use of reagents and analysis time), provide more reliable results and permit a tight sampling control.



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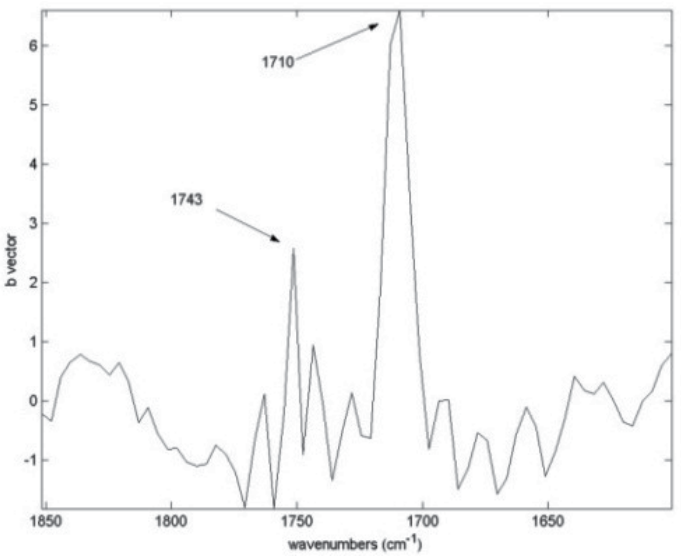

(b)

Fig. 6. (a) PLS1 regression relationship between actual and predicted value of olive oil acidity from the application of acidity calibration model and (b) the corresponding b vector plot (Reproduced with permission from Nunes et al. 2009 © Springer 2009).

7. Conclusions

The high sensitivity and reproducibility provided by the modern spectrometers allow in-depth studies of food systems, like olives and olive oil. The complexity of these matrices requires chemometric tools to extract both qualitative and quantitative information.

Infrared spectroscopic techniques have a potential in assisting and simplifying olive oil characterization. NIR spectroscopy in tandem with multivariate calibration models could provide a comprehensive chemical characterization of an olive oil sample for waxes, total sterols, sterol composition and free fatty acids composition.

NIR infrared may also contribute to the identification and qualification of adulterants in virgin olive oil (additions of refined olive oil, sunflower oil, maize oil and soya oil with "as low as 2% (w/w)).

Moreover, NIR and MID-IR spectroscopy as tool the advantage that it can be used to quantify oil and water content directly in olive and olive pomace and also to measure FFA directly in olives, allowing a quick quality evaluation that may reduce the processing time and cost.

The spectral profiles extracted from infrared spectra using chemometric methods could in many cases be a substitute for chromatographic and wet chemistry analysis, for olive oil overall characterization. Therefore, spectrometers of this type can be an important tool in modern olive oil analytical laboratory since they have so many advantages such as sensitivity, versatility (several type of analysis with only one equipment), real-time/in-line measurements, minimal sample preparation, relatively low cost implementation and high throughput.

8. Acknowledgment

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8

Innovative Technique Combining Laser Irradiation Effect and Electronic Nose for Determination of Olive Oil Organoleptic Characteristics

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1. Introduction

1.1 Olive oil

Olive oil has a characteristic flavor that distinguishes it from other edible vegetable oils. Its quality depends on the aroma, taste and colour, which in turn depend on many variables including location.

The International Olive Oil Council (IOOC,2001) Standards and European Commission regulations have defined the quality of olive oil based on parameters derived from spectrophotometric studies that include free fatty acid content, but these methods only give information about the samples' oxidation level. A specific vocabulary has been developed for virgin oil sensory descriptors (IOOC, 1987). The positive attributes are classified as fruty, bitter and pungent and negative attributes as fusty, musty-humid, muddy-sediment, winey-vinegary, metallic and rancid.

Odour is an important parameter determining the sensory quality of olive oils and it is therefore of interest to investigate if volatile compounds contributing to the characteristic odour can be measured.

In the last decades many efforts have been made to study the aromatic fraction of olive oils based mainly on chromatographic determinations (S. de Koning et al 2008, S. Mildner-Szkudlarz, H. H. Jeleń 2008, C. M. Kalua 2007). The presence or absence of particular volatile compounds is a good indicator of olive oil quality.

The aroma of olive oil is attributed to aldehydes, alcohols, esters, hydrocarbons, ketones, furans and probably, other volatile compounds, not yet identified. More than 120 volatile compounds that contribute both positively and negatively to the sensory properties of olive oil have been identified (Aparicio, R., Morales, M .T. & Luna, G. 2006). Table 1 lists some volatile compounds associated with negative attributes determined by Morales et al. in 2005.

Descriptor	Volatile compounds
Mustiness-humidity	1-octen-3-ol
Fusty	Ethyl butanoate, propanoic and butanoic acid
Winey-vinegary	Acetic acid, 3-methyl butanol and ethyl acetate
Rancid	Several saturated and unsaturated aldehydes and acids

Table 1. Volatile compounds associated with negative attributes of olive oils

Odour activity is a measure of the importance of a specific compound for the odour of a sample. It is calculated as the ratio between the concentration of an individual substance in a sample and the threshold concentration of this substance. The minimum concentration of a compound able to give rise to an olfactory response is the compound´s odour thereshold value. For this reason, high concentration of volatile compounds is not necessarily the main contribution to odour. For example, Reiners and Grosch reported a concentration of 6770 μg/g of trans-2-hexenal with an odour activity value of 16 whereas 1-penten-3-one with a much lower concentration of 26 μg/g had a higher odour activity value of 36 (C. M. Kalua, 2007).

According to the European Community Regulations (ECR 640/2008,ECR 1989/2008) olive oil can be classified in extra-virgin (high quality), virgin (medium quality) and lampante (lower quality). The first two categories can be bottled and consumed.

The quality and uniqueness of specific extra virgin olive oils is the result of different factors such as cultivar, environment and cultivation practices. The European Community (ECR 2081/1992) allows the Protective Denomination of Origen (PDO) labeling of some European EVOO with the names of the areas where they are produced.

1.2 Analytical techniques

The methods used and / or proposed to evaluate the oxidative deterioration of olive oil based on the determination of volatile compounds are HPLC / GC-MS, analytical methods associated with some headspace extractive techniques. The volatile profile of VOO closely depends on the extraction used (S. Vichi, 2010).

Some of the traditional distillation methods applied in the analysis of plant materials as steam distillation (SD), simultaneous distillation/extraction (SDE) and microwave-assisted extraction (MAE) were used for this purpose (Marriott, Shellie, & Cornwell, 2001).

Among these distillation techniques, SDE appeared to provide the most favourable uptake for mono- and sesquiterpenes, as well as for their oxygenated analogues (Marriott et al., 2001). Hydro distillation (HD) has been applied for the analysis of leaf, fruit and virgin oil volatiles of an Italian olive cultivar (Flamini, Cioni, & Morelli, 2003). With hydro distillation, the volatiles in the steam distillate are strongly diluted in water when collected in cold traps. This can be overcome in simultaneous distillation/extraction (SDE) via solvent extraction of the distillate.

Dynamic headspace techniques have been used to correlate the composition of the olive oil headspace to sensory attributes (Angerosa et al., 1996; Angerosa et al., 2000; Morales et al., 1995; Servili et al., 1995) and or flavors ''defects" (Angerosa, Di Giacinto, & Solinas, 1992; Morales, Rios, & Aparicio, 1997).

More recently, the solid-phase microextraction (SPME) technique has been introduced as a sample pre-concentration method prior to chromatographic analysis as an alternative to the dynamic headspace technique. Among other applications, SPME allowed the characterization of virgin olive oils from different olive varieties and geographical production areas (Temime et al., 2006; Vichi et al., 2003a), and the evaluation of varietal and processing effects (Dhifi et al., 2005; Tura et al., 2004). Since the SPME uptakes are strongly dependant on the distribution of analytes among the sample matrix, the gas phase and the fiber coating (Pawliszyn, 1999), some compounds present in virgin olive oil may remain undetected. In the case of other techniques such as SDE, the recovery of analytes is mainly related to their volatility.

These last techniques are complex, expensive and time-consuming. They generally highlight only one or few aspects of the oxidation process, providing only partial information. On the other hand, the olive oil industry needs a rapid assessment of the level of oil oxidation in order to predict its shelf-life. Consumers usually expect manufacturers and retailers to provide products of high quality and seek for quality seals and brands. Therefore, the development of innovative analytical tools for quick and reliable quality checks on extra virgin olive oil is required.

1.3 Electronic nose

Gardner and Barllet (1993) defined the electronic nose as an instrument which comprises an array of electronic chemical sensors of partial specificity and an appropriate pattern-recognition system, capable of recognizing simple or complex odours.

The sensors used in the array of an electronic nose should have the following characteristics: high sensitivity to chemical compounds, low sensitivity to humidity and temperature, medium selectivity, high stability, high reproducibility and reliability; short reaction and recovery time; robustness and durability; easy calibration and data processing and small dimensions (Schaller et al., 1998).

The chemical interaction between the odour compounds and the gas sensors alters the state of the sensors giving rise to electrical signals which are registered by the instrument. Since each sensor is sensitive to all odour components, the signals from the individual sensors determine a pattern which is unique for the gas mixture measured and that is then interpreted by multivariate pattern recognition techniques.

Nowadays, there are different gas sensor technologies available, but only four of them are currently used in commercialized electronic noses: metal oxide semiconductors (MOS); metal oxide semiconductor field effect transistors (MOSFET); conducting organic polymers (CP); piezoelectric crystals (Bulk Acoustic (Wave–BAW), Surface Acoustic (Wave SAW)). Others, such as fiber-optic, electrochemical and bi-metal sensors, are still in the developmental stage.

The processing of the multivariate output data generated by the gas sensor array signals represents another essential part of the electronic nose concept. The statistical techniques used are based on commercial or specially designed software using pattern recognition routines like principal component analysis (PCA), cluster analysis (CA), partial least squares (PLS), linear discriminator analysis (LDA) and artificial neural network (ANN).

The use of an electronic nose for quality evaluation as a means of olfactory sensing is becoming widespread due to its advantages of low cost, reliability and high portability. Electronic noses based on different sensor technologies and using different recognition schemes have been employed for this task.

When samples of olive oil are analyzed with an electronic nose, the standard procedure is to put a fixed quantity in a vial and sense the headspace. The main drawback of this method is that the concentration of some compounds in the headspace may be quite different from their concentration in the liquid phase. For example, the concentration of methanol and ethanol is usually much higher in the vapor phase than in the liquid. However, these substances have been found to be irrelevant in the definition of the olive oil characteristics (S. de Koning, 2008). On the other hand, substances such as hexanal and trans-2-hexanal which are responsible for the organoleptic properties, are more abundant than methanol and ethanol (Cosio et al, 2006, C. Di Natale et al, 2001) in the oil, but are scarcely present in the headspace. It is well known that the odour activity of hexanal and trans-2-hexanal is higher than that of those alcohols because of their low odour thresholds (Morales et al., 2005, J. Reiners, W. Grosch 1998, A. Runcio et al., 2008). Despite the abovementioned drawback, several efforts have been made to use the electronic nose for olive oil quality control (Guadarrama et al., 2000). The combination of electronic nose fingerprinting with multivariate analysis enabled the study of the profile of olive oil in relation to its geographical origin (Ballabio et al., 2006; Cosio et al., 2006). García Gonzalez and Aparicio, 2002, detected the vinegary defect in Spanish VOO with the use of metal oxide sensors. They used an Alpha MOS e-nose equipped with 18 MOS sensors distributed in three chambers, and heated the samples to 34 °C during 10 minutes before testing the headspace. Servili et al., 2009, reported the first study of the use of an Electronic Olfactory System (EOS 835) on-line to control the formation and evolution of the volatile compounds that characterize the most important sensory notes of VOO during the malaxation process.

In 2010, M. J. Lerma-García et al. compared the response of an electronic nose (EOS 507) to classify oils containing the five typical virgin olive oil sensory defects with that of a sensory panel. They demonstrated the usefulness of this tool when combined with panels to perform a fast screening of a large set of samples with the aim of discriminating defective oils. Each sample was incubated at 37°C for 7 minutes before injection.

In the same year, Massacane et al. proposed a method to improve the electronic nose performance for discriminating among different olive oils without changing the properties of the original oil sample. This task is carried out by IR laser vaporization (IRLV) which seems to be a promising technique that modifies only slightly the headspace by volatilizing certain organic compounds or by cracking them. Thus IRLV improves the selectivity of the overall response of the electronic nose. Due to the extremely low sample vaporization that it produces this method can be considered non-destructive as most ablation laser Analytical methods (C. A. Rinaldi and J. C. Ferrero, 2001).

1.4 Laser irradiation effect

Normal vaporization occurs when the vapour pressure in the ambient gas is lower than the saturation pressure of the liquid at the liquid temperature (Xu, X., and D. A. Willis, 2002). As the liquid's temperature increases, so do the saturation pressure and the rate of vaporization.

Laser vaporization (LV) produces a local heating of the irradiated liquid surface and, in consequence, some molecules are driven to the gas phase. This phenomenon can be produced by the use of either pulsed or continuous wave (cw) lasers. For a fixed wavelength, the main difference lies in the amount of energy emitted per unit time, or power. Pulsed lasers produce an increase of the liquid surface temperature without producing a significant change of the bulk volume temperature (Christensen, B., and M. S. Tillack, 2003). These lasers produce only a local heating of the surface allowing a large amount of vapour to be generated in a short time period.

Due to the intrinsic nature of the LV this surface effect is produced immediately after the irradiation time lapse. Therefore, it is common to speak of a "thermal spike" rather than simply "thermal heating", because of the transient nature of the high temperature. The characteristics of this spike are determined by the laser fluence and its pulse length (Taglauer, E, A. W. Czanderna and D. M. Hercules, 1991).

The cw lasers used to vaporize liquids can cause an increase of the bulk sample temperature and can induce chemical reactions, thus, modifying the sample's properties. However, the appropriate choice of the irradiation time lapse and the laser power make them suitable for this application.

In the present work the results of experiments carried out to illustrate the use of the combined techniques of electronic nose and pulsed or continuous wave laser irradiation for olive oil quality determination are reported.

2. E-nose + laser vaporization technique

2.1 Pulsed laser irradiation

In this experiment the Infrared Laser Vaporization, IRLV, properties to improve the e-nose selectivity are investigated. The role of the laser wavelength is additionally analyzed. This is due to the fact that the quality and the quantity of the chemical compounds incorporated the headspace depend on the laser parameters, particularly, the fluence and the pulse length, as it was mentioned in Section 1.4.

Two extra virgin olive oils produced in the same geographical region of Argentina (San Juan) classified as A and B, were tested. Three samples of 15 ml of each oil were subjected to three different analytical methods in order to compare the effects of the laser vaporization.

All analytical methods were carried out under the same temperature and humidity conditions, of 25 °C and 43%, respectively. The samples were introduced in 100 ml T-shaped Pyrex test tubes with screw-caps in air inlet and outlet channels and a CaF_2 window in order to allow the laser beam admission, referred to as vials.

The following analytical methods were undertaken:

Method I: Vial with oil sample A is kept closed during 2 minutes. Immediately afterwards the vial headspace is subjected to 35 seconds sampling with a Cyranose® 320. This procedure is repeated 5 times. The same operation is performed with oil sample B.

Method II: Vial with oil sample A is kept closed for 1min. The sample is subsequently irradiated with Nd:YAG laser pulses of 1064 nm at a repetition rate of 10 Hz during 1

minute. The laser is turned off and the vial headspace is immediately subjected to 35 seconds sampling with a Cyranose® 320. This procedure is repeated 5 times. The same operation is then performed with oil sample B.

The Nd:YAG laser (Continuum, Surelite I) has a pulse length of 5 ns and an output energy of 80 mJ. The laser beam is focused in order to obtain a spot size of 0.037 cm^2 at the surface of the sample so that a fluence of 2.14 J/cm^2 is therefore achieved.

Method III: The vial with oil sample A is kept closed for 1 minute. The sample is subsequently irradiated with a homemade TEA CO_2 laser (D. Petillo, J. Codnia, M. L. Azcárate, 1996) operating at 10.59 μm with a repetition rate of 1 Hz during 1 minute. The laser is turned off and the vial headspace is immediately subjected to 35 seconds sampling with a Cyranose® 320. This procedure is repeated 5 times. The same operation is made with oil sample B.

The TEA CO_2 laser has a pulse length of 100 ns and output energy of 1.45 ± 0.04 J/pulse. The beam is focused in order to obtain a spot size of 0.68 cm^2 at the surface of the sample; a fluence of 2.14 J/cm^2 is therefore achieved. The software provided by the Cyrano® 320TM electronic nose allowed the processing of the raw data given by the 32 sensors responses.

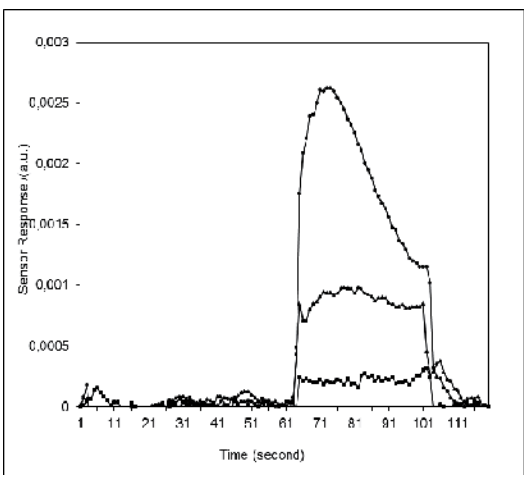


Fig. 1. Sensor response time dependence. (▪) Without laser, (▲) Nd:YAG laser, and (•) CO_2 laser. (Massacane et al., 2010) Permission?

Figure 1 shows the signals measured with one sensor corresponding to the samples of oil A subjected to the three analysis methods. We observe that the signal-to-noise ratio (S/R), is considerably increased by the laser vaporization. The highest S/N ratio is obtained when vaporization is performed with the CO_2 laser.

As it is well known, the fluence and the pulse length determine the laser radiation absorption mechanisms and these parameters modify the laser power. In this work the same power was used in both IRLV methods although different total energies were delivered to the sample in each analysis method: 47.5 and 87 J. in Methods II and III, respectively. This energy range produces a negligible temperature increment. Even assuming an ideal oil absorption of 100% of laser energy the sample temperature increment would be about 3°C,

as may be calculated from the volume of the sample and its average heat capacity. Therefore, the temperature of the sample remains constant throughout the experiment.

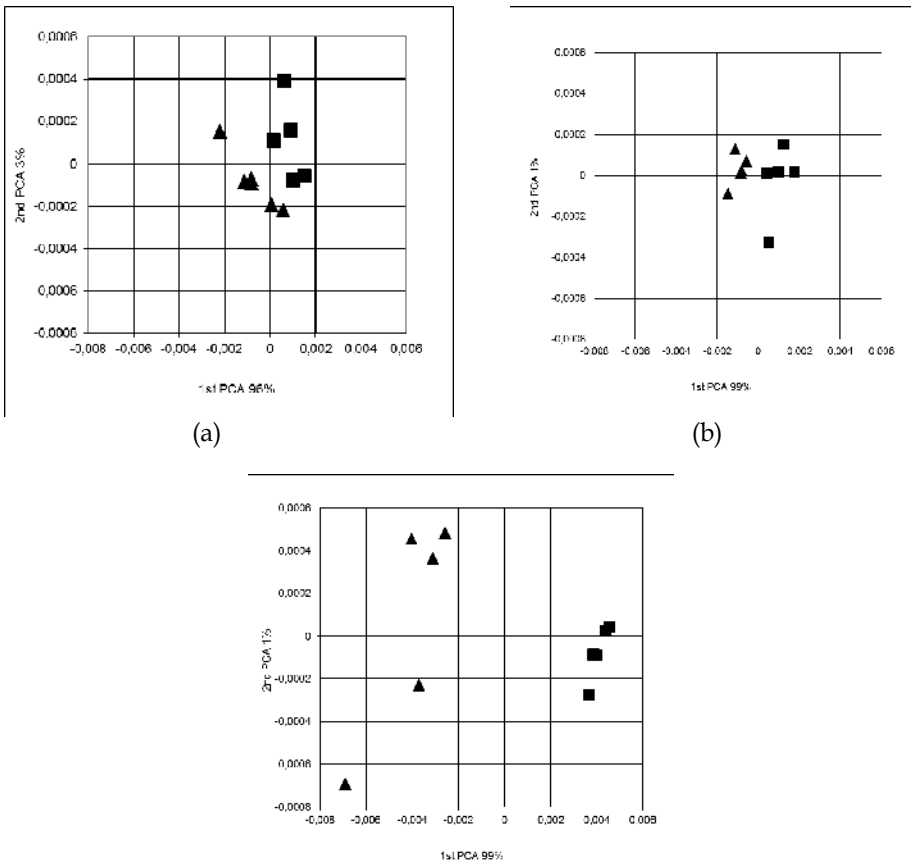


Fig. 2. PCA for olive oils (■:A; ▲: B) performed for experiments (a) without laser, (b) with Nd:YAG laser, and (c) with CO_2 laser. (Massacane et al., 2010) Permission

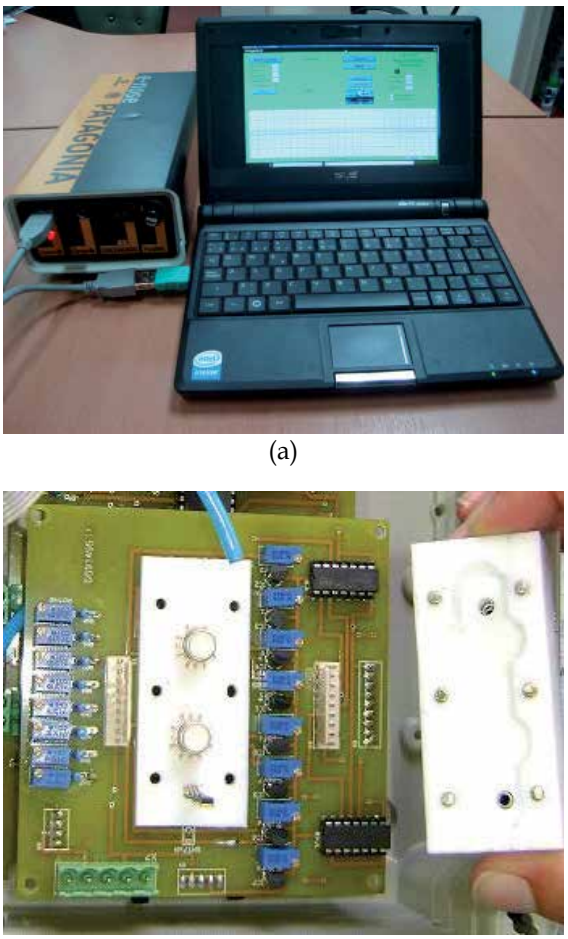
Not only the S/N ratio is increased by IRLV but also the discrimination of samples corresponding to different oils is dramatically increased. This fact can be verified by the results shown in Figures 2(a), 2(b) and 2(c). Thus the modification of the headspace is made evident by this result.

IR spectra of the liquid oil samples were registered both before and after methods II and III were performed. There were no significant differences between them indicating that IRLV does not produce changes in the liquid oil. On the other hand, IRLV does modify the vapour-liquid equilibrium conditions improving the selectivity of the electronic nose overall response. The only effect of IRLV is to increase the vapor concentration of the olive oil (Massacane et al., 2010)

It has been shown that the electronic nose selectivity is dramatically increased by the use of IRLV and that it is rather insensitive to the recognition pattern employed.

2.2 Continuous wave laser irradiation

A homemade portable electronic nose, Patagonia nose, was used in this study. The instrument comprises three parts: the automatic sampling system, the sensors' chamber with the sensor array, and the software for the e-nose control, data recording and processing. The first two are integrated in the same device and the software can be installed on any notebook (Figure 3)



(a)

(b)

Fig. 3. a) Homemade e-nose (Patagonia) b) Sensor chamber

The chamber contains 2 MOS commercial sensors (Silsens MSGS 4000, sensor array). Each sensor has four thin SnO_2 films, one of them is doped with Pd. Each thin film is maintained at the temperature range between 300 and 500 °C during all the measurements.

Two EVOO produced in neighboring geographical regions of Argentina (San Juan) were classified as A and B. About 15 samples of both A and B EVOO were tested. Each sample was divided into three 15 ml samples so that three sets of the 15 samples were obtained to be subjected to three different analytical methods.

All the analytical methods were carried out under the same temperature and humidity conditions: 22 ºC and 36 %, respectively. The samples were introduced in 100 ml T-shaped test tubes with screw caps, air inlet and outlet channels and BK7 upper windows to allow the laser beam admission referred to as vials.

The three analytical methods differ in whether the samples are irradiated or not and in the wavelength of the laser used for the irradiation. The vials were kept closed for about 90 seconds to allow the stabilization of the samples:

Method I: The vial headspace is subjected to a 15 seconds sampling.

Method II: After the headspace stabilization takes place, the closed vial is irradiated during one minute with a continuous wave diode laser emitting radiation of 98 mW at 450 nm. Immediately after the laser is turned off, the vial headspace is subjected to a 15 seconds sampling.

Method III: After the headspace stabilization takes place, the closed vial is irradiated during one minute with a continuous wave diode laser emitting radiation of 98 mW at 650 nm. Immediately after the laser is turned off, the vial headspace is subjected to a 15 seconds sampling.

Figure 4 shows a brief scheme of the experimental set up.

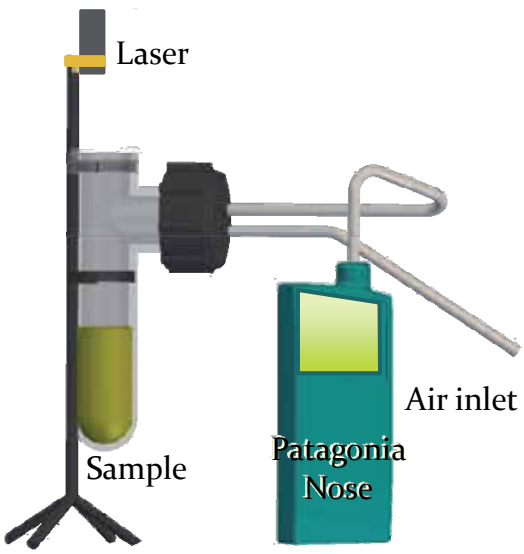


Fig. 4. Experimental set up

We have measured the V-UV spectra of the liquid oil samples before and after being irradiated during different time periods. We have noticed significant differences in the spectra of the liquid samples that had been irradiated during 5 minutes. Figure 5 shows the spectrum of each EVOO used before being irradiated.

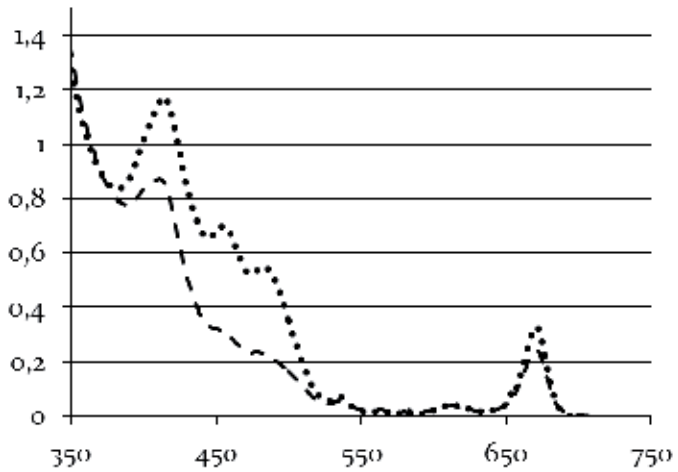


Fig. 5. V-UV Spectra of liquid EVOO A(- - - -) and liquid EVOO B (. . . .) before irradiation

Figure 6 shows the Principal Components Analysis (PCA) of the Patagonia Nose results obtained with Method I.

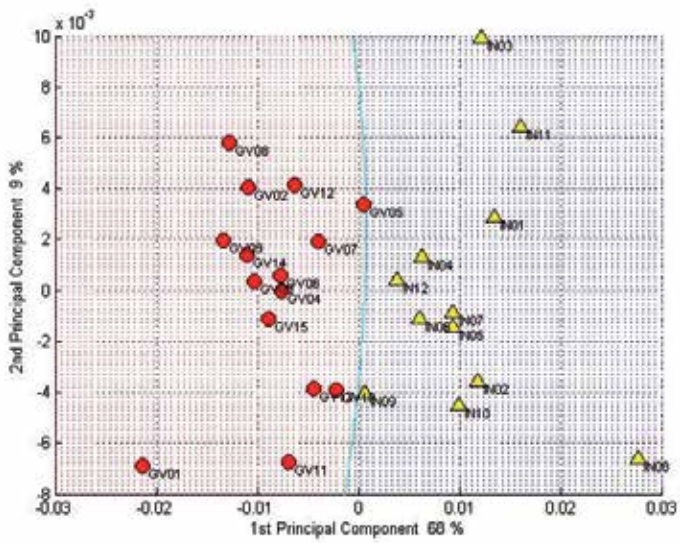
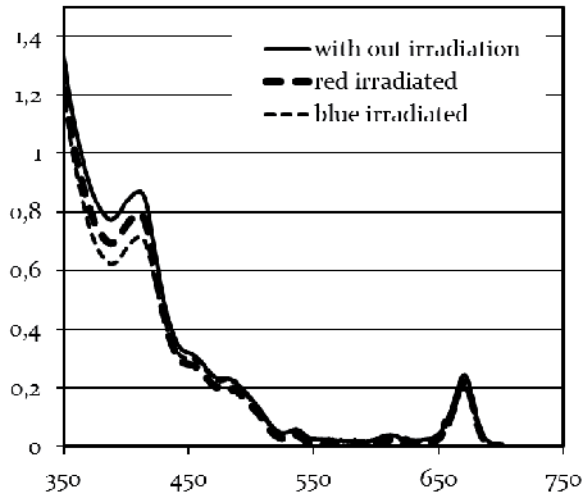
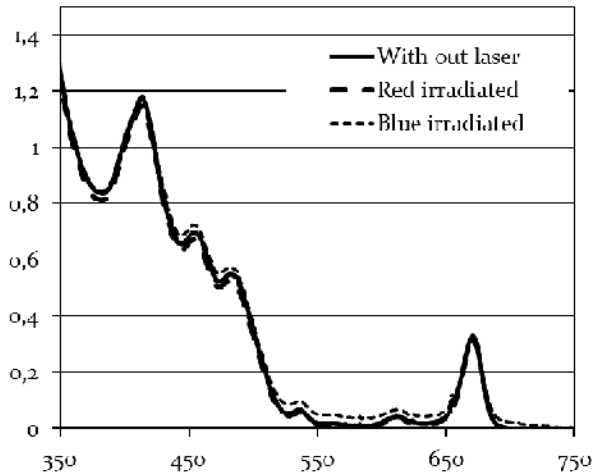


Fig. 6. PCA of EVOO A(GV) and B(IN) with Method I

Figure 7 shows the V-UV spectra registered for each liquid EVOO after the application of the three analytical methods. The experiment was repeated 5 times for each sample. It can be observed that the spectra of the irradiated samples of EVOO A exhibit significant changes with respect to the non-irradiated sample. The largest effect is produced by the irradiation with blue light (450 nm). This more energetic radiation affects the sample composition probably due to a photochemical effect. On the other hand, irradiation with the red light, gives rise to a thermal effect which causes the introduction of more molecules into the gas phase.



EVOO A



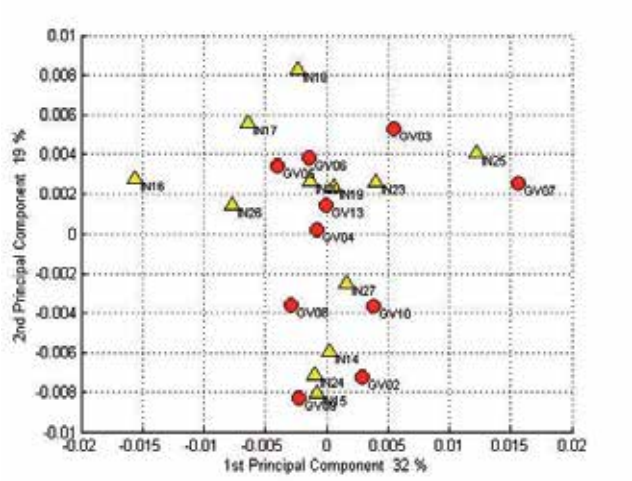
EVOO B

Fig. 7. V-UV spectra of EVOO (A) and (B)- Method I (_____) - Method II (- - - -) – Method III (- - - -)

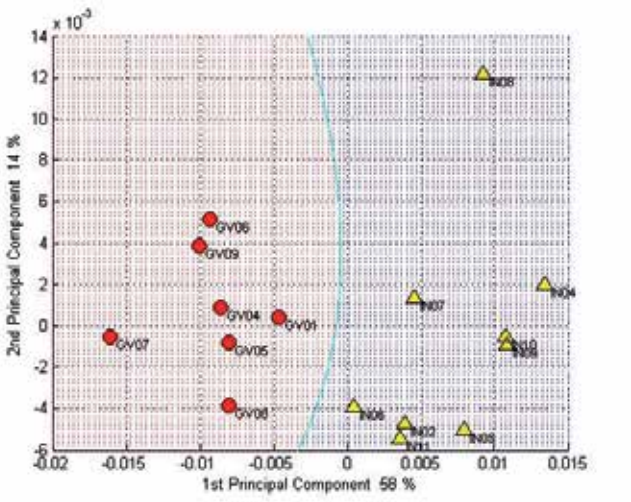
The discrimination ability of the three methods mentioned above was analyzed. For the data processing the absorption and desorption rates were taken into account in addition to the ratio of the peak value of each sensor response to the base-line value. Since different sensors have distinct desorption times, for each chemical compound, we have considered the integral of each time signal to the base line integral ratio.

The usual PCA of the data obtained with Methods II and III was performed. Figures 8 (a) and 8 (b) show the score plot obtained with both methods. It is evident that better discrimination is achieved with Method III.

A classification following the discrimination step was designed for Method III using about 70% of the available samples of both A and B oils.



(a)



(b)

Fig. 8. PCA (a) Method II (b) Method III (discriminating regions are shown).

For validation purposes, 30% of the measured samples were disregarded. Within the resultant restricted space, an unsupervised PCA was performed. It was then possible to establish multivariate confidence regions for the two identified classes: olive oil A and olive oil B. The assignment of a new multivariate measurement x to a given category k occurs when the quadratic discriminant $d_k(x)$ is maximized:

$$d_k(x) = -\frac{1}{2}\ln\left|\Sigma_k\right| - \frac{1}{2}m_k(x)$$

$$m_k(x) = (x - \mu_k)^T \Sigma_k^{-1} (x - \mu_k)$$

where $m_k(x)$ is the statical or Mahalanobis distance, μ_k is the mean value of the corresponding class and μ_k is the covariance matrix within each class sample.

3. Conclusions

An easy to implement method to improve electronic nose discrimination ability of a priori similar odours has been presented. This technique has been then applied to the case of virgin olive oils. The way laser vaporization of the samples improves this task has been additionally explored. The behaviour of the gas phase headspace following pulsed and cw laser irradiation with different wavelengths has been analyzed.

The use of pulsed IR lasers increases the sensitivity of the e-nose performance. Furthermore, the use of a CO_2 laser allows a better discrimination than the use of a Nd:YAG laser. When using the CO_2 laser, the signal-to-noise ratio (S/N) is increased by an order of magnitude with respect to the S/N without laser vaporization effect. The IR laser wavelength influences the discrimination capabilities of the method, probably due to the different IR absorption properties of the sample compounds. Further experiments in progress may shed some light on this question.

The use of continuous wave visible diode lasers (methods II and III) produces significant changes in the V-UV spectrum of one of the EVOO, (EVOO A). Irradiation with the diode laser at 450 nm produces larger changes than those produced by the 650 nm diode laser irradiation. The 450 nm laser induces chemical reactions in the liquid oil surface and as a result precludes the discrimination. On the other hand, Laser Vaporization at 650 nm modifies the vapour-liquid equilibrium conditions improving the selectivity of the electronic nose.

It is important to emphasize that although the discrimination obtained with IRLV is larger than that resulting from LV at 650 nm it is insensitive to the recognition pattern used. On the other hand, diode lasers are considerably cheaper than high power TEA CO_2 lasers and they produce very good results considering the benefit-cost ratio.

4. Acknowledgements

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9

Traceability of Origin and Authenticity of *Olive Oil*

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1. Introduction

Olive (*Olea europaea* L.) is one of the oldest agricultural tree crops worldwide and is an important source of oil with beneficial properties for human health. *Olive oil* is produced solely from the fruit of the olive tree (*Olea europaea* L.) and differs from most of the other vegetable oils in the method of extraction, allowing it to be consumed in crude form, hence conserving its vitamins and other natural healthy high-value compounds.

In comparison to commonly used vegetable oils, the cost of *olive oil* is higher. As such, *olive oil* is more prone against adulteration with other cheaper oils in order to increase profits. Several grades of *olive oil* are marketed which also command different prices. There is also the possibility of the addition of cheaper grades of *olive oil* to better graded ones for the same economic reasons. The presence in highly prized *olive oil*s of lower grade material is sometimes revealed by specific analytical methods.

Mixing low-grade sunflower, canola or other oil with olive's industrial chlorophylls, and flavouring it with beta-carotene has been brought to light and sold as *olive oil*. The FDA does not have the resources to test all the imported *olive oil* for adulteration, and some products are difficult to test. One can always expect adulteration and mislabeling *olive oil* products (Mueller, 1991). Christy et al., (2004) used near-infrared (NIR) spectra in the region of 12,000–4000 cm ($^{-1}$) to detect adulteration of *olive oil* with sunflower oil, corn oil, walnut oil and hazelnut oil. It has reported that adulteration of virgin *olive oil* with hazelnut oil could be detected only at levels of 25% and higher with Fourier transform infrared spectroscopy (Kumar et al., 2011). However, Rabiei (2006) has reported the use of molecular approach in revealing hazelnut-adulterated *olive oil* at level of less than 10% of hazelnut.

Several methods have been proposed for monitoring the adulteration of virgin *olive oil*s with other edible oils. In the last 10 years, technology and knowledge have undergone a great advance in the fight against adulteration; however, in the same way, knowledge of defrauders has also been increased. This enables them to prepare more sophisticated adulterations that make useless the methodologies proposed to detect them. Those oils normally added to virgin *olive oil* can be, either *olive oil*s of lower quality (e.g. olive-pomace *olive oil* or virgin *olive oil* obtained by second centrifugation of the olives), or seed oils (e.g. corn, soybean, palm or sunflower oil, among others) (Peña et al., 2005).

It has long been known that the chemical composition of virgin *olive oil* is influenced by genetic (variety) and environmental (climatological and edaphologic conditions) factors. So the olive production area is greatly responsible for the specific characteristics of *olive oil*.

The analytical analyses have their limits. This has promoted a growing interest towards the application of DNA-based markers since it is independent from environmental conditions. Specific protocols for DNA isolation from *olive oil* have been developed (Breton et al., 2004; Busconi et al., 2003; Consolandi et al., 2008; De la Torre et al., 2004). The application of DNA-based methods requests the knowledge on nucleotide sequences of olive. This information for olive is back to 1994, when the first *Olea europaea* L. sequence has deposited in NCBI. Table 1 provides brief information on olive genomics presented on NCBI from 1994 to July 2011 consist of Nucleotides, ESTs and GSS accessions.

T*raceability* in food is a recently developed concept of control of the whole chain of food production and marketing that would trace back to each step of the process. In a narrower sense, *genetic traceability* is performed to find out the genetic identity of the plant material from which the transformed products have originated. The recognition of the genetic background underlying food products aims to prove the authenticity of valuable food and to discourage from the adulteration with extraneous material of lower cost and value. Recently, Rotondi et al., (2011) has performed *olive oil* traceability by means of a combination of the chemical and sensory analyses with SSR biomolecular profiles. Her group demonstrated that the genetic (SSR analysis) component and the selected fatty acids (eicosenoic, linoleic, oleic, stearic, palmitic and linolenic), seems to represent a possible tool for inter- and intra-varietal characterisation and for monovarietal traceability.

Year	Olea europaea accessions on NCBI database*			
	Total nucleotide sequences	**Nucleotide**	**EST****	**GSS*****
1994	3	3		
1995	1	1		
1996	1	1		
1997	13	13		
1998	1	1		
1999	11	11		
2000	39	39		
2001	57	57		
2002	57	57		
2003	88	64	24	
2004	23	23		
2005	213	213		
2006	44	44		
2007	337	335		2
2008	186	186		
2009	4891	55	4,836	
2010	1871	690	1,159	22
07/2011	33			
Total sequences	7,869	1,793	6,019	24

*http://www.ncbi.nlm.nih.gov, **EST: expressed sequence tags, ***GSS: genome survey sequences

Table 1. Olive genomics information present on NCBI database from 1994 to July 2011

2. General description of olive plant

Olive (*Olea europaea* L.) is the main cultivated species belonging to the monophyletic *Oleaceae* family that comprises 30 genera and 600 species, within the clade of Asterids, in which the majority of nuclear and organellar genomic sequences are unknown. The Olea genus includes 30 species and has spread to Europe, Asia, Oceania and Africa (Bracci et al., 2011). The wild olive or oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris*) and the cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) are two co-existing forms of the subspecies *europaea* (Green 2002). Other five subspecies constitute the *Olea europaea* complex including *laperrinei*, present in Saharan massifs; *cuspidata*, present from South Africa to southern Egypt and from Arabia to northern India and south-west China; *guanchica* present in the Canary Islands; *maroccana* present in south-western Morocco; and *cerasiformis* present in Madeira (Green 2002).

The Mediterranean form (*Olea europaea*, subspecies *europaea*) includes the wild and cultivated olives is a diploid species (2n = 2x = 46) (Kumar et al., 2011). The origin of the olive tree is lost in time, coinciding and mingling with the expansion of the Mediterranean civilisations which for centuries governed the destiny of mankind and left their imprint on Western culture.

The common olive is an evergreen tree that grows up to ~12m in height with a spread of about 8 m. However, many larger olive trees are found around the world, with huge, spreading trunks. The trees are also tenacious, easily sprouting again even when chopped to the ground. Sometimes it is difficult to recognize which is the primary trunk. The tree can be kept at a height of about 5m with regular pruning. Olives are long-lived, with a life expectancy of greater than 500 years (Kumar et al., 2011).

Most olive-growing areas lie between the latitudes 30◦ and 45◦ north and south of the equator, although in Australia some of the recently established commercial olive orchards are nearer to the equator than to the 30◦ latitude and are producing a good yield; this may be because of their altitude or for other geographic reasons.

The olive fruit is termed a *drupe* botanically, which are green in color at the beginning and generally become blackishpurple when fully ripe. A few varieties are green even when ripe, and some turn a shade of copper brown. Olive fruits consist of a carpel, and the wall of the ovary has both fleshy and dry portions. The skin (exocarp) is free of hairs and contains stomata. The flesh (mesocarp) is the tissue that is eaten, and the pit (endocarp) encloses the seed. Olive cultivars vary considerably in size, shape, oil content and flavor. Raw olive fruits contain an alkaloid that makes them bitter and unpalatable. A few varieties are sweet enough to be eaten after sun-drying (Wiesman, 2009).

Olive cultivars are basically classified into “oil olives” and “table olives,” and oil cultivars predominate. Olive cultivars are also classified according to the origin of the cultivar – for example, Spanish, Italian, Greek, Syrian, Moroccan, Israeli, etc. The most popular cultivars are: Picual, Arbequina, Cornicabra, Hojiblanca and Empeltre in Spain; Frantoio, Moraiolo, Leccino, Coratina and Pendolino in Italy; Koroneiki in Greece; Chemlali in Tunisia; Ayvalik in Turkey; Nabali, Suori and Barnea in Israel and The West Bank; Picholin in France; Mission in California; and various varieties in Australia. The table olive cultivars include Manzanilla and Gordal from Spain; “Kalamata” from Greece; “Ascolano” from Italy; and “Barouni” from Tunisia (Jacoboni & Fontanazza, 1981; Weissbein, 2006).

The large number of cultivars, added to the many cases of synonymous and homonymous name, makes particularly difficult the description and classification of olive varieties (Fabbri et al. 2009). Notice that two varieties are synonymous when they have different names but the same profile of fingerprinting, and two varieties are homonyms when they have the same name but different fingerprinting profiles.

The size of olive germplasm is controversial: about 1,250 varieties (or in some other references 1,275 cultivars, Sarri et al., 2006), cultivated in 54 countries and conserved in over 100 collections, were included in the FAO olive germplasm database (Bartolini 2008), also if this number is certainly higher because the lack of information on many local cultivars and ecotypes (Cantini et al. 1999). The most part of these cultivars comes from southern European countries such as Italy (538 varieties), Spain (183), France (88) and Greece (52) (Baldoni & Belaj 2009). Due to this richness of the germplasm, olive is an unusual case among horticultural crops and its biodiversity can represent a rich source of variability for the genetic improvement of this plant.

3. Olive oil

It has been known that climate, soil, variety of tree (cultivar) and time of harvest account for the different organoleptic properties of different *olive oil*s. Two factors are influential: where the olives are grown and which harvesting methods are implemented. Certain locations yield more bountiful harvests; consequently their oil is sold for less. Olive trees planted near the sea can produce up to 20 times more fruit than those planted inland, in hilly areas like Tuscany. It is in these land-locked areas that the olive trees' habitat is pushed to the extreme; if the conditions were just a little more severe, the trees would not survive. Extra-virgin oils produced from these trees have higher organoleptic scores.

It is extremely difficult to establish the origins of *olive oil* using DNA technologies. One approach has been **to target yeasts associated with olives and** *olive oil*. Target for characterization was the LTR retrotransposon (Ty element) (Lenoir et al., 1997, as cited in Popping, 2002) using amplified fragment length polymorphism or similar techniques. This method has been more successful for olives, where different yeast strains are associated with olives and *olive oil*. The yeast strains in *olive oil* appear to be associated with the production site (fattoria) where the *olive oil* was produced. And since the number of production sites is limited, the *olive oil* can be traced back to the fattoria.

But this technique is not yet applicable for routine analysis. For the identification of the origin of *olive oil*, a second, non-DNA-based technology has proven very useful. The technology is called **site-specific natural isotope fractionation nuclear magnetic resonance (SNIF-NMR)** (Gonzalez et al., 1999; Martin et al., 1996).

The basis of this technology is that certain elements have naturally occurring stable isotopes (^{16}O and ^{18}O, ^{1}H and ^{2}H, ^{12}C and ^{13}C). The ratios of the different stable isotopes vary from one geographic location to another. These ratios are maintained in the organic material from that region, e.g. plants, animals etc. The SNIF-NMR technology allows measuring these stable isotope ratios at individual positions in a given molecule.

With an appropriate database listing the location and typical stable-isotope distribution, the origin of *olive oil* can be identified (Popping, 2002).

Other techniques such as proton transfer reaction mass spectrometry (PTR-MS), nuclear magnetic resonance spectroscopy (NMR) or high performance liquid chromatography (HPLC) has also been addressed using different methodologies (Luykx & van Ruth, 2008). However, the chemical composition of *olive oil* may differ among seasons and growing areas. Several investigations concerning the origin and authenticity of *olive oil* have shown that chemical analyses per se are not sufficient to assure *olive oil* authenticity or to reveal *olive oil* region (Gimenez et al., 2010). Christopoulou et al., 2004 expressed that no single known parameter could detect the presence of hazelnut and almond oils in *olive oil* which have many chemical characteristics (fatty acid profile, sterol composition, …) similar to *olive oil*.

Several Protected Denomination of Origin (PDO) *olive oil* regions have been established by legislation to ensure both producer's profits and consumer's rights. In this context, it is mainly the identification of the olive cultivar used for the oil production which is of importance for authentication (Luykx & van Ruth, 2008) since the contribution of cultivars is known for each designation (Gimenez et al., 2010). Different PDO labels such as "Oliva Cilento", "Colline Salernitane", and "Penisola Sorrentina", have been granted for the extra-virgin *olive oil* produced in Campania, and some others are in the process of assignment.

4. World olive oil production

According to the report of the International Olive Council (IOC) (the International *Olive oil* Council, IOOC, until 2006), Mediterranean countries account for around 97 percent of the world's olive cultivation, estimated at about 10,000,000 hectares. There are more than 800 million olive trees currently grown throughout the world, of which greater than 90 percent are grown for oil production and the rest for table olives. It is estimated that more than 2,500,000 tons of *olive oil* are produced annually throughout the world.

Since the mid-1990s, Spain has consistently been the largest producer; in the year 2004/05 it produced 826,300 tons of *olive oil* and it is expected a sum of 2,948,000 tons for total world olive oil production in 2010/11. The main producer is still European Union (EU), with 2.1 million tons, of which 1.2 from Spain (-14% from the previous campaign), 336000 tons produced by Greece, 480000 tons by Italy, 67500 tons by Portugal, 65000 tons by Cyprus and 6000 tons by France. Out of EU, IOOC estimated a production of 193500 tons from Syria, 160000 tons from Turkey, 12000 from Tunisia, 150000 from Morocco, 48000 from Algeria, 24900 from Palestine, 19000 from Jordan, 18000 from Australia, 17500 from Argentina, and 15000 from Lybia.

The world *olive oil* consumption (2010-2011) will reach 2.98 million tons, with a 3.65 % increase from the previous campaign 2009-2010 (*IOC website*).
IOOC has estimated that the world export will increase of 5.05% and reach 707000 tons, with EU (438000 tons) as the main commercial power, followed at great distance by Morocco (40000 tons), Syria (50000 tons), Tunisia (90000 tons), and Turkey (38000 tons).

The import for the period between October 1st 2010 and September 30th 2011 is estimated at 648000 tons, with a 2.93% increase from the previous year (*IOC website*).

The pattern of production of *olive oil* during these years shows big fluctuations from one year to the next; however, Spain, Italy and Greece remain the three largest *olive oil* producing countries, dominating the world annual *olive oil* production. This signifies a high level of uncertainty regarding production levels. In the year 2004/05, Spain, Italy and

Greece produced 32, 28 and 13.5 percent of the world's *olive oil*, respectively. However, the recent expansion of the *olive oil* industry and significant contribution to the global *olive oil* market by several other countries, such as Australia and the United States, may lead to stabilization of the market in the near future.

5. *Olive oil* traceability

Food traceability implies the control of the entire chain of food production and marketing, allowing the food to be traced through every step of its production back to its origin. The verification of food traceability is necessary for the prevention of deliberate or accidental mislabeling, which is very important in the assurance of public health. Thus, several regulations provide the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food.

In the case of *olive oil*s, the increase in the demand for high-quality *olive oil*s has led to the appearance in the market of *olive oil*s elaborated with specific characteristics. They include oils of certain regions possessing well-known characteristics, that is, *olive oil*s with a denomination of origin, or with specific olive variety composition, that is, coupage or monovarietal *olive oil*s. *Olive oil*s obtained from one genetic variety of olive or from several different varieties are called monovarietal or coupage, respectively. Monovarietal *olive oil*s have certain specific characteristics related to the olive variety from which they are elaborated (Montealegre et al., 2010). However, coupage *olive oil*s are obtained from several olive varieties to achieve a special flavor or aroma.

The appearance of denominations and protected indications of origin has promoted the existence of oils labeled according to these criteria. Regulation 2081/92 (2) created the systems known as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and the ''Traditional Speciality Guaranteed" (TSG) to promote and protect food products (Table 2).

General regimen	Origin	Characteristics	Restriction
Protected Designation of Origin (PDO)	In that region, specific place, or country	Quality essentially or exclusively due to a particular geographical area	Produced, processed and prepared in a given geographical area
Protected Geographical Indication (PGI)	In that region, specific place, or country	Slightly less strict; food reputation of a product from a given region is sufficient	One of the stages of production, processing, or preparation takes place in the area

*Council Regulation (EC) 510/2006, March 20, 2006.

Table 2. General regimen for food and certain other agricultural products based on Regulation 510/2006*

For example, an *olive oil* with a PDO denomination requires meeting precise definition of several parameters such as cultivar, geographical origin, agronomic practice, production

technology, and organoleptic qualities (Gimenez et al., 2010), and all of these parameters have to be investigated to study its traceability and to certify its quality. Among the above-mentioned factors, the two first are the most important (Montealegre et al., 2010).

Additionally, a Database of Origin and Registration (DOOR) was created to support these denominations (Montealegre et al., 2010). Based on the report of the *International Olive Council* (http://www.internationaloliveoil.org) gave the world production of *olive oil* in 2008/2009 as 2,669.5 million tons and it consumption for the same period as 2,831.5 million tons. It is quite clear that some of the *olive oil* sold has been mislabeled. *Olive oil* is priced from $13–105 for 500 mL where as canola oil and sunflower oils available from less than $1–10 for 500 mL.

The introduction of certifications of origin and quality for virgin *olive oil* as PDO makes necessary the implementation of traceability procedures. It seems that DNA analysis to be a promising approach to this problem, since it is less influenced by environmental and processing conditions in respect to other methods (i.e.; metabolites).

Any research dealing with *olive oil* traceability is focused on investigating the botanical or geographical origin. However, the concept of geographical traceability, in which the objective is the geographical location of the olive tree, is slightly different from the concept of botanical traceability, in which the olive used for the *olive oil* production is the aim. In both cases, the selection of the markers (compounds with discriminating power) to be studied is complicated because the composition of extra virgin *olive oil*s is the result of complex interactions among olive variety, environmental conditions, fruit ripening, and oil extraction technology (Araghipour et al., 2008).

5.1 Traceability to the botanical origin

The verification of the cultivars employed to produce an *olive oil* sample may contribute to address the oil origin. This fact may have commercial interest in the case of monovarietal *olive oil*s or *olive oil*s with PDO because these high-quality *olive oil*s may be adulterated by other oils of lower quality, using anonymous or less costly cultivars (Breton, 2004).

As the quality of an *olive oil* depends on the olive variety from which it is elaborated, the production of *olive oil*s from certain varieties has increased (Sanz-Cortes et al., 2003). The olive variety selection is based on its adaptation to different climatic conditions and soils. In addition, whereas some cultivars are characteristic of a given zone, others can be found in several countries (Japon-Lujan et al., 2006). As a consequence, one olive variety can be cultivated and nominated in a different way in distinct geographical locations, which makes the differentiation of olive varieties in *olive oil*s quite complex. (Montealegre et al., 2010)

Traditionally, differentiation among olive cultivars has been supported by numerous morphological (study of the form or shape) and pomological (the development, cultivation, and physiological studies of fruit trees) traits. Unfortunately, morphological traits have been difficult to evaluate, are affected by subjective interpretations, and are severely influenced by the environment and plant developmental stage (Sanz-Cortes et al., 2003). Nowadays, several efforts have been focused on the investigation of one or several compounds present in *olive oil*s usable to differentiate olive varieties (Montealegre et al., 2010). Compositional markers (substances that take part of the composition of the olive oils) include major and minor components. Major, (sterols, phenolic compounds, volatile compounds, pigments,

hydrocarbons, and tocopherols, fatty acids and triglycerides, components may provide basic information on olive cultivars. Minor components, can provide more useful information and have been more widely used to differentiate the botanical origin of *olive oil*s (Montealegre et al., 2010).

5.2 Compositional markers

There are several parameters, major and minor compositional markers, with varied discriminant power used for *olive oil* traceability according to the variety of olive participated in the production of the oil (Arvanitoyannis et al., 2007).

To relate the fatty acid composition of *olive oil*s with the cultivar, Mannina et al. (2003) studied *olive oil* in a well- limited geographical region, with no consideration of the pedoclimatic factor (soil characteristics such as temperature and humidity). A relationship between the fatty acid composition and some specific cultivars has been observed (Montealegre et al., 2010).

The *volatile fraction* in *olive oil*s, which represents one of the most important qualitative aspects of this oil, consists of a complex mixture of more than 100 compounds, but the most important substances useful for olive cultivar differentiation are the products of the lipoxygenase pathway (LOX). Only a subset of volatile compounds and a combination among them could provide valuable information for olive cultivar differentiation (Montealegre et al., 2010). Three volatile compounds [hexyl acetate, hexanal, and (E)-hex-2-enal] and the total concentration of ketones have nominated to distinguish the olive varieties (Tena et al., 2007) hich consequently could be used for olive oil, as well. However, it has been found that the level of (E)-hex-2-enal in the analyzed samples showed a variability that suggest an influence of genetic factors on the biosynthesis of this compound. In fact, genetic (Tura et al., 2008; Mahjoub-Haddada et al., 2007) and geographic (Mahjoub-Haddada et al., 2007) factors influence the volatile compound production of the olive fruits and affect the differentiation of *olive oil*s according to their olive variety. The volatile compound contents allowed differentiation among monovarietal *olive oil*s and even identification of the technique used for *olive oil* production (Torres Vaz-Freire et al., 2009).

The color of a virgin *olive oil* is due to the solubilization of the lipophilic chlorophyll and carotenoid pigments present in the fruit. The green-yellowish color is due to various pigments, that is, chlorophylls, pheophytins, and carotenoids (Cichelli & Pertesana, 2004). Chlorophyll a is the major chlorophyll pigment, followed by chlorophyll b. The carotenoid fraction is included lutein, violaxanthin, neoxanthin, β-carotene, β-cryptoxanthin, and luteoxanthin (Montealegre et al., 2010).

Several researchers reported the same qualitative composition in chlorophyll and carotenoid pigments, independent of the olive variety and the time of picking (Giuffrida et al., 2007 ; Roca et al., 2003).

Cerretani et al. (2006) showed that the carotenoid and chlorophyll content determination using UV-vis spectrophotometry was not useful to discriminate oils produced from different olive varieties. Lutein/β-carotene ratio has been reported as a tool to differentiate oils from a single cultivar.

Tocopherols and hydrocarbons are the compositional markers less studied to date to differentiate *olive oil*s. An important common aspect is that the content and composition of

these markers are highly affected by the environmental conditions, the fruit ripening, and the extraction technology (Montealegre et al., 2010).

6. DNA-based markers

Molecular markers are investigated as a diagnostic tool for food authenticity and traceability of variety/type composition of complex food matrices in an increasing number of projects (Palmieri et al., 2004). DNA-based methods make an important contribution to protect high-quality *olive oil*s.

Significant amounts of DNA are present in *olive oil* obtained by cold pressing (Consolandi et al., 2008). However, the filtration process lowers DNA concentrations, which tend to disappear due to nuclease degradation (De la Torre et al., 2004; Muzzalupo et al., 2002). On the other hand, the length of storage after milling of the oil can affect the use of DNA as an analyte for molecular traceability. Pafundo et al., 2010 observed a significant decrease of quality of DNA extracted from olive oil, with a consequent loss of information a month later from olive oil production.

Spaniolas et al. (2008b) has used lambda DNA as a marker to monitor the length of DNA fragments in olive oil during storage time when determined the varietal origin of olive oil. Lambda DNA is a linear molecule of approximately 50 kb, a length that probably resembles that of olive DNA present in olive oil. Based on the fact, PCR-based fingerprinting techniques, which require templates longer than 100 bp, might not be able to successfully amplify the target sequences from olive oil samples low in DNA content and stored for several months, they conduct their analyses. They have deduced the detection of polymorphic markers requiring DNA templates shorter than 100 bp might have a wider range of applications in DNA fingerprinting of olive oil.

In *olive oil*, once the barrier of DNA extraction has been overcome, several markers could be used to identify olive cultivars that made up a certain *olive oil*. (Consolandi et al., 2008)

DNA recovery methods from *olive oil* have been developed by many authors (Busconi et al. 2003; Doveri et al. 2006; Pasqualone et al. 2007; Consolandi et al. 2008). Several commercial kits, providing adapted protocols, were used in different works (Martins-Lopes et al. 2008; Spaniolas et al. 2008a; Ayed et al. 2009; Pafundo et al. 2010). All of these studies confirmed that the DNA of the cultivars is recoverable from extra virgin *olive oil*, but it has low quantity and quality. The first researches, carried out using genomic DNA extracted from drupes. That DNA had a good potential to amplify correctly using RAPDs markers (Cresti et al. 1997). By means of SCAR and AFLP markers, Busconi et al. (2003) were able to show that DNA recovered from *olive oil* had both organellar and nuclear origin. Pafundo et al. (2005) traced the cultivar composition of monovarietal *olive oil*s by AFLPs, suggesting that DNA extraction is the most critical step affecting the procedure. Pafundo et al. (2007), performed amplification of DNA isolated from *olive oil* using AFLPs. They have also developed some SCARs to amplify successfully the DNA extracted from *olive oil*. Using SSR analysis, Pasqualone et al. (2007) demonstrated that microsatellites are useful in checking the presence of a specific cultivar in a PDO oil, thus verifying the identity of the product. However, they obtained only the marker profile of the main cultivar in the oil: no signal was detected for the secondary varieties. Montemurro et al. (2008) analyzed ten virgin monovarietal *olive oil*s prepared in the laboratory by AFLP markers. They were able to

distinguish all the *olive oil*s examined, even if only a partial correspondence with the AFLP profile obtained from the leaves was obtained. Martins-Lopes et al. (2008) evaluated the efficiency of RAPD, ISSR and SSR molecular markers for *olive oil* varietal identification and their possible use in certification purposes (Bracci et al., 2011).

Consolandi et al. (2008) reported the development of a semi-automated SNP genotyping assay to verify the origin and the authenticity of extra-virgin *olive oil*s. The authors developed a Ligation Detection Reaction (LDR)/Universal Array (UA) platform by using several olive SNPs. They found that 13 accurately chosen SNPs were sufficient to unequivocally discriminate a panel of 49 different cultivars (Bracci et al., 2011).

Doveri et al. (2006) published a cautionary note on the use of DNA markers for provenance testing. Their observations were based on non-concordance between the genetic profiles of *olive oil* and fruit. They suggested that this could be due to the contribution of pollen donors in DNA extracted from the paste obtained by crushing whole fruits. They concluded that care needs to be taken in the interpretation of DNA profiles obtained from DNA extracted from oil for resolving provenance and authenticity issues (Martins-Lopes et al., 2008). It is to note that, the possible presence of additional alleles due to paternal contribution in oils extracted from entire drupes, should be taken into consideration for variety traceability purposes when comparing the amplification profiles of leaves with the corresponding oils for (Alba et al., 2009)

In a recent study, Pafundo et al. (2010) investigated the effect of the storage time on the degradation of the DNA purified from the oil; a negative correlation between storage time and quality–quantity of recovered DNA has been observed. The authors showed that 1 month after the production of the oil the degradation increases making harder traceability goals (Bracci et al., 2011). Table 3 presents a brief report on the application of DNA-based molecular markers for cultivar traceability in *olive oil*.

Molecular marker	Developers	References
RAPD (Random Amplified Polymorphic DNA)	Williams et al. (1990)	Muzzalupo and Perri (2002), Martins-Lopes et al. (2008)
AFLP (Amplified Fragment Length Polymorphism)	Vos et al. (1995)	Busconi et al. 2003, Pafundo et al. 2005
SCAR (Sequence Characterized Amplified Region)	Paran & Michelmore (1993)	De la Torre et al. (2004), Pafundo et al. (2007)
SSRs (Simple Sequence Repeats)	Morgante & Olivieri (1993)	Martins-Lopes et al. (2008), Alba et al. (2009), Rabiei et al. (2010)
ISSR (Inter Simple sequence Repeats)	Zietkiewicz et al. (1994)	Pasqualone et al. (2001), Martins-Lopes et al. (2008)
Chloroplast and mitochondrial (Direct sequencing)	Botstein et al. (1980)	Intrieri et al. (2007)

Table 3. Applications of DNA-based molecular markers for cultivar traceability in *olive oil*

6.1 Random Amplified Polymorphic DNA (RAPDs)

The applicability to the traceability of cultivars in the *olive oil* by means of RAPDs was evaluated by Muzzalupo & Perri (2002). This kind of markers was used, together with other DNA-based markers, in the construction of the first olive linkage maps (De la Rosa et al. 2004; Wu et al. 2004) as well.

6.2 Amplified Fragment Length Polymorphism (AFLPs)

AFLPs have been widely used for DNA fingerprinting of cultivars (Angiolillo et al. 1999; Owen et al. 2005), to analyse the relationships between wild and cultivated olive (Baldoni et al. 2006), for the construction of linkage maps (de la Rosa et al. 2003) and for cultivar traceability of *olive oil* (Busconi et al. 2003; Pafundo et al. 2005).

6.3 Development of sequence-characterized amplified regions (SCARs)

SCARs have been used for cultivar identification (Busconi et al. 2006) and in *olive oil* traceability (De la Torre et al. 2004; Pafundo et al. 2007). Putative associations of several SCAR markers with fruit characteristics (Mekuria et al. 2002) and resistance to pathogenic fungi (Herna´ndez et al. 2001) were found, suggesting the applicability of this type of marker for marker-assisted breeding programs (Bracci et al., 2011).

6.4 Simple Sequence Repeats (SSRs)

Many authors have reported on SSR development in olive and several of them are currently available for DNA analysis (Cipriani et al. 2002; De la Rosa et al. 2002; Rabiei & Tahmasebi Enferadi 2009; Sabino Gil et al. 2006; Sefc et al. 2000). This technique benefits from the use of microsatellites which are short stretches (1-6-bp long) of DNA, tandemly repeated several times. The number of repeats can vary from one individual to another. Besides, they are abundant in eucaryotic genomes. A combination of several SSRs loci allows virtually discrimination of individuals originated through sexual reproduction, especially in out-crossing species, where the level of heterozygosity is high.

In Olea europaea, these markers have been used for different applications such as cultivar discrimination (Sarri et al. 2006; Fendri et al. 2010), study of relationships between wild and cultivated olive tree (Belaj et al. 2007), construction of association maps (De la Rosa et al. 2003), paternity analysis (Mookerjee et al. 2005) and identification of *olive oil* varietal composition (Alba et al. 2009; Ayed et al. 2009; Rabiei et al. 2010). A list of recommended SSR markers and protocols for olive genotyping has been provided with the aim to develop a robust method to track the origin of olive cultivars (Doveri et al. 2008; Baldoni et al. 2009) (table 4).

Series	Number	Institute	References
ssrOeUA-DCA	15	Boku, Vienna, Austria	Sefc et al 2000
IAS-oli	5	CSIC, Córdoba, Spagna	Rallo et al 2000
GAPU	20	Agrobios, Matera, Italia	Carriero et al 2002
UDO	30	Università di Udine, Italia	Cipriani et al 2002
EMO	7	ETSIAM, Córdoba, Spagna	De La Rosa et al 2002
Totale	*77*		

Table 4. List of microsatellites isolated in olive which their related primers are available in the literature, most of them have been used in case of *olive oil* traceability (Bracci et al., 2011)

For studying the informative potential of the microsatellites, the observed (*Ho*) and expected (*He*) heterozygosities generally are calculated using the software POPGENE ver. 1.31 (Yeh et al., 1999, as cited in Alba et al., 2009). *He* values were estimated using the formula proposed by Nei et al. (1973):

$$H_e = 1 - \sum p_i^2 \quad (1)$$

where *pi* is the frequency of the *ith* allele. The power of discrimination (PD) [21] of microsatellite primer pairs are also calculated as reported by Cipriani et al. (2002), where the allele frequency of the *He* formula is replaced by the genotype frequency.(Alba et al., 2009)

A research carried on the use of SSRs as a tool to identify the genetic background of *olive oil* which was involved the analysis of DNA sequences using a panel of seven simple sequence repeats (SSRs) to provide genotype-specific allelic profiles (Rabiei et al., 2010). The amplified SSR fragments and the DNA profiles from the monovarietal oil corresponded to the profiles from the leaves of the same cultivar. The most reliable SSR in providing correct allele sizing in distinguishing either single cultivar *olive oil* samples or the different ratios of their blends are DCA3, DCA4, DCA16, DCA17, and GAPU101, while DCA9, GAPU59 produced less concordance against data obtained by the genetic analysis of leaf samples. Desalted PCR product has been analyzed on a MegaBACE 500 capillary sequencer (Amersham Biosciences) using Genetic Profiler v2.0 software to estimate allele sizes (figure 1).

Rabiei et al., 2010 concluded PCR product purification and selection of a set of markers with a highly robust amplification pattern is needed to have reproducible results in certify the genetic background of *olive oil*.

6.5 Inter simple sequence repeat (ISSR) polymorphisms

ISSRs are DNA fragments of about 100–3,000 bp located between adjacent, oppositely oriented microsatellite regions. These markers were used with success to distinguish 10 Italian varieties, by analysing genomic DNA extracted from the olive fruit (Pasqualone et al. 2001), and for the study of cultivar traceability in *olive oil* (Pasqualone et al. 2001, Martins-Lopes et al. 2008).

6.6 Chloroplast genome sequencing

A very important results, recently published, in *Olea europaea* L. genomic studies have been the DNA sequencing of the entire plastome of the Italian cultivar 'Frantoio' (Mariotti et al. 2010). This sequence has a length of 155,889 bp and showed an organization and gene order that is conserved among numerous Angiosperms. The olive chloroplast contains 130 genes and 644 repetitive sequences (among which 633 mono-nucleotide SSRs, 6 di-, 3 tetra- 2 penta-nucleotide SSRs were identified) (Bracci et al., 2011)

The annotated sequence was used to evaluate the content of coding genes, the extent, and distribution of repeated and long dispersed sequences and the nucleotide composition pattern. These analyses provided essential information for structural, functional and comparative genomic studies in olive plastids. Furthermore, the alignment of the olive plastome sequence to those of other varieties and species identified 30 new organellar polymorphisms within the cultivated olive. chloroplast DNA polymorphisms has been used as molecular markers to identify cultivars of *Olea europaea* L. (Intrieri et al. 2007).

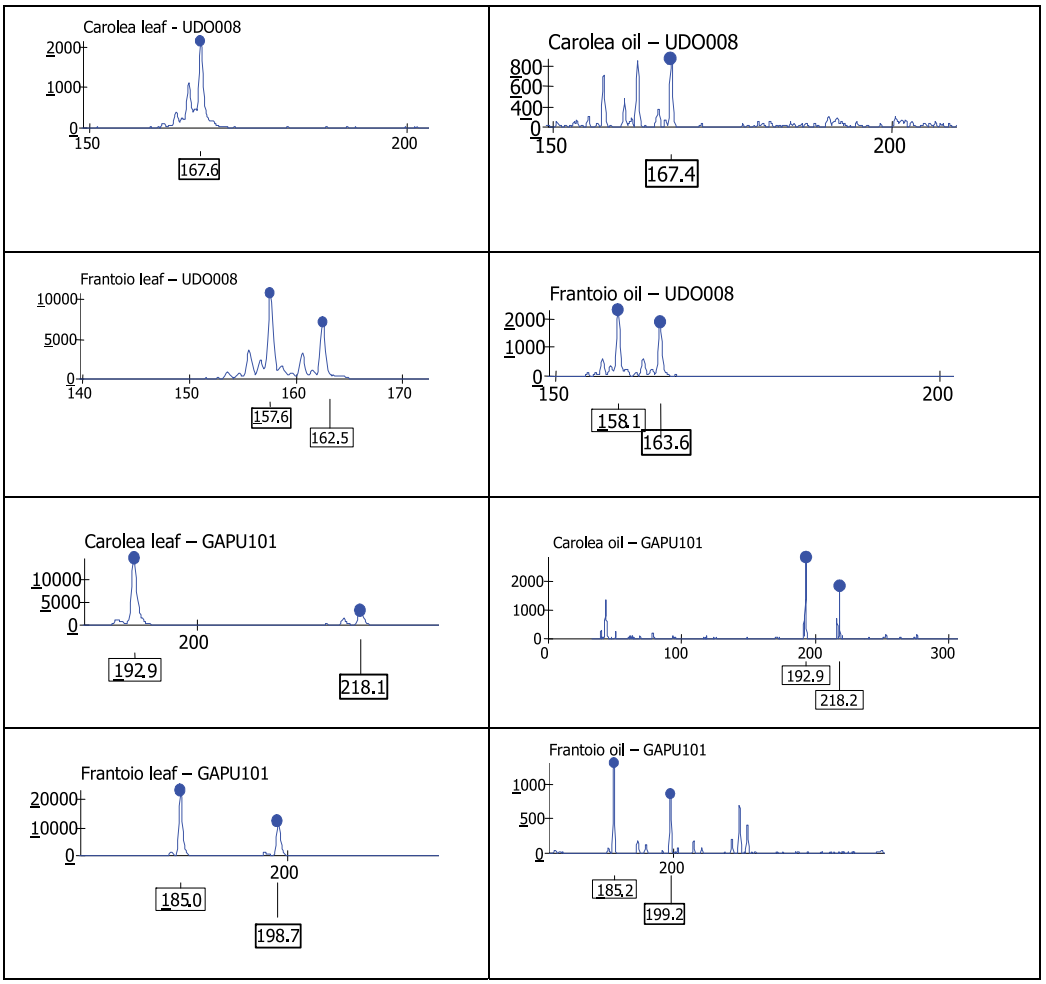

Fig. 1. Electropheregram of PCR products separated by capillary electrophoresis of microsatellite loci UDO008 and GAPU 101, obtained from DNA extracted from Carolea and Frantoio leaves and oils. Allele sizes are below the x axis. The scores on Y-axis are the intensity of amplified allele detection (Rabiei et al., 2010).

6.7 Expressed Sequence Tags (ESTs)

Understanding the function of genes and other parts of the genome is known as functional genomics. In olive, efforts to improve the identification and annotation of genes are prevalently based on EST identification, which are predominantly related to pollen allergens and characteristics of olive fruit (Bracci et al., 2011).

The first nucleotide sequences isolated in 1994 in olive coded for allergenic proteins (Villalba et al. 1994, as cited in Bracci et al., 2011) (Table 1).

6.8 Real time-PCR

The detection of frauds, either due to the mixtures with oils of other species such as hazelnut, or to the certification of PDOs would need quantitative tools. At its best, conventional PCR remains a semi-quantitative technique, and therefore, it is not optimal for authentication purposes when quantification is needed (Gimenez et al.; 2010).

The use of real-time chemistries allows for the detection of PCR amplification during the early phases of the reaction, providing a distinct advantage over detection of amplification at the final phase or end-point of the PCR reaction. qRT-PCR is a useful tool in the development of molecular markers for *olive oil* authentication since it allows inspecting the PCR efficiency. Besides qRT-PCR should be used for the optimisation of the amplicon size and the DNA isolation procedure which are critical aspects in *olive oil* authentication. The potential of cpDNA for *olive oil* authentication should be addressed in the future (Gimenez et al.; 2010).

6.9 DNA barcode

Several sequences from noncoding spacer region between psbA-trnH and partial coding region of matK of plastid genome provided a good discrimination of pure *olive oil* and its admixture by other vegetable oils such as canola and sunflower.

The plastid based molecular DNA technology has a great potential to be used for rapid detection of adulteration easily up to 5% in *olive oil* (Kumar et al., 2011).

7. Paternity analysis

Similar to other woody species, olive is characterized by a long juvenile phase that ranges between 10 and 15 years. This represents a great obstacle to breeding programs and makes the genetic improvement of olive very difficult and expensive. Although seedling-forcing growth protocols have been developed to reduce the length of this phase, the evaluation of the agronomic performance of mature olive plants still requires at least 5 years of experimentation (Santos-Antunes et al. 2005). For this reason, the application of molecular markers both to confirm the parental origins of the progeny and to select early agronomical characteristic-associated markers (Martı´n et al. 2005) can be very useful to reduce the time and cost of the development of new genotypes (Bracci et al., 2011).

With regard the paternity analysis, SSRs are the most suitable to trace the genetic contribution of alleles from the parents to the offspring, being co-dominant and highly polymorphic markers (Mookerjee et al. 2005). The effectiveness of SSRs in the identification of paternity contribution to progeny obtained from olive breeding programs has been demonstrated by several authors (De la Rosa et al. 2004; Diaz et al. 2007). The results demonstrated that SSR analysis is a convenient technique to routinely assess the crosses made in breeding programs and to for check self-incompatibility in olive cultivars (Diaz et al. 2006). These studies have highlighted that no contamination by self-pollen was found, indicating that placing pollination bags well before anthesis is important and that emasculation to avoid selfing is unnecessary (De la Rosa et al. 2004). The analysis also revealed that the main factor affecting the success of crosses seems to be the inter-

compatibility among the parental cultivars, since this significantly influences the rate of contamination from external pollen donors. These results indicate that knowledge of cross-compatibility among cultivars is necessary to plan efficient olive breeding crosses (Diaz et al. 2007).

The possibility of associating genetic characteristics and DNA-based molecular markers is very important to select the progeny showing interesting agronomical traits and even specific organoleptic characteristics at the first stages of development which may use as a marker for future *olive oil* identification. However, this technique, called marker-assisted selection (MAS), requires some knowledge on the co-segregation of molecular markers and genetic characteristics in the progeny. (Bracci et al., 2011)

8. Conclusion

For the inefficiency of analytical parameters in showing variability among samples of the same cultivar/blend due to the environmental conditions and pressing technologies, Several DNA-based technologies and traceability analysis has been used to reveal the different origin of lots that have contributed to the olive oil blend. In this regard, DNA-based methods make an important contribution to protect high quality brand names and in turn the consumer

The knowledge of genome nucleotide sequences also could be useful to identify new sequence polymorphisms, which will be very useful in the development of many new cultivar-specific molecular markers (e.g.; Single Nucleotide Polymorphisms, SNPs) and in the implementation of more efficient protocols for tracking and protect *olive oil* origin (in POD *olive oils*).

The greatest challenges one faces while using DNA technology is the low quality and highly degraded DNA recovered from the fatty matrices and the impact of oil extraction processing on the size of the recovered DNA. DNA of low, difficult to determine content and of unknown, variable quality would potentially lead to inconsistent and consequently inconclusive results. Although, the concentration of DNA did not appear to be limiting; rather, successful PCR amplification likely depended on the ability of the DNA extraction method to free DNA from inhibitors of PCR present in the *olive oil*.

It is to be considered if the DNA is damaged, it could be not properly accessible to the DNA polymerase, which stalls at the sites of damage and the reaction may be interrupted; this being able to influence the length and significance of the synthesized amplicons. The use of proteinase K during extraction process has recommended for a better protection of DNA from degradation and increase in DNA yield, as well.

Identification of molecular markers suitable for tracing the genetic identity of olive cultivars from which oil is produced, on the other hand, has a great importance**.** For making decision, which molecular markers will be more useful in obtaining reliable results through the numerous molecular markers existing in the literature, many of them have been practically examined (including RAPDs, AFLPs, SCARs, SSRs, ISSR, SNPs, …). A combination of molecular markers (RAPD, ISSR, and SSR) to establish a relationship between small-scale-produced monovarietal and commercial *olive oil* samples for certification purposes has been proposed.

Several authors recommended sequences of DNA that show polymorphism at low hierarchical level are therefore suitable for distinguishing between individuals within the same species. They clearly pointed non-coding nuclear DNA sequences could be the best choice. Among those sequences, the microsatellites are likely the most suitable ones. However SNPs that require shorter than 100 bp DNA templates, considered to be successfully used for a wider range of olive oil identification.

In some cases of using microsatellite, the microsatellite profiles obtained with the monovarietal oil-derived DNA were generally consistent with the cultivar used, although some ambiguities were recorded likely due to contamination in monovarietal oils by other cultivars grown in the same block or contaminations occurred at the mill. Moreover, in some cases the lack of matching in leaf and oil profiles has been reported that was due to the presence of embryos in berry seeds that brought the alleles of pollinators. Other cases of mis-amplification were recorded as a missing allele, due either to the preferential amplification of one of the two alleles in oil-derived DNA templates, or to the excess of degradation of the DNA template of the miss allele, that limited the production of a sufficient number of copies of that allele to be detected. In such a case, real-time PCR assay could possibly solve this kind of problems.

To trace out the adulteration in *olive oil* using combined approach of molecular biology and bioinformatics based on unique SNPs present in conserved DNA sequence of plastid genomes of sunflower, canola and olive has been already performed. In general, plastid/chloroplasts are miniature organelles (approx. 5 X 3 μm in size) enclosed in double layer membranes. They are present in abundance (10–100 per cell, and each plastid contains about 100 copies of circular plastid genomes, average size 150 kb) and there is probability that most of the plastid organelles may be left intact due to miniature size when cold pressed to extract the oil from seeds of olive, canola and sunflower.

Moreover, plastid DNA present in extracted oil could be safe from nucleases activities due to double layer membranes and present in large number of copies in comparison to 1–2 nuclear genomic DNA which may be more prone to degradation.

In addition, a new chloroplast marker represents a valuable tool to assess the level of olive intercultivar plastome variation for use in population genetic analysis, phylogenesis, cultivar characterisation and DNA food tracking is recommended.

In summary, molecular biological techniques have become an every-day tool to solve a number of problems and questions in the section of varietal/species identification, fraud, traceability and paternity analysis.

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10

Quality Assessment of Olive Oil by ^{1}H-NMR Fingerprinting

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1. Introduction

Olive oil is the oil extracted exclusively from the fruit of *Olea europea* L. only by means of mechanical methods or other physical procedures that do not cause any alteration of the glyceric structure of the oil thus preserving its characteristic properties. Olive oil is a highly appreciated edible oil, which is an important component of the Mediterranean diet, and is recognized for its potential health benefits.

The International Olive Council (IOC) establishes the definitions and classes of olive oils, based on methods of production and the free acidity of the oil, as well as the trade standard for their commercialization (International Olive Council, 2011). Much analytical work has been done on the authentication and quality assessment of this high added value agricultural product, as well as on the detection of its adulteration for both economic and health considerations (Frankel, 2010; Guillen & Ruiz, 2001). However, these issues continue to be major analytical challenges. In this context, the European Commission launched the TRACE project (http://www.trace.eu.org/) through the Sixth Framework Program under the Food Quality and Safety Priority with the aim of providing reliable analytical strategies to address this kind of problem.

This chapter reports research work on the use of ^{1}H-NMR fingerprinting, combined with pattern recognition techniques, for the quality assessment of olive oil. Two major issues have been studied, the geographical origin of virgin olive oil (VOO) and the stability of VOO at room temperature.

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The information contained in this chapter reflects the authors' views; the European Commission is not liable for any use of the information contained herein.

1.1 Geographical origin of virgin olive oil

At present, 75% of the global production of olive oil takes place in the Mediterranean basin, mainly in Spain, Italy and Greece (International Olive Council, 2011). VOO is permitted to be marketed under a Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), or Traditional Specialty Guaranteed (TSG) label, on the basis of its area and method of production [Council Regulations (EC) No 510/2006 and No 509/2006]. In this context, the characterization of the geographical origin of VOO is becoming increasingly important. According to the EU definition, PDO products are most closely linked to the concept of *terroir* — a sense of place discernible in the flavor of the food. PDO products must be produced, processed and prepared in a specific region using traditional production methods. The raw materials must also be from the defined area whose name the product bears. The quality or characteristics of the product must be due essentially or exclusively to its place of origin, i.e., climate, the nature of the soil and local know-how. Food products with a PGI status must have a geographical link in at least one of the stages of production, processing or preparation. The European Commission has already registered in the "Register of protected designations of origin and protected geographical indications" 86 PDO and 15 PGI olive oils, produced in Italy, Greece, Spain, France, Portugal and Slovenia. As can be expected, given the financial benefits associated with these prestigious labels, it is very likely that economic fraud occurs (e.g. labeling a non-PDO product as a PDO one or adulteration with olive oils that do not fulfill the PDO requirements).

Another fraudulent practice is the mislabeling of the geographical origin of olive oils. The EU established new labeling rules that make origin labeling compulsory for virgin and extra virgin labeled olive oil [Commission Regulation (EC) No 182/2009]. So, oil produced from olives from just one EU Member State or third country has to be labeled with the name of the country of origin. VOO produced from olives from more than one EU Member State has to be labeled as a 'blend of Community olive oils', while oil produced using olives from outside the EU would be labeled as a 'blend of non-Community olive oils' or 'blend of Community and non-Community olive oils', or a reference to the EU and/or third countries of origin. Therefore, analytical methods are urgently needed to guarantee the authenticity and traceability of PDO and PGI olive oils, as well as the country of provenance, to help prevent illicit practices in this sector, and to support the antifraud authorities dealing with these issues.

VOO is made up of triglycerides (more than 98%) and minor components (about 1-2%) such as squalene, α-tocopherol, phytosterols, phenolic compounds, carotenoids, and aliphatic and terpenic alcohols, which constitute the unsaponifiable fraction of the oil (Bortolomeazzi et al., 2001; Harwood & Aparicio, 2000). The chemical composition of this fraction may vary both qualitatively and quantitatively depending on vegetal species, climatic conditions, extraction and refining procedures and storage conditions (Canabate-Diaz et al., 2007; Harwood & Aparicio, 2000), which also greatly influence the organoleptic quality and stability of the oil. The diversity and interdependence between all these factors makes it highly unlikely that these influences would be the same in different regions. Hence, the geographical characterization of VOO addresses all these agronomic, pedoclimatic and botanical aspects which are unique to the oil of each origin (Aparicio et al., 1994).

A considerable number of sensorial (Pardo et al., 2007), physical (F. Marini et al., 2004; Federico Marini et al., 2006) and chemical (Aguilera et al., 2005; Boggia et al., 2002; Lanteri et al., 2002; Federico Marini et al., 2006; Federico Marini et al., 2007) approaches combined with

statistical analysis have been used to distinguish olive oils from different types, botanical, geographical origins and pedoclimatic conditions. For this purpose, fatty acids (Matos et al., 2007; Ollivier et al., 2006), triglycerides (Aranda et al., 2004), sterols (Alves et al., 2005; Matos et al., 2007; Temime et al., 2008), phenolic compounds (Lopez Ortiz et al., 2006; Vinha et al., 2005), and pigments (Cichelli & Pertesana, 2004) have been analyzed by conventional methods that usually require time-consuming pre-treatment methods (solvent extraction, isolation and/or derivatization) followed by chromatographic techniques (Aparicio & Aparicio-Ruiz, 2000) such as GC-MS and/or GC-FID (Bechir Baccouri et al., 2007; Haddada et al., 2007; Temime et al., 2006; Vichi et al., 2005) and HPLC-MS (Canabate-Diaz et al., 2007; Lopez Ortiz et al., 2006). PDO olive oils were distinguished using physicochemical parameters of the oils and chemometric class-modeling tools (Federico Marini et al., 2006), sensory parameters and fatty acid profiles of the oils (Ollivier et al., 2006), or the oil sterol composition (Alves et al., 2005). IRMS measurements of the alcohol and sterol fractions of olive oil also proved to be useful for its geographical characterization (Angerosa et al., 1999).

Fingerprinting techniques such as NMR (Mannina & Segre, 2002), NIR (Mignani et al., 2011), MIR (Reid et al., 2006), fluorescence (Kunz et al., 2011), FT-IR, FT-MIR and FT-Raman (Baeten et al., 2005; Lopez-Diez et al., 2003; Yang et al., 2005) spectroscopies, MS (Vaclavik et al., 2009), GC (Pizarro et al., 2011; Vaz-Freire et al., 2009) and DNA fingerprinting (Martins-Lopes et al., 2008; Ranalli et al., 2008) have been used for the determination of food authenticity (Reid et al., 2006). These types of techniques are particularly attractive since they are non selective, require little or no sample pre-treatment; use small amounts of organic solvents or reagents; and the analysis takes only a few minutes per sample. Chemometric analysis of NIR spectra of virgin olive oils allow us to determine its composition and geographical origin (Galtier et al., 2007). ^{1}H, ^{13}C and/or ^{31}P-NMR analysis of the bulk oil (Rosa M. Alonso-Salces et al., 2010b; Rosa M. Alonso-Salces et al., 2011b; Mannina et al., 2010; Petrakis et al., 2008; Rezzi et al., 2005) or the unsaponifiable fraction of olive oil (R. M. Alonso-Salces et al., 2010), in combination with multivariate techniques, have been used to distinguish VOOs from certain geographical origins. In section 3.2., the achievements of ^{1}H-NMR fingerprinting and chemometrics for the geographical characterization of VOO is reported. ^{1}H-NMR fingerprints of a statistically significant number of authentic VOOs from seven countries, namely Italy, Spain, Greece, France, Turkey, Cyprus and Syria and from three different harvests (2004/05, 2005/06 and 2006/07) were analyzed by pattern recognition and classification techniques, such as principal component analysis (PCA), linear discriminant analysis (LDA) and partial least square discriminant analysis (PLS-DA), to evaluate the best approach to identify the geographical origin at the national, regional and/or PDO level.

1.2 Stability of virgin olive oil

Another matter of major concern regarding the quality of edible oils is their oxidation, not only from the technological and economic point of view but also for safety reasons, due to the undesirable properties of some compounds produced during this process (Guillen & Ruiz, 2001).

The high resistance to oxidative deterioration of VOO is due to both its triglyceride composition, which is low in polyunsaturated fatty acids, and its antioxidant constituents, i.e. polyphenols and tocopherols. The oxidative stability of VOO has been evaluated by

several methods: Rancimat test (Di Lecce et al., 2009; Esquivel et al., 2009; Kamvissis et al., 2008; Mateos et al., 2006; Platero-López & García-Mesa, 2007), oxygen stability index (OSI) (Carrasco-Pancorbo et al., 2007; Ceci & Carelli, 2010; Cercaci et al., 2007; Gómez-Caravaca et al., 2007; Márquez-Ruiz et al., 2008), peroxide value (Carrasco-Pancorbo et al., 2007; Di Lecce et al., 2009; Márquez-Ruiz et al., 2008), AOCS method (Diraman, 2008), conjugated dienes (Deiana et al., 2002) and conjugated trienes (Hrncirik & Fritsche, 2005) analyses, K_{232} and K_{270} UV indexes (Antolin & Meneses, 2000; Cañizares-Macías et al., 2004a; Márquez-Ruiz et al., 2008; Platero-López & García-Mesa, 2007), thermogravimetry analyses (Coni et al., 2004; García Mesa et al., 1993; Gennaro et al., 1998; Santos et al., 2002; Vecchio et al., 2009), differential scanning calorimetry analyses (Vecchio et al., 2009), ^{13}C NMR on chromatographically enriched oil fractions (Hidalgo et al., 2002), high-resolution chromatographic techniques coupled with UV spectrometry or mass spectrometry (B. Baccouri et al., 2008; Gallina-Toschi et al., 2005; Tena et al., 2009), capillary electrophoresis (Carrasco-Pancorbo et al., 2007; Gallina-Toschi et al., 2005), ultrasound-assisted method (Cañizares-Macías et al., 2004b; Platero-López & García-Mesa, 2007), microwave-assisted method (Cañizares-Macías et al., 2004a), chemiluminescence (Navas & Jiménez, 2007), electron paramagnetic resonance (Papadimitriou et al., 2006), and ORAC assay (Ninfali et al., 2002), among others. Fingerprinting techniques such as NMR (Alonso-Salces et al., 2011a; Guillen & Ruiz, 2001, 2006), FTIR (Guillen & Cabo, 2000), and fluorescence (Guimet et al., 2005; Tena et al., 2009) spectroscopies, DNA fingerprinting (Spaniolas et al., 2008), electronic nose (Lerma-García et al., 2009), and Oxitest method (Kamvissis et al., 2008; Mora et al., 2009) have been also used successfully to study edible oil stability.

Most of the studies on the oxidative stability of olive oil employed questionably high-temperatures which, unfortunately, cannot be considered reliable to predict the stability of olive oils at room temperature (Frankel, 2010), i.e. under normal storage conditions. This is due to the fact that the mechanism of lipid oxidation changes at the elevated temperatures at which these experiments were run. In this sense, the rate of lipid oxidation is independent of O_2 pressure at ambient temperatures; whereas it does become dependent on O_2 pressures at elevated temperatures due to the decrease in solubility of O_2. This causes the O_2 concentration to become a significant limiting factor that increases with the degree of oxidation. For this reason, in oxidative stability studies the use of several temperatures, in a range as low as practical, preferably at or below 60 ^oC, is an important consideration. Moreover, polymerization and cyclization of PUFA, which mainly occurs at elevated temperatures, are not significant at room temperature. Furthermore, volatile acids that are measured by the Rancimat and OSI methods are produced only at elevated temperatures (Frankel, 2010). For all these reasons, the results of the studies on the oxidative stability of olive oil at high temperatures are neither relevant nor can be extrapolated to normal storage conditions at room temperature. Olive oil stability at room temperature is of great interest, for instance, to know its storage shelf-life. Because VOO is relatively stable to oxidation due to its particular chemical composition, there has been apparently little or no control of its stability under normal storage conditions in the past. To provide some knowledge on this issue, the stability of olive oil at room temperature while protected from light by ^1H-NMR fingerprinting was studied (Alonso-Salces et al., 2011a). The ^1H-NMR spectra of the VOO aliquots kept under these conditions, over a certain period of time, were analyzed by principal component analysis (PCA).

2. Experimental

2.1 Chemicals and plant material

Deuterated chloroform for NMR analysis (99.8 atom %D) was provided by Sigma-Aldrich Chemie (Steinheim, Germany).

Virgin olive oils (963 samples) from seven countries of the Mediterranean basin, namely Italy (661 VOOs), Spain (144 VOOs), Greece (97 VOOs), France (39 VOOs), Turkey (14 VOOs), Cyprus (6 VOOs) and Syria (2 VOOs), were collected directly from the producers (olive oil mills) from most of the main producing regions of these countries during three harvests (2004/05, 2005/06 and 2006/2007). The sample collection was carried out with the collaboration of Dipartimento di Chimica e Technologie Farmaceutiche ed Alimentari - Università degli Studi di Genova (Italia), Laboratorio Arbitral Agroalimentario (Ministry of Agriculture and Fishery, Spain), General Chemical State Laboratory D'xy Athinon (Greece), General State Laboratory (Ministry of Health, Cyprus), Departamento de Química Orgánica - Universidad de Córdoba (Spain), Istituto di Metodologie Chimiche - Laboratorio di Risonanza Magnetica Annalaura Segre – CNR (Italy), Fondazione Edmund Mach - Istituto San Michele all'Adige (Italy), and Eurofins Scientific Analytics (France), in the framework of the EU TRACE project. The true type (virgin or extra virgin) and origin of the olive oils at the national, regional and PDO level were assured. The Italian samples were representative of the olive oil producing areas, which are markedly influenced by pedoclimatic factors from the North to the South of the country.

For the study of VOO stability, about a liter of VOO was divided into aliquots contained in dark glass 40mL-vials completely filled and kept at -20°C in a freezer. Each month, over a period of more than 3 and half years (samples for the months 20th, 32nd, 38th and 42nd are missing), one vial was taken from the freezer and stored at room temperature (r.t.) in a closed box. A preliminary supposition was made; this considered that the degradation of VOO at -20°C is not significant and thus the last aliquot taken out of the freezer was time 0. All aliquots were analyzed by ^{1}H-NMR once the last sample was taken from the freezer.

2.2 NMR analysis

Aliquots of 40 μL of each VOO were dissolved in 200 μL of deuterated chloroform, shaken in a vortex, and placed in a 2 mm NMR capillary. The ^{1}H-NMR experiments were performed at 300K on a Bruker (Rheinstetten, Germany) Avance 500 (nominal frequency 500.13 MHz) equipped with a 2.5 mm broadband inverse probe. The spectra of the samples used for the study of the geographical origin of VOOs were recorded using a 7.5 μs pulse (90°), an acquisition time of 3.0 s (32k data points) and a total recycling time of 4.0 s, a spectral width of 5500 Hz (11 ppm), 64 scans (+ 4 dummy scans), with no sample rotation. The spectra of the samples used for the VOO stability study were recorded using a 6.7 μs pulse (90°), an acquisition time of 3.5 s (50k data points) and a total recycling time of 7.0 s, a spectral width of 7100 Hz (14 ppm), 32 scans (+ 4 dummy scans), with no sample rotation. Prior to Fourier transformation, the free induction decays (FIDs) were zero-filled to 64k and a 0.3 Hz line-broadening factor was applied. The chemical shifts are expressed in δ scale (ppm), referenced to the residual signal of chloroform (7.26 ppm) (Hoffman, 2006). The spectra were phase- and baseline-corrected manually, binned with 0.02 ppm-wide buckets, and normalized to total intensity over the region 4.10-4.26 ppm (glycerol signal). TopSpin 1.3 (2005) and Amix-Viewer 3.7.7 (2006) from Bruker BioSpin GMBH (Rheinstetten, Germany)

were used to perform the processing of the spectra. The region of the NMR spectra studied was 0-7 ppm for the geographical origin determination of VOOs, and 0-10 ppm in the VOO stability study. The data tables generated with the spectra of all samples, excluding the eight buckets in the reference region 4.10-4.26 ppm, were submitted to multivariate data analysis.

2.3 Data analysis

The data matrices, consisting of the ^{1}H-NMR buckets (variables) arranged in columns and VOO samples in rows, were firstly analyzed by univariate procedures (ANOVA, Fisher index and Box-Whisker plots), and afterwards, by the following multivariate techniques, already described in bibliography (Berrueta et al., 2007): unsupervised ones as principal component analysis (PCA); and supervised as linear discriminant analysis (LDA) and partial least squares discriminant analysis (PLS-DA). Statistic and chemometric data analysis were performed by means of the statistical software packages Statistica 6.1 (StatSoft Inc., Tulsa, OK, USA, 1984-2004), The Unscrambler 9.1 (Camo Process AS, Oslo, Norway, 1986-2004) and SIMCA-P 11.0 (Umetrics AB, Umea, Sweden, 1992-2005). Strategies used for variable selection in LDA and selection of the optimum number of PLS components in PLS-DA are described elsewhere (Rosa M. Alonso-Salces et al., 2010b).

For the geographical characterization of VOOs, the supervised techniques were applied to the autoscaled (or standardised) or Pareto-scaled data matrix of the VOO profiles following these steps: (*i*) the data set was divided into a training-test set and an external data set; (*ii*) the training-test set was subsequently divided into a training set and a test set several times in order to perform cross-validation; (*iii*) the training-test set was used for the optimization of parameters characteristic of each multivariate technique by cross-validation, for instance for variable selection in LDA or the number of PLS components in PLS-DA; (*iv*) a final mathematical model was built using all the samples of the training-test set and the optimized parameters; (v) this model was validated using an independent test set of samples (external data set), i.e. performing an external validation. During the parameter optimization step, the models were validated by 3-fold cross-validation (3-fold CV) or leave-one-out cross-validation (LOO). The reliability of the classification models achieved in the cross-validation was studied in terms of recognition ability (percentage of the samples in the training set correctly classified during the modeling step) and prediction ability (percentage of the samples in the test set correctly classified by using the models developed in the training step). The reliability of the final model was evaluated in terms of classification ability (percentage of the samples of the training-test set correctly classified by using the optimized model) and the prediction ability in the external validation (percentage of the samples of the external set correctly classified by using the optimized model) (Berrueta et al., 2007).

3. Results and discussion

3.1 ^{1}H-NMR spectra of virgin olive oil

^{1}H-NMR spectra of VOOs were recorded (Fig. 1). Olive oil is mainly made up of triglycerides, differing in their substitution patterns in terms of length, degree and kind of unsaturation of the acyl groups (Harwood & Aparicio, 2000). The chemical shifts of their ^{1}H signals are well known (Mannina & Segre, 2002; Sacco et al., 2000). However, the ^{1}H signals of the minor oil components, such as mono- and di-glycerides, sterols, tocopherols, aliphatic alcohols,

hydrocarbons, fatty acids, pigments and phenolic compounds (Harwood & Aparicio, 2000), are only observed by ^{1}H-NMR when their signals do not overlap with those of the main components and their concentrations are high enough to be detected (R. M. Alonso-Salces et al., 2010a; Rosa M. Alonso-Salces et al., 2010b; D'Imperio et al., 2007; Guillen & Ruiz, 2001; Mannina et al., 2003; Sacchi et al., 1996). Table 1 gathers the common ^{1}H-NMR signals of the major and some minor compounds together with their chemical shifts and their assignments to protons of the different functional groups. Several signals of minor compounds were found in ^{1}H-NMR spectra recorded because they were not overlapped by those of the triglyceryl protons: cycloartenol at 0.318 ppm and 0.543 ppm, β-sitosterol at 0.669 ppm, stigmasterol at 0.687 ppm, squalene at 1.662 ppm, sn-1,2 diglyceryl group protons at 3.71 ppm and 5.10 ppm, and three unknown terpenes at 4.571 ppm, 4.648 ppm and 4.699 ppm.

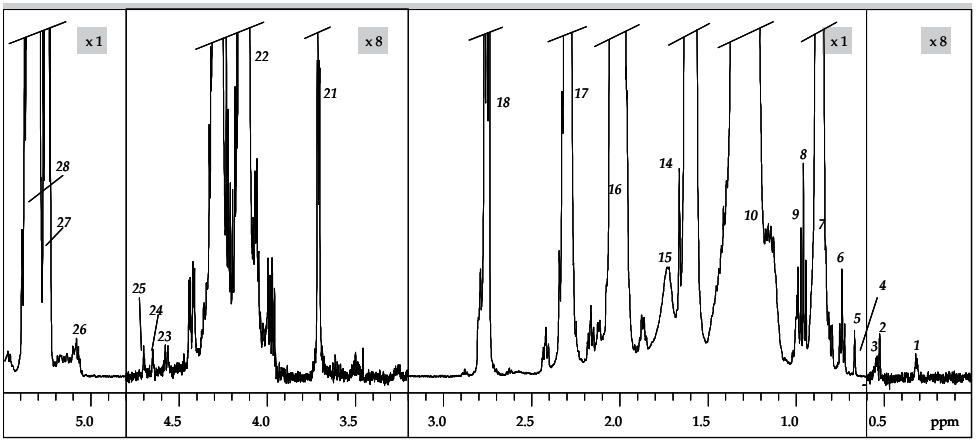

Fig. 1. ^{1}H-NMR spectra of a VOO (signal numbering, see Table 1).

3.2 Geographical origin of virgin olive oil

The large dataset of VOOs was studied regarding the situations that the antifraud authorities and regulatory bodies face. The PDO "*Riviera Ligure*", some Italian regions and the main countries that produce VOOs were used as examples to prove the potential of the tools to detect the mislabeling of non-PDO oils as PDO VOOs, or the mislabeling of the provenance of VOOs at the regional or national level. With this purpose in mind, several multivariate data analysis techniques, datasets, types of data scaling and cross-validation were evaluated to attain the best classification models for each case study.

After removing 28 extreme samples, the dataset (935 x 342) was analyzed by PCA. The four first principal components, accounting for 63% of total system variability (TSV), showed that samples were distributed in a compact cluster. However, some overlapping sub-clusters due to the harvest year were observed in the score plot of the samples in the space defined by PC2 (13% TSV), PC3 (11% TSV) and PC4 (7% TSV). Taking into account that 70% of the samples were Italian and the rest from countries in the Mediterranean region, seasonal aspects seem to affect all samples in the same way, independently of their geographical origin. Therefore, in the modeling for the authentication of agricultural food products, it is important to include chemical data of several harvests in order to obtain general classification models that allow for the seasonal variability.

#	Chemical shift (ppm)	Multiplicity[a]	Functional group	Attribution
1	0.318	d	-C*H*$_2$- (cyclopropanic ring)	cycloartenol
2	0.527	s		
3	0.543	d	-C*H*$_2$- (cyclopropanic ring)	cycloartenol
4	0.669	s	-C*H*$_3$ (C18-steroid group)	β-sitosterol
5	0.687	s	-C*H*$_3$ (C18-steroid group)	stigmasterol
6	0.740	t	-C*H*$_3$ (^{13}C satellite of signal at 0.87 ppm)	
7	0.866	t	-C*H*$_3$ (acyl group)	saturated, oleic (or ω-9) and linoleic (or ω-6)
8	0.960	t	-C*H*$_3$ (acyl group)	linolenic (or ω-3)
9	0.987	t	-C*H*$_3$ (^{13}C satellite of signal at 0.87 ppm)	
10	1.19-1.37		-(C*H*$_2$)$_n$- (acyl group)	
11	1.243		-(C*H*$_2$)$_n$- (acyl group)	saturated (palmitic, stearic)
12	1.256		-(C*H*$_2$)$_n$- (acyl group)	oleic
13	1.288		-(C*H*$_2$)$_n$- (acyl group)	linoleic and linolenic
14	1.51-1.65		-OCO-CH$_2$-C*H*$_2$- (acyl group)	
15	1.662	s	-C*H*$_3$	squalene
16	1.96-2.07		-C*H*$_2$-CH=CH- (acyl group)	
17	2.26-2.32	m	-OCO-C*H*$_2$- (acyl group)	
18	2.72-2.82		=CH-C*H*$_2$-CH= (acyl group)	
19	2.754	t	=CH-C*H*$_2$-CH= (acyl group)	linoleic
20	2.789	t	=CH-C*H*$_2$-CH= (acyl group)	linolenic
21	3.69-3.73	d	-C*H*$_2$OH (glyceryl group)	*sn* 1,2-diglycerides
22	4.09-4.32		-C*H*$_2$OCOR (glyceryl group)	triglycerides
23	4.571	d		terpene
24	4.648	s		terpene
25	4.699	s		terpene
26	5.05-5.15	m	>C*H*OCOR (glyceryl group)	*sn* 1,2-diglycerides
27	5.22-5.28	m	>C*H*OCOR (glyceryl group)	triglycerides
28	5.28-5.38	m	-C*H*=C*H*- (acyl group)	

[a] Signal multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet.

Table 1. Chemical shift assignments of ^{1}H-NMR signals of the main components in VOOs.

3.2.1 PDO virgin olive oils

Under the PDO of *Riviera Ligure*, only extra virgin olive oils produced in Liguria (Italy) that fulfill the PDO requirements related to olive varieties, farming practices, oil extraction procedures, bottling and labeling (Dossier Number: IT/PDO/0017/1540, Official Journal L22 24.01.1997) can be marketed. The ^{1}H-NMR dataset of VOOs from different geographical origins and PDOs was studied to create a classification model that differentiates between VOOs belonging to the PDO *Riviera Ligure* and those not belonging to this PDO.

Univariate data analysis (ANOVA, Fisher index and Box-Whisker plots) disclosed that any single variable could distinguish between Ligurian (belonging to the PDO *Riviera Ligure*) and non-Ligurian (not belonging to the PDO) samples. So, it was necessary to apply supervised pattern recognition methods to build classification models that can distinguish VOOs of this PDO from the rest. Several multivariate approaches (LDA and PLS-DA) were tested using balanced or unbalanced data sets, different cross-validation methods (LOO and 3-fold CV), different data scaling techniques (auto-scaling and Pareto-scaling) to find the best approach for the authenticity and traceability of PDO olive oils (Rosa M. Alonso-Salces et al., 2010b; Rosa M. Alonso-Salces et al., 2011b). Table 2 summarized the results of the best classification models achieved. Both supervised pattern recognition techniques performed better if using a balanced training-test set than an unbalanced data set (Rosa M. Alonso-Salces et al., 2010b; Rosa M. Alonso-Salces et al., 2011b). PLS-DA outperformed LDA. LDA achieved classifications of around 85% of hits for both categories. PLS-DA provided a model with 5 PLS components and the boundary at 0.540, that achieved slightly better results for the Liguria class (prediction ability in the cross-validation, 86-88%; classification ability of the final model, 92%; and prediction ability of the final model in the external validation, 88%) than for the non-Liguria VOOs (86-87%, 90% and 86% respectively). These results, together with the facts that in the cross-validation the recognition ability was higher but close to the prediction ability and the classification ability of the final model was also higher but close to prediction ability in the external validation, disclosed that the model achieved was feasible and not random, as well as being well-represented by the samples in the dataset.

Regarding the most important NMR variables on the classification models provided by these pattern recognition techniques, the variables selected in LDA were among the variables that presented the highest weighted regression coefficients (Esbensen et al., 2002) in the PLS-DA models (the larger the regression coefficient, the higher the influence of the variable on the PLS model). Thus, both pattern recognition techniques arrived at consistent results, and provided information about the most important features for the characterization of PDO *Riviera Ligure* VOOs. In this sense, the variables selected for the LDA model were five NMR buckets centered at the following chemical shifts: 6.61 ppm; 5.11 or 5.09 ppm; 4.57 ppm; 4.05 ppm; and 0.33 ppm. These buckets correspond to signals of the following VOO components: phenolic compounds and unsaturated alcohols, which present characteristic resonances in the spectral region 6-7.5 ppm (Owen et al., 2000) and 4.5-5 ppm respectively; sn-1,2-diglycerides (5.09-5.11 ppm) and sn-1,3-diglycerides (4.05 ppm), due to their CH glycerol protons; and cycloartenol (0.33 ppm), to the methylene proton of its cyclopropanoic ring (Sacchi et al., 1996).

In the PLS-DA models, the variables that presented the highest weighted regression coefficients were: 6.85-6.83 ppm, 6.75 ppm, 6.67 ppm, 6.59 ppm, and 6.23 ppm belonged to

signals of phenolic compounds; 5.15-5.07 ppm were due to the CH glycerol protons of *sn*-1,2-diglycerides; 4.99 ppm to unsaturated alcohols; 4.71 ppm, 4.65 ppm and 4.57 ppm, to terpenes; 2.79 ppm, to diallylic proton of linolenic acyl group; 1.29 ppm, to methylene proton of linoleic and linolenic acyl group; and 0.33 ppm, to cycloartenol.

		Cross-validation					Model		External Validation	
			% Recognition		% Prediction		% Classification		% Prediction	
	N						132	135	67	601
	prior prob						0.49	0.51		
Technique	Miscellaneous	Validation	Lig	Non-Lig	Lig	Non-Lig	Lig	Non-Lig	Lig	Non-Lig
LDA[b]	5 NMR buckets selected: 6.61, 5.11, 4.57, 4.05 and 0.33 ppm; autoscaling	3-fold CV	84.1	85.9	84.1	83.7	82.6	85.2	86.6	79.7
PLS-DA[b]	5 PLS components selected; boundary: 0.540; autoscaling	3-fold CV	91.3	92.6	87.9	86.7	91.7	90.4	88.1	85.5
PLS-DA[c]	5 PLS components selected; boundary: 0.540; autoscaling	3-fold CV	-	-	86.4	85.9	91.7	90.4	88.1	85.5
PLS-DA[c]	5 PLS components selected; boundary: 0.540; autoscaling	LOO	-	-	87.1	85.9	91.7	90.4	88.1	85.5
PLS-DA[d]	5 PLS components selected; boundary: 0.540; autoscaling	3-fold CV/	-	-	-	-	91.7	90.4	88.1	85.5
PLS-DA[d]	4 PLS components selected; boundary: 0.520; Pareto scaling	3-fold CV/	-	-	-	-	87.1	83.0	80.6	81.0

[a] Abbreviations: N, number of samples; prior prob, prior probability; Lig, Liguria; Non-Lig, Non-Liguria; LDA, linear discriminant analysis; PLS-DA, partial least square discriminant analysis; Class codes: Liguria, 1; non-Liguria, 0.
[b] Statistica.; [c] The Unscrambler.; [d] SIMCA-P.

Table 2. Classification results obtained by supervised pattern recognition techniques for the authentication of VOO of the PDO *Riviera Ligure* using ^{1}H-NMR spectral data (balanced data set).[a]

3.2.2 Virgin olive oils from different regions

The large sample set of VOOs available was also studied from the point of view of the authentication of VOOs at the regional level, in particular, VOOs produced in certain Italian regions. The regions selected were those best represented in the dataset: Umbria (which is also a registered PDO: PDO *Umbria*), Sicily (6 PDOs: *Monte Etna*, *Val di Mazara*, *Valli Trapanesi*, *Valle del Belice*, *Valdemone* and *Monti Iblei*), Puglia (4 PDOs: *Terra d'Otranto*, *Collina di Brindisi*, *Dauno* and *Terra di Bari*), Lazio (3 PDOs: *Tuscia*, *Canino* and *Sabina*), Garda (3 PDOs: *Garda*, *Laghi Lombardi* and *Veneto Valpolicella, Veneto Euganei e Berici, Veneto del Grappa*), Campania (3 PDOs: *Peninsola Sorrentina*, *Colline Salernitane* and *Cilento*) and Calabria (3 PDOs: *Lametia*, *Alto Crotonese* and *Bruzio*). The binary classification models created for these regions were developed using an auto-scaled balanced training-test set by PLS-DA and LOO cross-validation (Rosa M. Alonso-Salces et al., 2010b). The final models were also evaluated by external validation. The results are summarized in Table 3.

Origin	Cross-validation			Model	External Validation		
Binary model[b]	***N***	***prior prob***	**% Prediction**	**% Classification**	***N***	***prior prob***	**% Prediction**
Umbria	*35*	*0.45*	71.4	82.9	*12*	*0.014*	50.0
Non-Umbria	*43*	*0.55*	74.4	79.1	*845*	*0.986*	74.8
Sicily	*54*	*0.47*	92.6	98.1	*24*	*0.029*	87.5
Non-Sicily	*62*	*0.53*	85.5	88.7	*795*	*0.971*	85.8
Puglia	*47*	*0.42*	68.1	72.3	*22*	*0.027*	81.8
Non-Puglia	*64*	*0.58*	62.5	71.9	*802*	*0.973*	65.1
Lazio	*40*	*0.49*	80.0	97.5	*19*	*0.022*	73.7
Non-Lazio	*41*	*0.51*	68.3	90.2	*835*	*0.978*	69.3
Garda	*36*	*0.46*	72.2	91.7	*13*	*0.015*	69.2
Non-Garda	*43*	*0.54*	74.4	90.7	*843*	*0.985*	80.1
Campania	*21*	*0.43*	71.4	81.0	*7*	*0.008*	57.1
Non-Campania	*28*	*0.57*	64.3	78.6	*879*	*0.992*	62.9
Calabria	*17*	*0.38*	70.6	94.1	*5*	*0.006*	60.0
Non-Calabria	*28*	*0.62*	85.7	96.4	*885*	*0.994*	79.9

[a] See abbreviations: Table 2; Models obtained by PLS-DA using autoscaling, LOO and The Unscrambler; Class codes: "Region", 1; "non-Region, 0.
[b] Binary models: Umbria *vs.* non-Umbria: 2 PLS components, boundary at 0.525; Sicily *vs.* non-Sicily: 3 PLS components, boundary at 0.460; Puglia *vs.* non-Puglia: 2 PLS components, boundary at 0.4435; Lazio *vs.* non-Lazio: 4 PLS components, boundary at 0.515; Garda *vs.* non-Garda: 3 PLS components, boundary at 0.555; Campania *vs.* non-Campania: 2 PLS components, boundary at 0.430; Calabria *vs.* non-Calabria: 3 PLS components, boundary at 0.445.

Table 3. Classification results obtained by supervised pattern recognition techniques for the authentication of VOO from certain Italian regions using ^{1}H-NMR spectral data.[a]

The model obtained to authenticate VOOs from Sicily recognized 98% of the Sicilian oils and 89% of the non-Sicilian ones and managed to correctly predict in the cross-validation step 93% and 86% of Sicilian and non-Sicilian oils respectively. Since this model achieved similar predictions in the external validation (higher than 85% of hits for both categories) to those in the modeling step, it can be considered stable and robust. In contrast, the models created for other regions such as Lazio, Garda and Calabria, were not so satisfactory: although the classification abilities were close to 90% of correct hits or even higher, the prediction abilities in the cross-validation were from 10 to 24% lower, which meant that the classification results were very dependent on the samples included in the training set in the modeling step. This also occurred for Umbria and Campania, but the models achieved about 80% of correct classification for the training set, and predictions on the test set were more than 10% lower, except for the oils belonging to the non-Umbria category (5% less). The external validation

of some models (only 50% of Umbria, 57% of Campania, and 60% of Calabria VOOs were correctly predicted) confirmed that the classes were not well represented in the modeling step. Puglian VOOs, as well as non-Garda VOOs, were much better predicted in the external data set (82% of hits) than in the cross-validation (68% and 72% of hits respectively). This was probably due to the way samples were divided into the training-test set and the external set: the PCA scores of all the VOOs were regarded to select samples from the whole cloud of points including the borders. This procedure assured that the training-test set was representative of all the samples (at least of the 3 harvests studied), however the predictions on the external set could be overoptimistic.

Regarding the most influential variables, i.e. those with the highest weighted regression coefficients, on the binary PLS-DA models achieved for each region are the following. The signals due to cycloartenol (0.31-0.33 ppm) and *sn*-1,2-diglycerides (5.07-5.15-ppm) were important for all models except for Garda, as well as the resonances in the phenolic region at 6.73-6.79 ppm, which only did not influence the model for Sicily. The acyl group methylene protons of saturated fatty acids (1.23 ppm), ^{13}C satellite of signal at 4.09-4.32 ppm (α-methylene protons of the glyceryl group of triglycerides) at 3.97 ppm and the signal at 5.57 ppm were important specifically for the Umbria model; the signals at 0.53 ppm and 0.79 ppm, for the Sicily model; the methylic proton of the C18-steroid group of β-sitosterol (0.67 ppm) and the terpene signal at 4.57-4.59 ppm, for the Puglia model; the signal of the cycloartenol at 0.55 ppm, ^{13}C satellite of signal at 2.26-2.32 ppm (α-methylene protons of the acyl group) at 2.15 ppm, the glycerol proton of *sn*-1,2-diglycerides (3.71 ppm) and signals at 6.19 ppm and 6.15 ppm in the phenolic region, for the Lazio model; signals in the region 1.35-1.43 ppm, 2.35-2.39 ppm, and 4.33-4.35 ppm, the α-methylene protons of the acyl group (2.29 ppm and 2.33 ppm), the signal at 3.75 ppm, the α-methylene protons of the glyceryl group of triglycerides (4.27 ppm) and the signal at 6.15 ppm in the phenolic region for the Campania model; and the signal at 5.93 ppm for the Calabria model. The glyceryl protons of *sn*-1,3-diglycerides (4.05-4.07 ppm) and triglycerides (5.25 ppm, 5.29 ppm) were influent for the models of Umbria, Lazio, Umbria and Campania respectively; signals in the phenolic region at 6.25-6.29 ppm for the models of Puglia and Calabria; signals in the phenolic region at 6.63-6.65 ppm and 6.69-6.71 ppm for the models of Umbria and Campania ; signals in the phenolic region at 6.45-6.47 ppm for the models of Umbria and Garda.

These results disclosed that ^{1}H-NMR spectra of VOOs contained information related to the region of provenance of the oil, nonetheless further studies should be carried out with a considerably larger sample set for each region, and even for each of their PDOs, in order to guarantee the detection of counterfeit VOOs. In this regard, Sicily, which is an island at the southernmost part of Italy, produces an olive oil which is markedly influenced by pedoclimatic factors, in accordance with its geographical position. It is therefore coherent that the VOO produced on this island presents a characteristic chemical composition that allows one to distinguish it from all other VOO coming from different geographical regions.

3.2.3 Virgin olive oils from the main producing countries: Spain, Italy and Greece

The adulteration of VOOs from a certain country with VOOs produced in another country at a lower cost, or the false labeling of the VOOs as coming from a certain country when they

were actually produced in another, are actual events that the antifraud authorities have to deal with regularly. The need for chemical approaches to detect these fraudulent activities is evermore apparent.

The ^{1}H-NMR data of the VOOs from the main olive oil producing countries, i.e. Spain, Italy and Greece, were analyzed by multivariate techniques with the purpose of creating classification models to distinguish the geographical origin of VOOs from these three countries (Rosa M. Alonso-Salces et al., 2010b). PLS-DA, using LOO cross-validation, was applied to the autoscaled data to provide binary classification models (country *vs.* non-country), which were also evaluated by external validation (Table 4). The model 'Greece *vs.* non-Greece' distinguishes Greek VOOs from all the rest of the VOOs; it classified properly more than 97% of the samples of both categories, Greece and non-Greece, and predicted correctly more than 90% of the samples in the test set of the cross-validation, as well as in the external validation. The binary models for Italy and Spain presented classification abilities of 89% for the Italian oils and the Spanish oils, 84% for the non-Italy category and 85% for the non-Spain category. The prediction abilities in the cross-validation for the model for Spain were ca. 80% of hits for both classes; whereas the predictions in the external validation were considerably different, for the Spanish VOOs it was overoptimistic (92%), and for the non-Spanish VOOs it was considerably low (67%). In the model for Spain, the variability of the non-Spain category was under-represented in the training-test sets. As a result, this model did not provide good predictions for this category in the external set. The model for Italy provided prediction abilities in the cross-validation of ca. 76% for both classes; and in external validation, close to this value. So, these predictions were substantially lower than the recognition ability of the model, indicating that the model was dependent on the samples included in the training set.

Origin	**Cross-validation**			**Model**	**External Validation**		
Binary model[b]	***N***	***prior prob***	**% Prediction**	**% Classification**	***N***	***prior prob***	**% Prediction**
Italy	*72*	*0.35*	75.0	88.9	*568*	*0.78*	75.7
Non-Italy	*135*	*0.65*	77.0	84.4	*160*	*0.22*	71.9
Spain	*71*	*0.34*	78.9	88.7	*70*	*0.10*	92.9
Non-Spain	*136*	*0.66*	80.9	85.3	*658*	*0.90*	67.2
Greece	*64*	*0.31*	92.2	98.4	*31*	*0.04*	96.8
Non-Greece	*143*	*0.69*	93.7	97.9	*697*	*0.96*	90.0

[a] See abbreviations: Table 2; Models obtained by PLS-DA using autoscaling, LOO and The Unscrambler; Class codes: "Country", 1; "non-Country, 0.
[b] Binary models: Italy *vs* non-Italy: 4 PLS components, boundary at 0.4020; Spain *vs* non-Spain: 3 PLS components, boundary at 0.3563; Greece *vs* non-Greece: 5 PLS components, boundary at 0.4725.

Table 4. Classification results obtained by supervised pattern recognition techniques for the authentication of VOO from the main producing countries, i.e. Italy, Spain and Greece, using ^{1}H-NMR spectral data.[a]

The most influential variables, i.e. those with the highest weighted regression coefficients, on the binary PLS-DA models obtained for each country are due to signals in the phenolic regions at 6.45-6.47 ppm and 6.83-6.85 ppm, which were important for the three models (Rosa M. Alonso-Salces et al., 2010b). In contrast, the model for Spain was particularly influenced by the methylic proton of the C18-steroid group of β-sitosterol (0.67 ppm), the β-methylene protons of the acyl group (1.59 ppm, 1.67 ppm), the allylic protons of the acyl group (1.99-2.07 ppm), the diallylic protons of the acyl group of linoleic (2.73-2.77 ppm) and linolenic (2.77-2.81 ppm), the glycerol proton of *sn*-1,2-diglycerides (3.71 ppm), *sn*-1,3-diglycerides (4.05-4.07 ppm) and triglycerides (5.25 ppm, 5.29 ppm), the olefinic protons of the acyl groups (5.37 ppm), the signals in the phenolic region at 6.37 ppm, 6.61 ppm and 6.71 ppm, and the signals at 0.53 ppm, 1.75-1.77 ppm, 2.35 ppm. Among the most important variables, those who only affected the model for Greece were the methylic proton of the linolenic acyl group (0.97 ppm), and the terpene signal at 4.55-4.57 ppm, and the signals at 0.77 ppm and 3.81 ppm. The resonances of cycloartenol (0.31-0.33 ppm and 0.55 ppm) and phenolic compounds at 6.23 ppm and 6.27 ppm were important for the models of Italy and Greece.

These results show that ^{1}H-NMR fingerprinting of VOOs can be a useful tool to assure authenticity and traceability of VOOs at the national level. From this study, a stable model was achieved to distinguish Greek VOOs from oils from other countries. However, for Italian and Spanish VOOs further studies should be performed with a larger balanced data set, in which all categories will be well represented, to obtain robust models. In the present data set, Spain was clearly under-represented, being the main producer (50% of EU production of olive oil); and Italy, even though it was quite well-represented, the number of samples were very unbalanced regarding the other countries, and so few Italian samples were used in the modeling step. The classification results might therefore be very dependent on the samples in the training-test set.

3.3 Stability of virgin olive oil

Regarding the importance of oil stability on its quality and nutritional properties, the stability of VOO was studied over a period of 43 months in the dark at room temperature by ^{1}H-NMR. The high stability of VOO is mainly due to its relatively low degree of fatty acid unsaturation and to the antioxidant activity of some of the unsaponifiable components. For instance, the oxidative susceptibility of olive oil is related to the antioxidant activity of α-tocopherol, which also showed a synergistic effect in association with some phenolic compounds with significant activity (Deiana et al., 2002).

In contrast with previous studies on the oxidative stability of edible oils, which were performed at high temperatures (Frankel, 2010); we studied VOO stability at r.t. (Alonso-Salces et al., 2011a). The ^{1}H-NMR spectra of 40 VOO samples in the spectral region 0-10 ppm (492 buckets) were analyzed by PCA. The samples represented in the PCA score plot of the first two principal components (Fig. 2) are distributed in the direction of the first principal component (PC1) in clusters, partially overlapped, according to the length of time they had been at r.t. So, PC1, accounting for 15% TSV, contains information related to the change and evolution of the chemical composition of VOO during storage in the dark at r.t., and hence, about the degradation of olive oil under these conditions.

The most influential features, i.e. buckets of ^{1}H-NMR spectra, on PC1 are those with the highest loadings in absolute value, and are shown in Table 5. Some of these chemical shifts correspond to ^{1}H-NMR signals of compounds involved in the hydrolytic and oxidative degradation of VOO. During the oxidation process, hydroperoxides (primary oxidation compounds) are produced (Guillen & Ruiz, 2001, 2006) , which may degrade into secondary oxidation products such as aldehydes, ketones, lactones, alcohols, acids, etc. The oxidation of edible oils is a matter of major concern also from a safety point of view because some oxidation products such as aldehydes are toxic (Guillen & Ruiz, 2001, 2006). Furthermore, several saturated and unsaturated aldehydes have been found to be responsible for rancid sensory defect in VOO (Morales et al., 2005), as well as for off-odours (Kalua et al., 2007), altering its organoleptic properties.

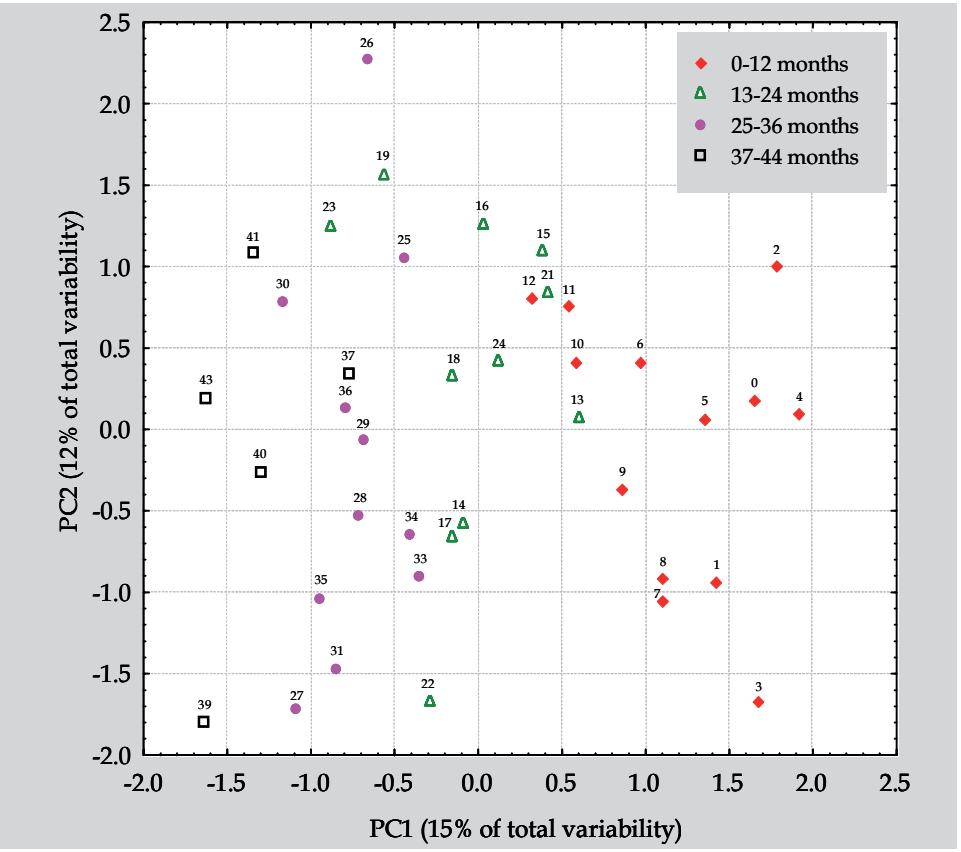

Fig. 2. PCA score plot of the samples used to study the stability of VOO on the space defined by the two first principal components. Samples are numbered according to the time (months) that they had been at r.t. in the dark before analysis.

Bucket (ppm)	Loadings	Multiplicity[a]	Functional group	Attribution
8.19 8.17 8.15 8.13 8.11 8.09	-0.807 -0.860 -0.823 -0.820 -0.749 -0.716	broad signal	-OO*H* (hydroperoxide group)	hydroperoxides
6.97	0.766	s	-Ph-*H* (phenolic ring)	phenolic compounds
6.75	0.811	d	-Ph-*H* (phenolic ring)	phenolic compounds
6.57 6.55 6.53 6.51	-0.792 -0.838 -0.834 -0.727	t	-C*H*=C*H*-C*H*=C*H*- (*cis, trans* diene system)conjugated	hydroperoxides
6.01 5.99 5.97 5.95	-0.833 -0.854 -0.860 -0.822	t	-C*H*=C*H*-C*H*=C*H*- (*cis, trans* conjugated diene system)	hydroperoxides
5.57 5.55	-0.824 -0.776	m	-C*H*=C*H*-C*H*=C*H*- (*cis, trans* conjugated diene system)	hydroperoxides
5.25	0.880	m	>C*H*OCOR (glyceryl group)	triglycerides
4.45	0.726	m	-CH_2OCOR (^{13}C satellite of signal at 4.09-4.32 ppm, glyceryl group)	triglycerides
4.37 4.35	-0.782 -0.786		>C*H*-OOH (methine proton of hydroperoxide group)	hydroperoxides
4.27	0.770	m	-CH_2OCOR (glyceryl group)	triglycerides
4.09 4.07 4.05	-0.795 -0.931 -0.875	q	>C*H*-OH (glyceryl group)	*sn* 1,3-diglycerides
3.89 3.87 3.85	-0.745 -0.802 -0.715	broad signal		saturated alcohols
3.59	-0.708	broad signal		saturated alcohols
2.79	0.924	t	=CH-CH_2-CH= (acyl group)	linolenic
2.77	0.839		=CH-CH_2-CH= (acyl group)	linolenic and linoleic
2.75	0.870	t	=CH-CH_2-CH= (acyl group)	linoleic
2.21 2.19 2.17 2.15	-0.740 -0.885 -0.913 -0.885	m	-OCO-CH_2- (^{13}C satellite of signal at 2.26-2.32 ppm, acyl group)	
2.03	0.792		-CH_2-CH=CH- (acyl group)	linoleic and linolenic
1.29 1.27	0.819 0.852		-(CH_2)$_n$- (acyl group)	linoleic and linolenic
1.25	0.784		-(CH_2)$_n$- (acyl group)	oleic

[a] Signal multiplicity: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet.

Table 5. Stability of VOO: Loadings of the most influential variables on the first principal component, and chemical shift assignments of the ^{1}H-NMR signals.

The presence of hydroperoxides in the samples which had been stored at r.t. and protected from light for more than one year was confirmed by the ^{1}H-NMR signals at 8.09-8.19 ppm due to hydroperoxide protons; 6.51-6.57 ppm, 5.95-6.01 ppm and 5.55-5.57 ppm due to protons of the conjugated diene systems; and 4.35-4.37 ppm due to the methine proton of the hydroperoxide group, as observed by other authors (Guillen & Ruiz, 2001). All these signals presented very small intensities in comparison with characteristic VOO signals, indicating that the oxidative degradation was taking place at a very low rate and yield. This was also supported by the fact that characteristic resonances of aldehydes (9.3-9.9 ppm), the main secondary oxidation products, were not detected in the VOO over the 3 and half years of storage at r.t., so the secondary oxidation process had not yet occurred. These facts agree with the recognized high oxidative stability of VOO. Some minor signals at 6.97 ppm and 6.75 ppm were assigned to phenolic compounds (Owen et al., 2003). The decrease or disappearance, respectively, of these signals during storage at r.t. was in agreement with the role that these substances play as antioxidants during the oxidative degradation process of VOO.

During hydrolytic degradation of olive oil, triglycerides hydrolyze thereby increasing the content of free fatty acids and consequently, the acidity of the oil, which means deterioration in the oil quality. Several resonances indicated the occurrence of hydrolytic degradation. In this sense, slight changes in the intensity of the tryglyceride signals at 5.25 ppm, 4.45 ppm and 4.27 ppm and the α-methylene protons of the acyl group (^{13}C satellite of the signal at 2.26-2.32 ppm) at 2.15-2.21 ppm were observed. Moreover, a slight decrease in the intensity of the signals at 2.75-2.79 ppm of the diallylic protons and at 2.03 ppm of the allylic protons of linoleic and linolenic acyl groups, and at 1.25-1.29 ppm of the methylene proton signal of oleic, linoleic and linolenic acyl groups, during storage at r.t., revealed that tryglycerides were degrading. The increase in the intensity of the signal at 4.05-4.09 ppm, assigned to the proton of the glyceryl group of *sn*-1,3-diglycerides, was indicative of the loss of quality and freshness of the VOO (Guillen & Ruiz, 2001). Young, good quality olive oils contain mainly native *sn*-1,2-diacylglyceride and only small amounts of *sn*-1,3-diacylglyceride. The increase in the latter was observed after one year of storage at r.t., which seems to be caused by intermolecular transposition and/or lipolytic phenomena (Sacchi et al., 1996). Moreover, in the samples stored for longer than 18 months, a broad signal also appeared in the region of saturated alcohols at 3.85-3.89 ppm, which can arise from lipolysis (Sacchi et al., 1996).

4. Conclusion

^{1}H-NMR fingerprinting of olive oil is a valuable analytical tool for the traceability of VOOs from different points of view, i.e. food authentication and food quality.

For authentication purposes, ^{1}H-NMR fingerprints of VOOs analyzed by supervised pattern recognition techniques allow the determination of their geographical origin at the national, regional and/or PDO level. PLS-DA afforded the best model to distinguish the PDO *Riviera Ligure* VOOs: 88% of the Ligurian and 86% of non-Ligurian oils were correctly predicted in the external validation. At the regional level, a stable and robust PLS-DA model was obtained to authenticate VOOs from Sicily, predicting well the origin of more than 85% of the samples in the external sample set. At the national level, Greek and non-Greek VOOs were properly classified by PLS DA: >90% of the oils were correctly predicted in the crossvalidation and external validation.

Regarding quality control, ^{1}H-NMR fingerprinting enables us to control the stability of VOO since this technique can detect its compositional changes due to oxidative and hydrolytic degradation. Under normal VOO storage conditions, i.e. at room temperature and protected from light, none of the signals present in the ^{1}H-NMR spectra of VOO at time zero disappeared or experienced significant decreases or increases over a period of more than 3 and half years. Only small changes in the signals and the appearance of some low intensity signals indicate that some oxidative and hydrolytic degradation of the VOO started after one year. These results confirm the high oxidative stability of VOO at r.t., and supports the best-before date for VOO that is normally between one and one and a half years, depending on the type of container and the olive variety used. Moreover, they show that VOO during this time period does not experience any significant changes which could render its consumption hazardous. In addition, aliquots (even small aliquots of 40 mL) can be preserved at r.t. in the dark (amber glass) until analysis for at least one year, which is of great interest to control laboratories of VOO with regard to storage space and expense. Furthermore, this research is a proof-of-concept that ^{1}H-NMR is a useful tool to study and evaluate the oxidative stability of edible oils in a quality control context at any temperature, since any toxic substances that may be generated during the degradation process can be detected and even quantified. Further studies would be needed to validate quantitative methods for this purpose.

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6. Abbreviations used

VOO, virgin and extra virgin olive oils; PDO, Protected Designation of Origin; NMR, nuclear magnetic resonance; ANOVA, analysis of variance; PCA, principal component analysis; PC, principal component; LDA, linear discriminant analysis; PLS-DA, partial least squares discriminant analysis; TSV, total system variability; CV, cross-validation; LOO, leave-one-out cross-validation; r.t., room temperature.

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11

Cultivation of Olives in Australia

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1. Introduction

Australia, by European standards, is a very young country with the first European settlers arriving as recently as 1788. Olives were not native to Australia but it took only a short time before the species was introduced. The first introduction of an olive tree to Australia was in 1800 (Spennemann, 2000), 12 years after the country was settled. Other importations have been recorded into New South Wales (NSW) including a tree planted by John Macarthur, one of Australia's pioneers and a man considered to be the father of the Australian sheep wool industry. A remaining olive tree still stands at Elizabeth Farm where he lived.

Despite the early start in the new settlement in NSW, little development occurred in that state over subsequent years. As the colony moved to other areas in Australia, olive production was spurred on by European immigrants particularly in the states of South Australia and Victoria. The NSW Department of Agriculture was formed in 1890 with an agenda to introduce new and useful species and study orchard farming and animal husbandry. The Department established experimental farms at sites throughout NSW including Wollongbar and Hawkesbury which became sites for evaluating olive production. In 1891 several Department of Agriculture research stations established schools and experimental farms including one at Wagga Wagga in Southern NSW, which included olive growing.

One of the most significant early developments for the olive industry was through the efforts of Sir Samuel Davenport (1818 – 1906), one of the early settlers of Australia, who became a landowner and parliamentarian in South Australia. His father was an agent of the "South Australia Company" in England and purchased land in South Australia. Samuel and his wife Margaret went to Australia in 1843 and ventured into mixed farming, almonds and vines. He tried sheep-farming and in 1860 he bought land near Port Augusta, SA, and turned to ranching horses and cattle. Davenport strongly promoted agriculture in South Australia and between 1864 and 1872 he published a number of papers, some concerning the cultivation of olives and manufacture of olive oil (en.wikipedia.org). In 1891 Davenport provided the NSW Department of Agriculture and other parts of the colony with olive cuttings from four cultivars, Verdale, Pigale, Blanquette and Bouquettier, from the south of France which were trialled for fruit production at the experimental farms.

In 1894, the farm at Wagga Wagga established orchards for evaluation of various fruits including plums, pears, persimmons and others. It was decided to establish a complete collection of olive cultivars within that orchard (Wagga Wagga Advertiser, 14 June 1894

from Spennemann 2000). Spennemann reports (2000) that by 1895, 8 acres of olives had been sown in Wagga Wagga "which now had the finest collection of cultivars in Australia" including many from California. By the turn of the century approximately 60 cultivars were present in the Wagga Wagga collection.

In future years significant studies were carried out on oil production and fruit pickling based on cultivars including *cvv*: Amelau, Blanquette, Bouquettier, Boutillan, Corregiola, Cucco, Dr Fiaschi, Gros Redondou, Macrocarpa, Nevadillo Blanco, Pigalle and Pleureur. Small scale commercial production and sales occurred after 1900 with the sale of olive oil and the distribution of olive cuttings for orchard development.

New cultivars continued to be introduced and the grove at Wagga Wagga expanded over subsequent years with several lines brought from Spain in 1932. Despite the excellent collection which had been established at Wagga Wagga, in 1959 it was decided to remove many of the trees due to low demand for the product. Although one of each of the cultivars was to be retained, subsequent loses through trees dying or being removed resulted in confusion about tree identification.

Fig. 1. One of over 100 trees and 60 cultivars planted at the Wagga Experimental Farm in 1891.

There was resurgence in interest in olive production in 1995 with the formation of the Australian Olive Association. At that time, Charles Sturt University, which had taken over ownership of the olive collection, made an attempt to resurrect the grove. The trees were severely pruned back from the massive size to which they had grown. A project funded by Rural Industries and Research Organisation (RIRDC) (Mailer & May, 2002) analysed DNA from leaves of the individual trees using RAPD analysis to attempt to identify the collection. This study was successful in naming many of the trees but for others there were no matches and identification was not possible. Some of the trees by this time had been named by areas in which the cuttings had been taken, such as Pera Bore or Hawkesbury Agricultural College, although logically, they were of European origin. At the same time, research on yield, oil content and oil quality was being carried out.

Based on this research, together with data from the original maps and planting diagrams, the Wagga Wagga orchard became the source of cuttings for some of Australia's leading

nurseries. Many trees were propagated and sold to new growers. Despite the best attempts to ensure correct identification, many of these new trees were misidentified and created problems for new orchardists in future years.

Amelon	Dr Fiiaschi	Pecholine
Arecrizza	Frantojo	Pecholine de St Chamis
Atro Violacca	Gros Redoneaux	Pendulina
Attica	Hardy's Mammoth	Pera Bore
Attro Rubens	Hawkesbury Agric. College	Pigalle
Barouni	Large Fruited	Polymorpha
Belle d'Espagne	Lucca	Praecox
Big Spanish	Manzanillo No.14	Regalaise de Languedoc
Blanquette	Manzanillo No.2.	Regalis
Borregiola	Marcocarpa	Rubra
Bouchine	Nevadillo Blanco	Saloma
Bouquettier	O de Gras	Sevillano
Boutillon	Oblitza	Tarascoa
Columella	Oblonga	Verdale
Corregiolla	Oje Blanco Doncel	
Cucco	Olive de Gras	

Table 1. Olive Cultivars included in the historic Wagga Wagga Olive Grove. NB. *Names and spelling of cultivars are from the Spennemann report (1997). Some names are descriptive (e.g. large fruited) or the source of cuttings (e.g. Pera Bore).*

Despite an early start, for over 100 years olive production showed only minor indications of becoming a substantial crop in Australia. Olive oil production remained only a boutique industry with the bulk of olive products being imported, almost entirely from Spain, Italy and Greece. There were several feasibility studies carried out which indicated a potential for an olive industry. These included a report published by Farnell Hobman (1995), a Senior Research Officer with the South Australian Department of Primary Industries, on the economic feasibility of olive growing. This reported stimulated further interest.

Olives today are planted throughout Australia, from the most southern point of Western Australia to the northern tropical areas of Queensland (Fig. 2.). The trees have been found to be capable of surviving in a wide range of environments from hot tropical regions to the cold areas of Tasmania. Over many years, birds have spread seeds across the land around many of these established orchards and numerous feral trees now grow throughout olive production areas, reinforcing the suitability of the Australian environment to grow olive trees. Studies to select for new cultivars from these wild trees (Sedgley, 2000) failed to establish any outstanding new cultivars. These wild trees are now considered a pest to native flora and in some States have been declared noxious weeds.

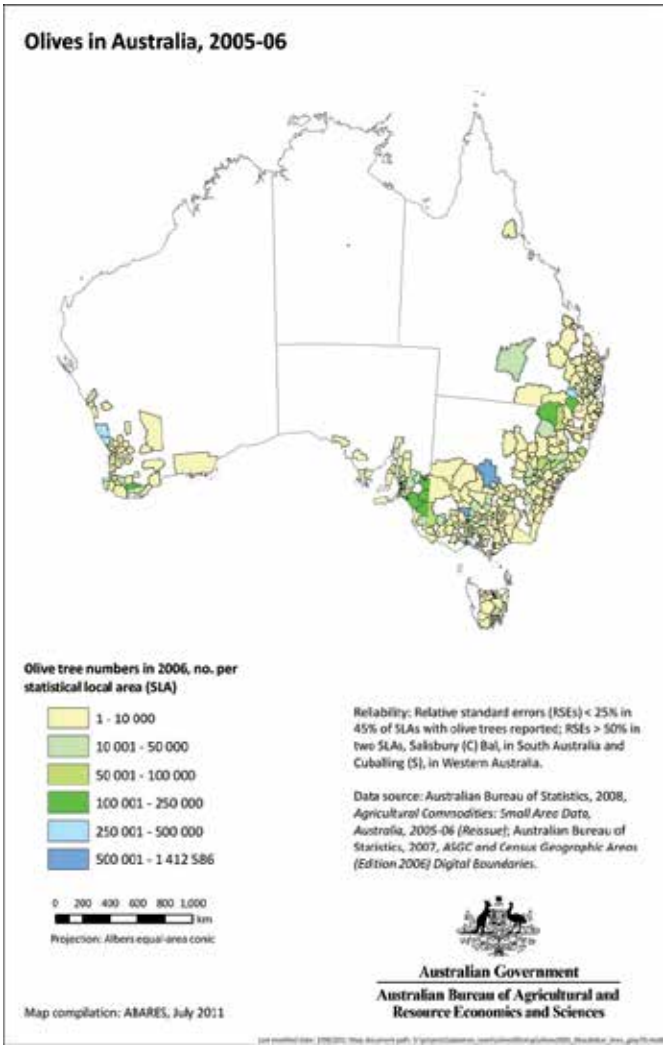

Fig. 2. Olive growing regions and intensity in Australia.

Today, the Australian olive industry is a modern production system for excellent quality oil. High yields have been achieved with low production costs. It is estimated that in the late 1990s, Australia had only 2,000 hectares of traditional olive groves, producing about 400 tonnes of oil. By 2008, Australia produced approximately 12,000 tonnes of oil. By 2013 it is expected that this production will have doubled. Most of this new oil production comes from 30,000 hectares of modern olive groves planted since 2000. There have been significant improvements in mechanical harvesting to achieve high levels of efficiency and economy which is comparable with any in the world. In traditional olive growing regions mechanical harvesting using trunk shakers was once considered as the best and most reliable method for reducing labour costs over the past decade. Today, continuous straddle harvesting machines are used which have been adapted or developed for Australian conditions with great success. These are currently used for more than 75% of Australian production.

Australia produces mostly extra virgin olive oil. The natural diversity of the Australian environment along with the selection of the most productive cultivars, harvested and processed under optimal conditions, is responsible for the exciting range of high quality olive oil products from Australia.

2. Formal development of an Australian Olive Association

The first national symposium on olive growing was held at the Roseworthy Campus of the Adelaide University in 1994, with strong interest spurred on by the economic feasibility report by Farnell Hobman (1995). The symposium was attended by over 100 participants. A decision was made to form an "olive industry group". Over the next two years this olive group drafted a constitution which was to become the Australian Olive Association (AOA).

The AOA committee had identified several issues which were critical to the development of a new industry (Rowe and Parsons 2005). These included:

The lack of any Australian or State quality standard for olives
A lack of knowledge about cultivars suited to the large range of environments
Strong optimism about growing olives in Australia
A network needed to be established for the free transfer of information.

The constitution was adopted by the committee in Mildura in May, 1995. Of the 100 participants at that meeting, 65 became members of the new AOA. The committee adopted a number of objectives:

a. To promote interest in olive growing and processing
b. To foster cooperation between regional groups
c. To facilitate research
d. Encourage education and information
e. Develop and distribute superior genetic olive material
f. Market research and promotion
g. Quality assurance

Following the formation of the AOA, several State industry organisations were then formed. The first publication of the AOA magazine, the Olive Press, was printed in 1995. By the end of 1995 regional grower groups had been established in Queensland (Qld), New South Wales (NSW), South Australia (SA) and Tasmania (Tas).

The International Olive Oil Council (IOC) provided assistance to the developing industry in Australia. The IOC funded olive experts to attend workshops held in Wagga Wagga NSW and Roseworthy, SA and provided information to further encourage the industry in Australia. This included the development of Australia's first organoleptic panel, in South Australia in 1997. The AOA and State Departments also held industry and grower workshops throughout the country on oil quality and production.

The AOA developed a five year strategic plan for the industry in 1997. This plan described the AOA as an "umbrella organisation" with a national industry structure (Rowe & Parsons 2005) overseeing State grower groups. In 1999 the Association was well established with the creation of 27 Regional Olive Associations and 1000 members.

The AOA made a commitment to establish an Australian standard. Existing international standards were based on oil produced under limited environmental variation, particularly in Mediterranean climates, and failed to recognise the natural variation in Australian olive oil. The AOA, together with the Australian Government and international organisations has been able to illustrate the high quality of Australian oil and the limitations of existing standards. The Australian standards were approved by Standards Australia in June 2011.

Today the Australian Olive Association Limited is recognised as the Peak Industry Body in Australia for olive growers. In its own words "*The Australian Olive Association (AOA) is the national body representing the Australian olive industry. Formed in 1995 as a result of a burgeoning industry that recognised the need for collective action in developing and supporting the industry, it now represents over 800 people nationwide. Members are involved in all aspects of the Australian olive industry, from grower (small and large), processors to end-user and associated business partners and service industries*" (http://www.australianolives.com.au).

The Australian Olive Association exists to:

- set and maintain quality standards for Australian-produced olive products
- provide quality research & development to create and maintain a sustainable integrated olive industry
- implement an ongoing consumer awareness programme to promote the benefits, and create a preference for Australian olive products
- provide a focal organisation which facilitates progressive direction for stakeholders in the olive industry.

The AOA holds an annual conference (Expo) within Australia to address the latest technology and research. The National Extra Virgin Olive Oil and the Australian Table Olive Competitions are held concurrently with the Expo to highlight the quality of Australian olive products.

3. The code of practice

One of the major outcomes of the AOA has been a Code of Practice. This was developed to ensure to consumers that signatories to this Code have undergone rigorous procedures to certify that the contents of a bottle of olive oil being sold is indeed Australian extra virgin olive oil. Signatories to the Code of Practice are listed on the Australian Extra Virgin website. To conform to the code of practice, producers must apply for registration and have their oil tested to ensure it meets specific criteria.

Australian extra virgin olive oil must have the following characteristics:

- be produced only from olives grown in Australia
- have a free fatty acid content of not more than 0.8 grams per 100 grams (as oleic acid)
- have a peroxide value of less than 20 (mEq peroxide oxygen per kg of oil)
- not exceed the following extinction coefficients for ultra-violet absorbency tests:
  - An absorbance value at 270nm of no greater than 0.22
  - An absorbance value at 232nm of no greater than 2.5
  - A ΔK value of no greater than 0.01

- have been assessed organoleptically by a person or persons accredited by the Australian Olive Association Ltd or in accordance with processes determined by the Australian Olive Association Ltd as having positive attributes such as fruitiness and not having any defects including fusty, muddy, musty, rancid or winey characteristics.

The chemical analyses for these purposes shall be undertaken by a person or organisation accredited by the Australian Olive Association. A sample from each batch identified on labels needs to meet the above tests before the claim that the oil is Australian extra virgin olive oil can legitimately be made. Inclusion of a 'best before' date on a label shall not be more than the equivalent of 30 days for every hour of the oil in Rancimat® at 110°C. Where the oil is a blend and the constituent oils have been tested separately the 'best before' date shall be that for the lowest scoring constituent.

In 2010 there were 230 Australian producers signed up to the Code of Practice including grocery retailers who use the Code as an internal standard for extra virgin olive oils (http://www.australianolives.com.au/).

4. Interaction with the International Olive Council

The Australian olive industry has learnt and benefited from input from the IOC and continues to work with their members. Both chemists and producers utilise the IOC website and advice from the IOC technical experts. Australia has two chemical laboratories and sensory laboratory which continue to participate in the IOC proficiency program. The laboratories utilise IOC methods of analysis and generally follow the limits of IOC standards.

The IOC initially provided funding for representatives to attend IOC meetings and during the 1990s the Australian Government Analytical Laboratories (AGAL), Sydney, gained IOC accreditation. Unfortunately, this provided no support for the industry due to a lack of contact between the two organisations.

In 1996 the NSW Government laboratory at Wagga Wagga pursued accreditation for the laboratory and in 2005, with funds from Horticulture Australia Limited, the organoleptic laboratory was also accredited (Mailer, 2005a). In 2007 the Modern Olives laboratory in Victoria also gained IOC accreditation. The sensory panels and chemical laboratories provide the industry with a resource to monitor quality and to meet the stringent requirements of the IOC and international standards.

During the period through 1995 - 2000, the IOC helped raise awareness of the health benefits of olive oil. Partly, as a result of the Olive Council's interaction, olive oil imports increased in Australia from 7 million litres in 1978 to 15 million litres in 1992 and to 30 million litres by 2000. The IOC continues to play a role in the Australian industry. Accredited Australian chemists are invited to attend chemists meetings in Madrid and the laboratories are invited to participate in proficiency programs and ring tests in the development of new methods.

5. Codex alimentarius

During the early years of the olive industry, although Australian growers were producing high quality olive oil, it was recognised that there were minor differences in the chemical profile of oil from olives grown across the range of Australian environment. These oils had a

spectrum of flavours and qualities not apparent in olives grown in the limited environmental fluctuations of the Mediterranean climate. The first workshops in Wagga Wagga in 1996, identified linolenic acid levels from 0.6 – 1.8% whereas the IOC standard for olive oil was <1.0%. Many studies have shown that fatty acid profiles are strongly influenced by environment, particularly the temperature during fruit development. Although insignificant in value, and no problem in terms of nutritional quality, this factor needed to be considered within international standards.

Further studies at WWAI in subsequent years showed other chemical parameters to sometimes vary from the existing and restrictive limits of the IOC standards (Mailer, 2007). Some cultivars being grown in Australia, particularly *cv* Barnea, from Israel, contained up to 5% campesterol whereas the limit imposed by IOC was <4.0%. These limits, developed as a means to detect adulteration, have no bearing on the nutritional value in olive oil. These components became a limitation for exporters of Australian olive oil but it became apparent that not only did Australian oils fail to meet these standards but many countries from the Southern Hemisphere (Argentina, Australia, Chile and New Zealand) also failed.

Through Food Standards Australia and New Zealand (FSANZ) Australia has attempted to have these limitations in the standards amended so that the standards represent olive oil grown throughout the world. Over several years Australia has sent representatives to Codex meetings to discuss these issues. IOC have responded vigorously, defending the standards on the basis that changes will encourage fraud, despite the fact that genuine oils, such as those growing in countries in the Southern Hemisphere, fail these limits. Due to the lack of agreement Codex have failed to come to a conclusion and particularly, an accepted level for linolenic acid in virgin olive oil has not been agreed upon.

6. An Australian standard

The lack of an Australian standard left Australia exposed to a lack of control of imported product as well as limitations for the domestic industry. Until recently no testing was done within Australia on imported olive oil. Several recent studies of imported olive oil products taken from supermarket shelves has illustrated that a significant proportion of it does not meet the international trade standards of IOC and Codex Alimentarius. Similar recent studies have shown that many imported oils into the USA do not meet European and international standards (Frankel et al 2011).

Surveys of Australian olive cultivars have been used to determine compliance with international standards (Mailer et al, 2002). Reports show the effects of olive cultivars, the influence of harvest timing and the changes to quality as a result of site and seasonal growing conditions.

From the first inception of the Australian Olive Association, it has been recognised that Australia must have its own standards for olive oil. The standards are required to set guidelines for Australian producers to ensure the oil was authentic and of acceptable quality. It was also critical to allow authorities to determine if the imported and local product meets the quality levels demanded by the industry and the consumers. The

standard was created with consultation within the industry including producers, marketing and exporters. It needed to address issues of authenticity, to detect any efforts to blend or mislabel the product. It needed also to be able to detect oil which had been heated and/or refined or if the oil had exceeded its potential use by date.

The standard was established with the support of Australian Standards organisation (www.standards.org.au). A wide spectrum of representatives from the industry contributed. A final draft was made available to the public for comment in early 2011. The draft drew both praise and criticism from all aspects of the industry both domestically and internationally. Ultimately it had strong support and was accepted with the final standard approved in July 2011 for release during 2011.

The new standard is similar in many ways to that of the IOC. The standard allows for a higher level of linolenic acid and campesterol, reflecting the actual properties of the Australian product. It has also included some new tests developed by the German DGF which allow traders to identify fresh oil from old oils or oil which has undergone heating, such as in refining. The standard is available from Standards Australia.

7. Consumption and production

7.1 Development of a boutique industry

In the early years of Australian settlement, there was not a strong demand for olive oil. Olives were grown for personal use or for a small boutique industry. The major edible oils used continue to be refined sunflower, cottonseed and canola oil. It was not until the late 20th Century that the olive industry began to grow. Australia had an increasingly cosmopolitan population including a large portion of Greek and Italian migrant workers who increased the demand for olive oil production. Despite this, the industry continued for many years as a boutique industry with small farms of only a few trees in which people produced their own oil or sold small quantities to others. Olive oil was imported from Spain, Greece and Italy for many years and by the 1990s the value of the imported olive oil products was in excess of $100 million dollars per annum.

7.2 Australian consumption

Outside the Mediterranean region, Australia is currently the largest consumer of olive oil per capita, consuming about 32,000 tonnes of olive oil in 2008. The demand for olive oil continues to grow, creating a good opportunity for the domestic market. The increased demand is highlighted by the increase in total imports of olive products in the last five years (Table 2).

7.3 Australian production

Australia currently has about 10 million olive trees spread across approximately 30,000 ha. Although the initial plantings of olives in Australia included a large number of cultivars, today about 90 percent of Australian olive oil is produced from 10 major cultivars including Arbequina, Barnea, Coratina, Corregiola, Frantoio, Koroneiki, Leccino, Manzanillo, Pendolino and Picual. These cultivars have been found to be agronomically suitable while at

the same time producing a good range of oil types. Barnea, a cultivar from Israel, is a recent addition to the other predominantly European cultivars but is today the most prolific.

Commercial production increased rapidly from the mid 1990s onwards, designed using state of the art equipment and methodologies. From an almost non-existent crop prior to 1990, olive oil production in Australia reached 12,000 tonnes in 2008. Due to the modern technologies used, that production is almost 100% extra virgin olive oil with no facilities or requirements for solvent extraction and only limited refining capacity for the oil. Only rarely do harvest conditions produce poor quality fruit which requires refining. These refined oils would generally be marketed as alternative products. Hence, Australian olive oils in supermarkets are all extra virgin olive oil. Additionally, around 10 per cent of Australian groves have organic certification, representing an increase of 60 per cent since 2006 (Australian Olive Association).

Most of Australia's olives are grown in the east, south and west of the country. Although South Australia was originally the largest producer, with 39% of total production in 2003, Victoria has become the leader with 48% of the production in 2009. New South Wales, Queensland, Western Australia and South Australia share the other half of production. The main harvest time is May to July although Queensland tends to harvest first due to climate, although harvest time vary across the states.

Australia's share of the world's extra virgin olive oil production has grown from only 0.31 percent in 2006, to 3 per cent of the world market with a 2008 harvest of 12,000 tonnes. By 2014, production is expected to reach 25,000 tonnes.

	Production		EVOO		Table Olives	
Year	Table Olives	Olive Oil	Imports (tonnes)	Exports (tonnes)	Imports (tonnes)	Exports (tonnes)
2001		500	27,680	385	11,545	74
2002		750	28,987	300	12,618	199
2003		1,500	28,447	278	14,483	138
2004	2,000	2,500	32,657	501	13,711	265
2005	2,700	5,000	29,062	1,652	15,143	215
2006	3,200	8,650	34,511	2,988	15,608	230
2007	2,500	9,250	43,404	2,502	16,364	207
2008	2,200	12,000	23,952	4,169	17,542	239
2009	3,000	15,000	31,169	6,960	16,210	366

Table 2. Extra virgin olive oil and table olive production, imports and exports (www.australianoliveoil.com)

The majority of olives grown are for oil production. Much of the production is from a few large producers although there are a large number of small producers spread throughout the growing regions of Australia. Despite the rapid increase in production, Australians are continuing to increase their consumption of olive oil and imports have been maintained at around 20-30,000 tonnes per annum. It would seem however, that there is some import

replacement with imports of 43,000 tonnes in 2007 being reduced to around 31,000 tonnes in 2009.

Fig. 3. a. Harvesting at night at Boundary Bend and b. aerial photograph of olive harvesting at Boundary Bend (photo courtesy of Boundary Bend)

7.4 Australian imports / exports of extra virgin olive oil

Australia imports in excess of 31,000 tonnes of olive oil per annum. Despite that, an increasing percentage of olive oil is being exported. In 2004, 501 tonnes, or 20 per cent of Australian production was exported while in 2009, 6,959 tonnes, or around 46 per cent, was destined for the export market, an average annual increase of 85 per cent. The value of exports in 2009 was $37.8m (Source ABS).

The top five countries buying Australian extra virgin olive oil have been the United States, England, China, Singapore and Japan. The Australian customers are changing over time, with the United States and Italy now being major destinations of Australian olive oils. Exports to China are also increasing albeit from a very low base.

The export figures from Table 2 indicate that there is a demand and an opportunity for Australian olive producers to continue to sell olive oil overseas. Despite that, a significant level of import replacement is a long term goal for the Australian olive industry and is on track to being achieved. The amount of Australian produced olive oil that is consumed domestically is now one quarter of the sum total of olive oil that is imported. The increased

percentage of Australian olive oil that is being consumed domestically has occurred in the context of fairly static import volumes over the last 5 years. Further increases in market share for Australian extra virgin olive oil in the domestic market will require further investment in consumer education.

7.5 Table olives

Data about table olive production in Australia is less well known than for olive oil. Although olives for oil production have been increasing rapidly, table olives have not had such success with production figures increasing from 2000 tonnes in 2004 to only 3000 tonnes in 2009 (Table 2). Although there are many boutique operations, a few operations have the capacity to process hundreds of tonnes of olives. Table olives are appearing more on domestic and export markets but large-scale production is still limited by the costs of harvesting. Despite the limited increase in production of table olives, the demand for them continues to increase. Imports of table olives have increased from 12,000 tonnes in 2001 to 16,000 tonnes in 2009 (Table 2.). Exports of Australian table olives have remained steady over the past few years, with around $800,000 worth of table olives having been exported in 2007.

By far the greater amount of research on olives has been directed toward the production of high quality olive oil. However, in addition to this research on oil and applications in Australia, some work has also been carried out on table olives (Kailis & Harris, 2004). The Australian table olive industry and trade currently has no nationally accepted guidelines for ensuring the quality and safety of processed table olives and the Kailis report was prepared for olive growers and processors from both national and international viewpoints.

		Percentage of production for Australian States					
Year	**Olive Oil (tonnes)**	**NSW**	**Qld**	**SA**	**Vic**	**Tas**	**WA**
2001	500	-	-	-	-	-	-
2002	750	-	-	-	-	-	-
2003	1,500	11	12	39	28	1	9
2004	2,500	12	8	16	47	1	16
2005	5,000	12.1	5	16.2	40.1	0.4	26.2
2006	8,650	8.3	4	18.2	53.9	0.2	15.4
2007	9,250	8	4.1	14.6	49.2	0.2	23.9
2008	12,000	7.7	4.1	19.5	53	0.2	15.5
2009	15,000	9.0	4.3	14.5	48.0	0.2	24

Table 3. Percentage of olive oil produced per State.

8. Quality

Australian olive oil quality is generally of high quality using modern technology for growing, harvesting, processing and packaging. The majority of the crop is mechanically harvested and transported to processing facilities within a few hours. Everything from the

machines used to harvest the fruit through to the temperature controlled stainless steel storage vessels are built on new technology. Oil extractors are generally centrifugal machines which are kept hygienically clean and housed in temperature controlled facilities.

The oil produced is almost entirely extra virgin olive oil and is ensured through the Australian Olive Association's "Code of Practice". The code requires olive growers to have their oil tested to ensure EVOO quality at the time of bottling. It also requires that the oil remains within specifications throughout the oils "best before" date to provide the consumer with confidence that the product meets the label qualifications. Only fruit that may have been damaged through frost, insect or disease generally fails EVOO quality. In these cases the oil is refined and redirected toward alternative uses.

Australia maintains two IOC accredited laboratories and sensory panels which advise the industry on oil quality. There is a continuous educational program through workshops and conferences to inform producers and consumers to help them understand defects and attributes in olive oil. The AOA presents several industry awards to olive oil producers at the annual AOA Expo. In addition, many regional growers groups have their own olive competitions judged by trained sensory personnel. All of the olive competitions demand that the oil passes basic chemical requirements.

8.1 Fatty acids

For commercial samples, the majority of oil analysis is carried out by the two Australian IOC accredited laboratories. This allows the laboratories to keep accurate records of the quality of oil being produced in Australia from year to year.

Free fatty acid value (FFA) of olive oil is a general indicator of how sound the olive fruit was at harvest and how carefully it was processed into oil. Table 4 shows a typical range for free fatty acids (FFA) and peroxide value (PV). The range shows that the majority of oils are well within acceptable limits with the median value of 0.18 FFA and 8 mEq oxygen/kg. Occasionally, due to fruit damage or climatic factors, oils may be outside of acceptable standards. However, less than 3.3% of FFA samples and 2.2% of PV samples failed to meet the IOC limits in 2006.

2006	Free Fatty Acid	Peroxide Value
Minimum	0.05	0
Maximum	3.48	48
Average	0.26	9
Median	0.18	8
No of samples	585	501

Table 4. Typical range of free fatty acids and peroxide value in Australian olive oil based on commercial samples in 2006.

The quality of Australian extra virgin olive oil has improved over the last decade. A summary of the FFA's of olive oils submitted to the Australian National EVOO Competition since 1997 (Table 5) shows how oil quality has improved. Between 1997 and 2002, only 34%

of the oils were less than 0.19% free fatty acids. In the following six years, from 2003 to 2009, 62% were less than 0.19% FFA.

1997-2002		2003-2009	
<0.19	34%	<0.19	62%
0.20-0.29	33%	0.20-0.29	26%
0.30-0.39	14%	0.30-0.3	8%
>0.40	19%	>0.40	4%

Table 5. Average free fatty acid levels of Australian olive over two periods (AOA)

The variable Australian climate and differences in temperature during fruit development has a strong influence on fatty acid profiles (FAP) as shown for oil analyses carried out in 2006 (Table 6). The profile of the fatty acids covers the full IOC range for acceptable limits but exceeds that range in several instances. Although the range is not indicative of nutritive value, the issues of compliance to international standards are significant.

Sample	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0
IOC limits	7.5- 20.0	0.3-3.5	≤ 0.3	≤ 0.3	0.5 - 5.0	55.0 - 83.0	3.5 - 21.0	≤ 1.0	≤ 0.6	≤ 0.4	≤ 0.2
Average	12.3	0.9	0.1	0.1	2.1	74.1	8.9	0.7	0.3	0.3	0.1
Min	7.4	0.4	0.0	0.0	1.1	55.7	2.7	0.4	0.2	0.1	0.0
Max	18.3	1.9	0.4	0.6	4.0	84.9	23.4	1.5	0.5	0.5	0.2

Table 6. Average, minimum and maximum limits for FAP of Australian olive oil in 2006 (n=468).

The range of fatty acids is further demonstrated with the analysis of samples from New Zealand, a cooler climate to that of the Australian olive producing areas. The FAP of 56 randomly selected samples in 2006 (Table 7) show that the oleic acid level often (23%) exceeds the IOC values which suggest these oils are nutritionally superior to those with high levels of saturated fat. However, these oils would officially fail the IOC standard. Many samples are lower (17%) than the IOC standard for palmitic (saturated) acid.

Fatty Acids	**C16:0**	**C16:1**	**C17:0**	**C17:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**	**C20:1**	**C22:0**
Max	12.4	0.9	0.06	0.11	2.59	**85.5**	7.6	0.9	0.4	0.4	0.2
Min	**6.4**	0.3	0.03	0.06	1.12	78.2	3.0	0.5	0.2	0.2	0.1

Table 7. Fatty acid profile of 56 randomly selected New Zealand oils from 2006.

8.2 Phytosterols

There is also a significant range in the phytosterol content and profile in Australian olive oil (Table 8). In particular, the level of campesterol often exceeds 4.0%, generally due to the production level of *cv* Barnea which is higher in campesterol than other cultivars. Due to the

suitability of this cultivar to the Australian climate and its high production rate, this cultivar will continue to be a significant portion of the Australian crop. As for other parameters, these components may exceed the international limits.

Sterols (%)	Cholesterol	Brassicasterol	24-Methylene-cholesterol	Campesterol	Campestanol	Stigmasterol	D-7- Avenasterol	D-7- Stigmastenol	D-7-Campesterol
Average	0.08	0.00	0.09	3.61	0.16	0.63	0.50	0.19	0.13
Min.	0.03	0.00	0.02	2.27	0.10	0.34	0.22	0.00	0.00
Max.	0.16	0.02	0.48	4.89	0.25	1.41	1.00	0.52	0.59
Median	0.07	0.00	0.07	3.49	0.15	0.56	0.47	0.19	0.06
	β-Sitosterol	**D-5- Avenasterol**	**D-5,23-Stigmastadienol**	**Clerosterol**	**Sitostanol**	**D-5,24-Stigmastadienol**	**Apparent β sitosterol**	**Diols**	**Total Sterols (mg/kg)**
Average	85.08	7.34	0.01	0.58	0.93	0.68	94.62	1.11	1537.8
Min.	79.45	5.21	0.00	0.20	0.28	0.21	93.83	0.64	1131.7
Max.	88.24	13.66	0.13	0.93	2.51	1.27	96.38	3.09	2153.8
Median	85.75	6.81	0.00	0.60	0.48	0.58	94.56	1.06	1520.9

Table 8. Phytosterols profile in Australian olive oil showing the range and the average and median values for each component.

9. Research

9.1 Funding

A new agricultural industry requires significant research and development support to optimise the industry. Such was the case with the awakening of the olive industry in Australia. It created a need for Australian research scientists to develop an understanding of the agronomy and the chemistry of the crop, essential for producing the highest yield with the best quality. The research effort has been supported strongly by the Australian Olive Association and financial support from some of the larger producers. Much of the financial support has come from the Federal Governments "Rural Industries Research and

Development Council" (www.rirdc.gov.au) which has consistently supported projects the olive industry considered to be of significant value.

Although most Australian agricultural industries pay a levy to the Federal Government to support research, olives have always been considered a new crop and have been exempt from a levy. However, in 2011, through support from the Australian Olive Association, the industry has agreed to contribute to a crop levy. This guarantees ongoing funding for the research and development of this industry in the future.

9.2 Cultivar selection

Determining which cultivars to grow was an early requirement for growers. At the early stages of development one of Australia's best resources was the historic olive orchard at Wagga Wagga. This orchard, with over 50 cultivars and trees which, in some cases, were over 100 years old, provided an ideal resource for study. Such was one of the first research projects funded by RIRDC (Ayton et al., 2001) in which oil content, oil quality and initial attempts to identify cultivars by DNA were carried out. The range of trees, some of which were grafting experiments and others with varying levels of irrigation, appeared to be an ideal study. Although the trees were producing reasonable crops due to poor maintenance for such a long period, and the variable conditions under which each of the trees were grown, the use of the data was limited.

There remained considerable confusion about cultivars being grown in Australia and if they were true to type. After many years, maps of the grove had been altered and many trees removed. Using RAPD DNA methods to discriminate between the cultivars (Mailer & May, 2002), dendrograms were constructed showing the relationship of the cultivars to each other. Although some trees were identified, it was not possible to obtain reference standards for many of the cultivars and they remained unknown. Errors in this labelling on the map became evident as shown by the dendrogram of trees labelled as *cv* Manzanillo in Fig 4. The comparison of trees, reportedly to be the same cultivar, was clearly different, based on DNA patterns and seed morphology.

There was little data on the performance of any olive variety for optimal yields and quality under Australian conditions and the industry has relied on information from the Northern hemisphere, particularly from Mediterranean sources. Performance characteristics of cultivars are the basis on which a selection is made for a particular use or physical situation. The National Olive Variety Assessment (NOVA) project was established to help resolve the confusion in olive variety identity and to evaluate the performance, in different climatic regions of Australia, of the majority of known commercial olive varieties. (Sweeney, 2005). The establishment of a national varietal grove at Roseworthy provided an opportunity for growers to evaluate different cultivars, grown at that site.

At the same time studies were being undertaken on wild olive trees which had become established in the Adelaide Hills to attempt to identify feral olives which may be better adapted to the Australian conditions (Sedgley, 2000). Despite these investigations, the Australian industry has been established on common European cultivars and some more recently introduced including *cv* Barnea from Israel.

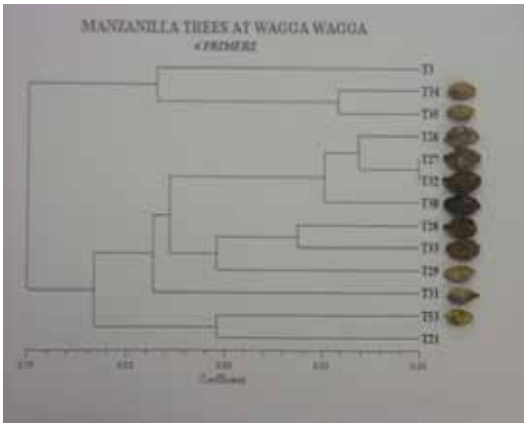

Fig. 4. Dendrogram created with 10 RAPD primers illustrating differences in seed morphology of 11 trees identified by the historic map as *cv* Manzanillo (Mailer unpublished).

9.3 Environment

Perhaps the main issue for Australian oils was the very variable environmental conditions under which the crop was being grown. Oil quantity and quality rely heavily on crop management, moisture availability, harvest timing, processing methodology and storage. As a result of these differences Australian oils showed a wide range in the fatty acid profiles (Mailer, 2005b). The diversity in other quality characteristics and sensory analysis were also significant.

In the initial stages of the development of this industry, there was little understanding of the intricacies of these crop management practices and the resultant crop yield, chemical quality and sensory attributes such as taste, colour and odour. For these reasons, several studies have been undertaken to look at the relationship between oil quality and harvest timing, irrigation treatments, yield and sensory characteristics (Mailer 2007).

As a result of the low rainfall and unpredictable nature of the Australian environment, almost all Australian olive groves are irrigated. Irrigation provides more predictable yields and harvest timing unlike dryland groves. Due to the importance of irrigation, research has focussed on water requirements, particularly in the stages from planting to commercial harvesting (De Barro, 2005). This research has been aimed at increasing the understanding of olive water use and requirements in the period from planting to early fruit bearing. As most Australian olive groves are irrigated several studies have focussed on variation in maturity, yield, oil quality and sensory attributes under variable moisture availability (Ayton et al., 2007) and with different harvest times.

Environmental effects on oil production have created unexpected issues for Australian producers. The variation in quality and sensory has created a new spectrum of oils with unique qualities and sometimes more variable attributes than has been produced in Mediterranean climates. This sometimes results in the oil being outside the limited range of existing international standards (Mailer, 2007).

9.4 Harvesting and processing

Along with the determination of the best cultivars, the methods of harvesting and processing have been evaluated. Many types of harvesters and extractors, generally from European manufacturers, were being used throughout the industry. Hand harvesting and many types of vibrating rakes, tree shakers and accessories were unsuitable for large scale production. Larger producers investigated straddle harvesters (Fig. 3) which underwent several modifications to make them suitable. These are used widely today.

Processing also went through stages. Some producers tried mechanical mat presses or stone mills (Mailer & Ayton, 2004) in the early stages but these were never used for large scale production The majority of processors have installed modern two or three phase centrifugal extraction mills.

Fig. 5. One of the early harvesting methods adopted in an Australian olive grove.

9.5 Quality analysis

As the industry developed, a need for quality evaluation increased. Using the resources of the International Olive Oil Council, Australian laboratories were able to set up methods to determine oil quality. Many of the existing methods were time consuming, reasonably difficult to carry out and expensive. This prompted the investigation of more rapid methods such as the near infra red spectrometry (Mailer, 2004), a rapid screening analytical tool whilst more intensive, wet chemistry methods were maintained as checks where necessary.

Minor compounds in olive oil were recognised as the basis of the sensory attributes, nutritional value and stability, or antioxidant capacity, of the oil. Additionally, minor compounds are used to ensure authenticity in Australian research, particularly the sterol profile. As Australian oil has a wide spectrum for each of the sterol components, which may lie outside the limits of the IOC standards, this has been an important focus for Australian scientists (Guillaume et al., 2010). Environmental effects and irrigation on polyphenols both showed a significant effect (Mailer et al 2007). The influence of frost on these compounds and the resulting changes in sensory and chemical characteristics has also been investigated (Guillaume et al., 2009). Frost is one of the most important weather related hazards for the Australian olive industry and has caused significant economic losses during the past decade. Its impact on oil quality has been significant in 2006 with more than 20 per cent of Australian

oil of that year being affected to some degree. Early frosts will normally affect the fruit leading to significant changes in the chemical and organoleptic characteristics of the oils.

9.6 Shelf life

Although oil may be acceptable when it is processed, maintaining the quality after processing became a major consideration. Two studies carried out in 2008 by the Australian Olive Association on supermarket oils (AOA Report – unpublished) included 22 oils initially and later, a further 33 oils of random brands. The reports revealed that many oils would not pass IOC tests most likely because of poor storage or old age, although some oils were clearly adulterated. The AOA and RIRDC have funded long term storage studies under extremes of temperature, light and oxygen exposure to determine potential shelf life and develop an understanding of methods used to advise marketing on potential shelf life.

Shelf life depends heavily on the type of material the oil is stored in. Although most experts would recommend the use of glass or stainless steel, often oil is stored temporarily in plastic bottles or collapsible bags. Studies on the effects of the different types of containers used for transport and sometimes for long term storage have been published (Mailer & Graham, 2009). The study reinforces that the best storage conditions for olive oil is in opaque, impervious and inert containers, stored at cool temperatures. Metallised flexible bags used for short term transport may provide reasonable protection. Storage in clear plastic, particularly in the light and at elevated temperatures, is unacceptable and results in loss of extra-virgin olive oil quality within a short period. Re-use of these containers is highly undesirable and would cause more rapid degradation.

9.7 Pest and disease

Australia has been free of many cosmopolitan olive pests due to its isolation but the rapid expansion of the olive industry in all mainland states has led to increased problems with pests and diseases. The control of these problems became a focus for all growers. A report on sustainable Pest & Disease Management in Australia Olive Production (Spooner-Hart, 2005) describes sustainable management, monitoring pest and beneficial species in groves and identified a number of previously unreported pests and diseases. Further publications have included a field guide to olive pests, diseases and disorders in Australia (Spooner-Hart et al., 2007) designed as a quick reference to take into the grove and use to identify pests and diseases and the damage they cause.

9.8 Waste management

Dealing with by-products of olive oil processing is another important issue in modern agriculture. A study on recycling of solid waste from the olive oil extraction process (Tan & Markham, 2008) and a subsequent report outlines methods for developing an environmentally sustainable system to manage solid waste from the 2- and 3-phase olive oil mill extraction processes. The expanding Australian olive industry over recent years, with significant increase in fruit production, has resulted in vast quantities of solid and liquid wastes generated to the detriment of the environment. The industry is been faced with the

challenge to manage these wastes in order to achieve sustainable production under a clean environment. The research work provided the industry with a tool to recycle processed oil mill waste to improve the health of the crop and the status of the soil.

9.9 Reviews

The revival and development of the olive industry stimulated wide areas of research over a relatively short period. The quest for information has been intense. This has led to the publication of several reviews being carried out, particularly regarding the potential for olive production in Australia. These include studies by McEvoy et al. (1999) in which the market for the development of an olive industry in Australia was examined based on analysis of: trends in international production and trade; consumer segments and product characteristics; whether Australia could compete with imported olive products.

Another review contains detailed steps required to establish an olive grove in Australia and is a comprehensive survey of the Australian Industry (Meyers Strategy Group, 2010). It was developed as a method of establishing how Australia could compete in a rapidly growing olive industry worldwide.

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12

Consumer Preferences for Olive-Oil Attributes: A Review of the Empirical Literature Using a Conjoint Approach

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1. Introduction

1.1 Olive oil: Some general aspects

During the last decade, olive oil consumption has experienced a major breakthrough in the world, not only in producing countries but also among those who are not. Undoubtedly, this growth in consumption is a consequence of the consolidation of a cultural phenomenon established between the main producing countries (Spain, Italy and Greece), owing to the so-called Mediterranean diet[1]; a food concept that provides important health benefits and of which olive oil is one of the main components. The recent recognition of United Nations Educational, Scientific and Cultural Organization (UNESCO) — it has declared to the Mediterranean diet 'the intangible cultural heritage of humanity'— offers promising perspectives for the Mediterranean diet in the coming years.

The major producer of olive oil in the world is the European Union (EU), which produces 80 per cent and consumes 70 per cent of the world's total olive oil production (European Commission, 2010). Italy and Spain are the major producers and can influence the prices of olive oil (Blery and Sfetsiou, 2008). Greece takes third place in world production after Spain and Italy (Sandalidou and Baourakis, 2002) and first place in olive oil consumption

[1] The Mediterranean Diet is a way of eating based on the traditional foods (and drinks) of the countries surrounding the Mediterranean Sea. The principal aspects of this diet include high olive oil consumption, high consumption of pulses unrefined cereals, fruits and vegetables, as well as moderate consumption of dairy products (mostly as cheese and yogurt), moderate to high consumption of fish, low consumption of meat and meat products, and moderate wine consumption. Olive oil is particularly characteristic of the Mediterranean diet. It contains a very high level of mono-unsaturated fats, most notably oleic acid, which epidemiological studies suggest may be linked to a reduction in the risk of coronary heart disease. There is also evidence that the antioxidants in olive oil improve cholesterol regulation and 'Low-density lipoprotein' (LDL) cholesterol reduction, and that it has other anti-inflammatory and anti-hypertensive effects.

(Hellonet, 2006). Olive farming provides an important source of employment in many rural areas of the Mediterranean, including many marginal areas where it is either a principal employer or an important part-time employer which can be combined with other activities, such as tourism. Olive farming is also an important part of local rural culture and heritage in many areas, and is maintained and 'valorized' through labelling schemes in some cases. Olive production is an important economic sector in many rural areas of the Mediterranean. In some areas, it is the principal economic activity and the basis for other sectors (Beaufoy 2002:11). The greatest concentration of oil production is found in two Spanish provinces, Jaén and Córdoba in Andalusia, which between them account for over one-third of EU output. Olive farming has both positive and negative environmental effects. As Beaufoy's (2002:30) report indicates, such effects depend on several factors, including prevailing environmental conditions in and around the plantation (soil type, slope, rainfall, adjacent land uses, presence of water bodies, etc.) and farm management, pest control, irrigation and the type of land (and previous land cover) on which new plantations are established. In particular, Beaufoy (2002) identified the following as the main categories of actual and potential environmental effects associated with the management of olive plantations: soil, water, air, biodiversity (flora and fauna), and landscape.

Among the EU non-producing countries of olive oil, Germany and the UK are the main consumers (de la Viesca *et al*., 2005), although the US is the most important market outside the Mediterranean basin (Zampounis, 2006). In the US, interest in and consumption of olive oil has been growing exponentially over the last 20 years (Delgado and Guinard, 2011). Indeed, the US ranks fourth in olive oil consumption after Italy, Spain and Greece. US consumption went from 88,000 tons in 1990 to 260,000 tons in 2009; an increase of 228% (International Olive Council, 2008). Something similar is happening in China, where the demand for olive oil is expected to increase significantly in the next few years (Soons, 2004). According to this author, Chinese tourism to Mediterranean countries will affect the general awareness of the healthy Mediterranean kitchen and its use of olive oil in a positive way.

The increasing preference for olive oil worldwide denotes a change in consumer behaviour, either by strengthening the role of it in their diet or by incorporating it in a novel way. The set of tangible and intangible attributes that consumers believe to particularly meet their needs, is a concept of product marketing. From this point of view, the concept is intended to reflect two fundamentally different approaches: a) considering the product itself as a sum of characteristics or physical attributes; or b) considering the needs of the consumer, where the buyer's choice rests not with the product, but with the service they expect to receive or the problems it can solve.

During the purchase process, consumers form their preferences based on the best combination of attributes, evaluating the brands that are part of their evoked sets, or are considered important in terms of attributes such as price, country of origin, quality or design, among others. Olive oil, like any other commodity, is not immune to this stage of the buying process, despite the uniqueness of its attributes that determine the degree of preference for the consumer. In this chapter, we aim to describe what attributes assume greater importance, and therefore are preferred, by the consumer. In order to do so, a review of the previous literature focusing on this stage of olive oil consumer-buying behaviour is developed.

The rest of the chapter is structured as follows. Section 2 discusses the importance of culture as a factor in the formation of consumer preferences with respect to olive oil. In Section 3, we discuss consumer preferences for olive oil from the literature review, with reference to the methodology on conjoint analysis. Finally, we present the findings of the work.

2. Consumer behaviour in purchasing food: The role of culture in the consumption of olive oil

The study of consumer behaviour and marketing discipline has focused on analysing how individuals make decisions to spend their resources in categories related to consumption (Schiffman and Kanuk, 2001). The act of purchase is considered as an activity aimed at solving a problem (Howard and Sheth, 1986). Typically, the consumer is faced with a multitude of decisions to make, whose complexity varies depending on product and purchase situation (Lambin, 1995). Consequently, understanding consumer behaviour requires assessing how people made and make their purchasing and consumption decisions (Blackwell et al., 2001), considering that a decision is the result of selecting a choice from two or more alternative possibilities (Schiffman and Kanuk, 2001).

In the context of food products, Steenkamp (1997) proposes a conceptual model of consumer behaviour in which four stages in the purchase decision process are identified: (1) problem recognition; (2) information search; (3) evaluation of alternatives; and (4) choice. In addition, there are three groups of factors that influence this process: a) properties of foods; b) Individual-related factors (e.g., biological, psychological and demographic); and c) environmental factors (i.e., economic, cultural factors and marketing aspects (see Figure 1).

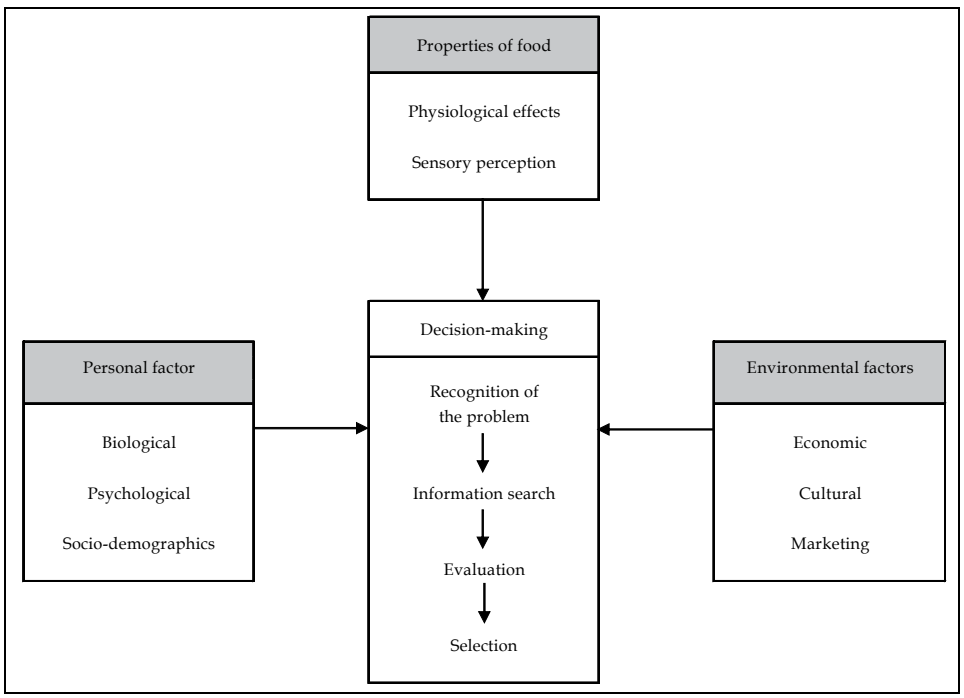

Fig. 1. Model of consumer behaviour for food (Steenkamp, 1997)

As Figure 1 shows, culture is a key concept for understanding consumer behaviour. According to Solomon *et al*. (1999), this is a consequence of culture representing the collective memory of society and the prism through which such society develops its perception. Culture includes a complex of values, ideas, attitudes and other meaningful symbols that allow humans to communicate, interpret and evaluate as members of a society (Blackwell *et al.,* 2001). According to Schiffman and Kanuk (2001), it is the sum of learned beliefs, values and customs helping to determine the behaviour of members of a given society as a consumer. Undoubtedly, culture is a key element not only because it affects all stages of consumer choice, but also because it exerts a major influence on the reasons why people of different cultures buy and consume products (Blackwell *et al*., 2001). In this manner, culture helps to explain the behavioural differences between them. Specifically, during the evaluation stage, culture mainly influences the way in which consumers assign a greater value to certain attributes of the product over others. As Solomon and colleagues (1999) indicate, a consumer culture determines the priorities of certain products and the success or failure thereof.

When consumers buy a product, they expect it to perform their need. But these needs are different between cultures. This is, for instance, the case of olive oil. There are big differences between olive oil producing, Mediterranean countries and non-producing countries. For the former, olive oil can be considered as a traditional food product. In this respect, the literature shows some important associations between the consumption of such traditional products and cultural aspects such as values, beliefs and life-style orientations (Vanhonacker et al., 2010). In this respect, as noted by Govers and Schoormans (2005), some studies have tested how consumers prefer products or brands with a particular symbolic meaning, compatible with the image they wish to convey of themselves. In some cases, this is intended to resemble the kind of people who normally use the product (Heath and Scott, 1998). Thus, traditional food consumers are generally not caught up in modern ways of life (Guerrero et al., 2009), where time pressure, business and convenience orientation dominate. Housewives are usually portrayed in the literature as typical consumers. In addition, traditional food consumers are also portrayed as liking the familiar; one expression of this preference being the consumption of familiar dishes (Dagevos, 2005). According to this author, these consumers have fairly conservative food habits, maintaining culinary customs across generations. In addition, they are concerned about their health. In this context, olive oil plays an important role.

There exist several studies analysing the role of culture and food habits in the behaviour of consumers regarding such products. Thus, Nielsen *et al.'s* (1998) cross-cultural study showed that there were large differences in the perceptions of virgin olive oils across UK, Denmark and France. Olive oil users from all three countries agreed on the health benefits of virgin olive oil, which led to the feeling of good health and a long life. Therefore, both hedonistic and sensory aspects of virgin olive oil appeared the most varied between countries.

In the UK, García *et al.* (2002) used focus groups and conjoint analysis to analyse the product attribute trade-offs that consumers make when choosing olive-oil products. One of this paper's main findings was that British consumers continue to regard olive oil as a set of individual attributes (e.g., size, taste and health) instead of a product that is perceived as encapsulating all these attributes. They found that price was one of the most influential

factors on consumers' preferences for basic olive oil, followed by size of container. The main role of price was recently pointed out by Dekhili and d'Hauteville (2009), who found that price was the most important choice attribute in both producing (e.g., Tunisia) and non-producing (e.g., France) countries. In this respect, although fair prices can be charged for olive oil compared with other vegetable oils, there is a limit to the price many consumers are willing to pay (Mili, 2006). The use of price as a choice criterion for consumers is a consequence of the variety of olive-oil brands. Nevertheless, it is true that aspects such as colour, packaging and product labelling are helping producers to differentiate their brands from those of competing suppliers in the distribution chain for olive oil (Van der Lans et al., 2001).

Moreover, aspects related to the origin of olive oil are becoming more important in consumers' choice behaviour. Dekhili and d'Hauteville (2009) showed that the region of origin was relevant in explaining consumer behaviour. Such a regional image has three components: (i) local agronomic conditions (soils, climate); (ii) traditional human know-how; and (iii) raw product characteristics (variety). Thus, these authors found important differences between France and Tunisia in giving credence to the role of an olive-oil-specific regional image. In particular, these authors found quite significant differences regarding the relative weights of the attributes of this image valued in each country. In this respect, there is a growing segment of consumers who prefer quality food with certification of origin (both Protected Designation of Origin [PDO] and Protected Geographical Indication [PGI]). Dekhili *et al*. (2011) found that these 'official cues' are more important for those consumers belonging to non-producing olive oil countries (e.g., France), whereas in producing countries (e.g., Tunisia) consumers tend to choose olive oil based on origin and 'sensory cues' (e.g., colour and appearance). For instance, in Spain there are 32 PDOs for olive oil; that is, Andalusia, in southern Spain, the geographical area with the highest number of certifications of origin. Sanz and Macías (2005) confirmed the strategic role of Spanish olive oil PDOs. Thus, these authors found that such PDOs, effectively, add greater value to local production systems and so enhance the final quality and market differentiation of a specific-origin olive oil. In this respect, Scarpa *et al*'s. (2005) study in the context of three products (table grapes, oranges and olive oil), confirmed the importance of PDOs. According to these authors, the role of PDOs was stronger for olive oil compared to the other two categories analysed.

Thompson et al. (1994) used Ajzen & Fishbein's (1980) *theory of reasoned decision* (TRA) successfully, as a mean of identifying the major issues influencing olive oil choice in the UK. These authors found that attitudes were strongly related to the user or non-user of olive oil. In this respect, the most significant attitude related to the flavour-improving attributes of olive oil (e.g., improving the taste of salads and cooked meals).

In Mediterranean countries, Saba & Di Natale (1998) surveyed 909 Italians in order to assess their attitudes towards fats and food choice. The researchers also used Ajzen & Fishbein's (1980) TRA, combined with a measure of 'habit', as a theoretical framework. The findings suggested that in Italy, culture and food habits might predict intention to consume fats and oils better than TRA. Saba *et al*. (2000) recently re-confirmed this attitudinal TRA model in the Italian context.

Another interesting aspect related to culture and purchase habits is the place of purchase. Delgado and Guinard's (2011) study of US consumers, reported that the majority of them bought olive oil (extra-virgin) primarily at the supermarket (68%), specialty stores (50%) and

farmers' markets (43%), in contrast with the ways in which Mediterranean consumers most frequently buy their olive oil. Thus, Fotopoulos and Krystallis (2001) reported that 41% of Cretan consumers buy olive oil at the supermarket, while 38% buy in bulk directly from the producer or farm, and 21% make oil from their own olive orchards. Similar figures can be associated with other Mediterranean producing countries such as Italy or Spain. This habit is a consequence of the consumer experience, of belonging to producing countries and the role of olive oil in their intrinsic cultures. Thus, in buying olive oil at supermarkets or hypermarkets, consumers are not exposed to the sensory properties of the product, as they are at farmers' markets or direct from the producers or farms, and so their decisions are based on extrinsic factors such as packaging materials, bottle material and label design (Delgado and Guinard, 2011). This is the case of US consumers.

This is probably the reason why US consumers prefer Italian oil more than Spanish oil. In our opinion, Italian oil possesses a lower quality than Spanish olive oil. However, Italian oil's marketing strategy, from a general point of view, is stronger than the strategies used by Spanish producers. In contrast, when consumers buy oil in bulk directly from the producer, as is the case with consumers belonging to producing countries, they experience the properties of the oil and can make purchasing decisions based on sensory factors. This is the case for consumers living in Mediterranean countries. Even, nowadays these 'experienced consumers' show a greater interest in organic olive oil, given the increasing interest of consumers in ecologically clean products for health and environmental reasons (Gavruchenko et al., 2003). Consumers' need for safer, good quality food has increased over the last years and thus, healthiness and nutritional value are the basic reasons given by consumers for purchasing organic olive oil. In this way, more consumers are willing to pay a higher price, since they take into account organic olive oil's contribution (Sandalidou et al., 2002:405). Nevertheless, Sandalidou *et al*. (2003) pointed out that there is a large number of people who still do not know what an organic product is. For this reason, these authors suggest that the systematic provision of information, mainly through advertising, is necessary, in order to enhance consumers' awareness of organic olive oil's features and nutritional content.

3. Analysis of consumer preferences of olive oil

What motivates consumers to prefer and purchase olive oil is not clear (Delgado and Guinard, 2011:214). As has been indicated before, some authors highlight, as the main motivators behind consumption, the oil's region of origin, focusing on the influence of PDO designation and the degree to which an oil typifies the characteristics of that particular region. This is especially true for those consumers who are experienced, local or familiar with a particular region of origin, whereas these factors do not seem to affect urban, less knowledgeable and less experienced consumers (Caporale *et al*., 2006).

Other authors focus on olive oil's health benefits and flavour (including its use to enhance the taste of recipes) as main motivators for olive oil consumption. Thus, olive oil is promoted as beneficial for health, and industrial strategies and advertising are often based on health claims (Duff, 1998) although, nowadays, EU regulation has imposed the use of 'nutritient profiles', which are already in use in the USA and Canada, and which are under development (Blery and Sfetsiou, 2008:1151). However, many critics argue that this aspect would mean that products such as olive oil should not carry health claims. At present, there is no harmonized legislation at EU level to ensure the scientific accuracy and

appropriateness of such claims. Nevertheless, homogeneous regulation is expected to set clear parameters across Europe for health claims, and they will be allowed only if they are substantiated scientifically (Tamsin *et al.,* 2005). Given the increasing number of countries being integrated into the EU in the last few years, this seems to be even more important for the success of European olive oil production.

New olive oil consumers seem to be more interested in olive oil for two main reasons: health benefits and flavour (Santosa, 2010). Olive oil is claimed to be beneficial for health, as it is rich in vitamin E and it does not contain preservatives (Blery and Sfetsiou, 2008). Among health benefits, lowering the risk of coronary disease, preventing certain kinds of cancer and reducing inflammation have been highlighted (Medeiros and Hampton, 2007). For these reasons, Duff (1998) pointed out that the preference for olive oil is a result of health reasons because the replacement of saturated fats by olive oil results in a lowering of the rate of heart disease. Nevertheless, it is true that there other cheaper seed oils being used as substitutes (Bernabéu et al., 2009). In this respect, olive oil has a high price, although it depends on its origin and its quality (Bourdieu, 1984). For instance, virgin and extra-virgin olive oils are more expensive than standard olive oil. With regard to flavour, Santosa and Guinard (2011) recently reported that this is an important aspect in both the consumption and purchase motivations for olive oil, especially for extra-virgin olive oil, where sensory characteristics are even more important. According to Thompson *et al.* (1994), this is also a consequence of improving the taste of salads and meals.

3.1 Study of consumer preferences of olive oil through conjoint analysis

The measurement of attitudes/preferences using a multi-attribute methodology, especially a conjoint Analysis of Multivariate technique, is most appropriate.

In fact, this methodology has become an important tool to assess the preferences that a consumer assigns to the various attributes of a specific product/brand (Ruiz and Munuera, 1993). Hair *et al.* (2009) define conjoint analysis as:

'a multivariate technique used specifically to understand how respondents develop preferences about products or services, and is based on the simple premise that consumers assess the value of a product/service idea (real or hypothetical) combining separate amounts of value provided by each attribute. The utility, which is the conceptual basis for measuring this value, is a subjective preference unique to each individual which includes all the features of a product or service, both tangible and intangible, and as such, is the measure of overall preference'.

The most direct application of conjoint analysis is as a tool to find the weight or importance that different levels or categories of product attributes play on the formation of consumer preferences (Múgica, 1989). Therefore, conjoint analysis seeks to establish the relative importance of attributes and levels, inferring the utility (satisfaction) that consumers express when they are presented with a series of product concepts that vary in a systematic way (Walley *et al.,* 1999). The application of this methodology in the field of food has, until recently, been quite limited (Van der Pol and Ryan, 1996), starting in the 1990s when it began to generate a relevant scientific production. This confirms the suitability of this methodology to improve knowledge about consumer behaviour when purchasing food. Thus, when reviewing the literature, it appears that it has only been in recent years that

there has been further development in this field of research, ranging from the wine, to the meat, dairy, fruit and vegetable industries. In the case of olive oil, although the literature is not extensive, there are several studies that have examined consumer preferences in deferent countries, as shown in Table 1.

Despite the tradition existing in the consumption of olive oil in the main producing countries, studies carried out to analyse consumer preferences in these countries have been scarce. Probably, this is a consequence of the olive oil's difficulties for differentiating itself in order to better meet the needs of consumers.

One of the first studies that examined consumer preferences is Fotopoulos and Krystallis (2001) that analysed Greek consumer preferences based on two attributes: price and character of protected designation of origin (PDO). Van der Lans *et al.* (2001), in turn, focus their analysis of preferences on extra-virgin olive oil, a variety that is characterized by acidity (oleic acid) to a maximum of 0.8 g per 100 g. According to these authors, the selected attributes were price, colour, origin and appearance, like the unit sample in two Italian regions.

Garcia *et al.* (2002) provide the first work that analyses consumer preferences of olive oil in a non-producing country such as the UK. Following this study, other countries have been used (e.g., Japan (Mtimet *et al.*, 2008) and Canada (Menapace *et al.*, 2011)).

Therefore, in analysing consumer behaviour for olive oil, there are various areas of analysis that take us beyond the attributes that have been considered in each study. In this respect, preference will be conditioned by the variety of olive oil covered by the study, and analysed even if the consumer resides in a country producing this product or not.

A first result that emerges from the literature review is that the extrinsic attributes of olive oil (e.g., price, origin or variety) are the most important when consumers face the act of purchase. Instead, intrinsic attributes, such as colour or flavour, are relegated to second place, with the exception of Mtimet *et al.* (2008), who analysed the Japanese consumer, for whom colour comes first.

Focusing specifically on the extrinsic attributes, a second interesting result refers to the importance of price when buying olive oil. Indeed, in five of the nine studies analysed this is the attribute with the highest relative importance. It should also be noted that this is true for consumers belonging to both producer and non-producer countries, and is also irrespective of the variety of olive oil.

In addition, our analysis shows that origin of oil is also an extrinsic attribute of interest to the consumer. Thus, in a majority of the papers analysed, it is the first or second attribute in order of importance, either in the consumer's conceptualization of country of origin, the region of origin or as part of a Protected Geographical Indication.

With regard to the variety of olive oil, another interesting finding is that,for the case of extra-virgin olive oil, price is always the most important attribute.

Finally, with regard to the sampling unit of analysis, it can be observed that among consumers of non-producing countries, the origin of oil is not the primary attribute on which they make their purchasing decision. Indeed, in two of the three studies analysed, the origin of the oil is the second attribute in importance. In the third study, this factor was not considered. This conclusion is very different among consumers of producer countries, probably as a consequence of the fact that they are more familiar with the product.

	Type	Country	Producing	Attribute	Rank (relative importance) (%)
Fotopoulos and Krystallis (2001)	Olive	Greece	Yes	PDO Price	1st - (55.51) 2nd- (44.49)
van der Lans, van Ittersum, De Cicco and Loseby (2001)	Extra-virgin olive	Italy	Yes	Price Colour Origin Appearance	1st- (n.d.) 2nd- (n.d.) 3rd- (n.d.) 4th- (n.d.)
García, Aragonés and Poole (2002)	Olive	UK	No	Price Size Packaging	1st- (37.40) 2nd- (33.48) 3rd- (29.12)
Scarpa and Del Giudice (2004)	Extra-virgin olive	Italy	Yes	Price Quality Certification Origin Appearance	1st- (44.44) 2nd- (28.60) 3rd- (25.41) 4th- (1.54)
Krystallis and Ness (2005)	Olive	Greece	Yes	Origin Organic label Health info Quality certifications (HACCP, ISO) PDO label Price Glass bottle	1st- (21.71) 2nd- (19.07) 3rd- (16.96) 4th- (11.11) 5th- (9.58) 6th- (8.10) 7th- (7.17) 8th- (6.29)
Mtimet, Kashiwagi, Zaibet and Masakazu (2008)	Olive	Japan	No	Colour Origin Price Olive oil type Taste	1st- (30.14) 2nd- (29.06) 3rd- (20.50) 4th- (10.34) 5th- (9.94)
Bernabeu, Olmeda, Diaz y Olivas (2009)	Olive	Spain	Yes	Oil type Origin Price Production System	1st- (41.09) 2nd- (33.35) 3rd- (25.35) 4th- (0.20)
Chan-Haldbrent, Zhllima, Sisior and Imami (2010)	Olive	Albania	Yes	Price Olive oil type Origin Taste Place purchase	1st- (34.70) 2nd- (22.16) 3rd- (20.96) 4th- (18.66) 5th- (3.52)
Menapace, Colson, Grebitus and Facendola (2011)	Extra-virgin olive	Canada	No	Price Origin Production system Geographic identification Appearance Colour	1st- (36.88) 2nd- (26.54) 3rd- (23.72) 4th- (12.49) 5th- (0.35) 6th- (0.00)

Table 1. Analyses for consumer preferences of olive oil.

4. Conclusions and recommendations

From a marketing point of view, consumers' purchasing behaviour is affected by the presence of heterogenous preferences that are derived from their own needs. This is especially important when the consumer is faced with new products or innovations. With regard to olive oil, an agrofood product that is one of the main components of the well-known Mediterranean diet, this heterogeneity is still greater, given the lower consumer knowledge in relation to such a product. Nevertheless, it is not exclusive of markets in the first stages of adopting olive oil, but is also, to a lesser extent, present in countries with a stronger tradition of olive oil consumption. Thus, the majority of studies report a stronger role of extrinsic aspects over intrinsic attributes. In this context, price is the major factor affecting consumer behaviour. Nevertheless, when the consumer is familiar with the product, the country of origin is the most influential aspect in determining consumer purchases.

The results obtained here lead the authors to offer a number of implications for marketing management in the olive oil sector. Given the strong presence of olive oil in international markets, as well as production systems strongly linked to a specific area of origin, promoting familiarity or experience of the products could provide firms with an effective source of competitive advantage, because of the positive consequences for the product image.

In particular, for those markets with less knowledge of olive oil (e.g., the US or China) communication actions will be critical for increasing consumers' familiarity and consequently their knowledge about these products. Thus the objective of commercial communication should consider not only different perceptions of the product cues, but also the differences in product familiarity. In particular, for countries in which product knowledge is greater and consumers have greater knowledge of the intrinsic characteristics of the product, communication campaigns should aim to reinforce consumers' image of the product. In contrast, in countries where the product knowledge is more limited, communication should focus on raising the consumers' level of familiarity and knowledge. This will raise the consumer confidence and, by extension, their purchase intention (Laroche *et al*., 1996). In general terms, such campaigns should be divided into four consecutive stages:

1. *Campaign to raise awareness among producers and exporters of olive oil*. This first stage should focus on informing producers/exporters about the positive consequences that a favourable image of the area of origin can have for each firm in particular, and for the sector in general. It is necessary to improve training in marketing and communication, for producing and commercial firms, so that they adopt a market orientation approach. For instance, this is the case for Spanish olive oil. Such campaigns are necessary if Spanish olive oil wants to improve its international market penetration, in relation to Italian olive oil.
2. *Educational campaign aimed at consumers*. The main objective at this stage should be to inform the consumer about the area of origin, the olive oil's area characteristics, the production techniques, and so forth. In short, the objective should be to communicate to the consumer the sector's enormous experience in the cultivation of olive oil, and the quality and safety processes used to obtain the end product. This stage of the campaign should also include communication actions aimed at prescribers (e.g., restaurant owners, food specialists) so that they recommend the product to the end consumers (current and potential consumers).

3. *Campaign aimed at distinguishing the most important product cues in the formation of the image in each target market*. The advertising messages should be adapted to each market as a function of its preferences, and the tools and communications media chosen should be consistent with the messages. The messages should transmit the idea that olive oil from the area of origin has attributes that are the most important for that particular consumer.
4. *Campaign to increase consumption and maintain loyalty to the brand.* After achieving the positioning, the consumer should be reminded of the benefits of consuming olive oil from the specific area of origin. At this stage, the communication campaigns must consolidate the product image and be aimed at different segments. Likewise, in the distribution channel, the communication should be flexible and adapted to the particular needs of each channel agent.

In developing all these communication campaigns, public institutions and governments can play an important role. Given the importance of olive oil sector in their socio-economic context, this is especially relevant for the case of Mediterranean olive oil producing countries. Therefore, public institutions are advised to collaborate with their own countries' olive oil sector.

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Part 2

Olive Oil Extraction and Waste Water Treatment – Biotechnological and Other Applications

13

New Olive-Pomace Oil Improved by Hydrothermal Pre-Treatments

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1. Introduction

The health properties of virgin olive oil (VOO) are well known in the Mediterranean Diet, in which VOO is the main source of fat (Boskou, 2000). The Mediterranean area provides 97% of the total olive production of the world and represents a major industry in the region (Aragon & Palancar, 2001). The fatty acid composition is not the only healthy component of olive oil; in addition, minor components have high biological activities (Pérez-Jiménez et al., 2007). From the olive oil by-product, the olive-pomace oil (OPO) is obtained. Recent studies have demonstrated the positive benefits of OPO on health, and these effects are due mainly to the presence of minor components (Ruiz-Gutiérrez et al., 2009). The new olive oil extraction processes in the olive mills make the extraction of OPO and the general utilisation of wastes more difficult. New thermal systems are proposed to pre-treat the olive oil wastes to facilitate their utilisation and OPO extraction.

1.1 Olive oil extraction systems

The manufacturing process of olive oil has undergone evolutionary changes. The traditional discontinuous pressing process was initially replaced by continuous centrifugation, using a three-phase system and later a two-phase system. Depending on the different olive oil production method, there are different kinds of wastes. The classic production of olive oil generates three phases and two wastes: olive oil (20 %), solid waste (30 %) and aqueous liquor (50 %). The solid waste (olive cake or "orujo") is a combination of olive pulp and stones. The aqueous liquor comes from the vegetation water and the soft tissues of the olive fruits, with water added during processing (so-called "alpechin" or "olive-mill waste water"). The presence of large amounts of organic substances (oil, polyphenols, protein, polysaccharides, etc.) and mineral salts represents a significant problem for the treatment of wastewater (Borja et al., 1997).

The use of a modern two-phase processing technique to which no water is added generates oil and a new by-product that is a combination of liquid and solid waste, called "alperujo", "alpeorujo" or "two-phase olive mill waste". This by-product is a high-humidity residue

with a thick sludge consistency that contains 80 % of the olive fruit, including skin, seed, pulp and pieces of stones, which is later separated and usually used as solid fuel (Vlyssides et al., 2004). In Spain, over 90 % of olive oil mills use this system, which means that the annual production of this by-product is approximately 2,5-6 million tons, depending on the season (Aragon & Palancar, 2001).

1.2 Utilisation of olive oil wastes

Alperujo presents many environmental problems due to its high organic content and the presence of phytotoxic components that make its use in further bioprocesses difficult (Rodríguez et al., 2007a). Most of these components mainly phenolic compounds, confer bioactive properties, to olive oil. The extraction of the phenolic compounds has a double benefit: the detoxification of wastes and the potential utilisation as functional ingredients in foods or cosmetics, or for pharmacological applications (Rodríguez et al., 2007a). Although olive mill wastes represent a major disposal problem and potentially a severe pollution problem for the industry, they are also a promising source of substances of high value. In the olive fruits, there is a large amount of bioactive compounds, many of them known to have beneficial health properties. During olive oil processing, most of the bioactive compounds remain in the wastes or alperujo (Lesage- Meessen et al., 2001).Therefore, new strategies are needed for the utilisation of this by-product to make possible the bioprocess applications and the phase separation of alperujo.

Until now, efforts focussed on detoxifying these wastes prior to disposal, feeding, or fertilisation/composting, because they are not easy degradable by natural processes, or even used in combustion as biomass or fuel (Vlyssides et al., 2004). However, the recovery of high value compounds or the utilisation of these wastes as raw matter for new products is a particularly attractive way to reuse them, provided that the recovery process is of economic and practical interest. This, added to the alternative proposals to diminish the environmental impact, will allow the placement of the olive market in a highly competitive position, and these wastes should be considered as by-products (Niaounakis & Halvadakis, 2004).

1.3 Olive-pomace oil

After VOO extraction, the residual oil, or crude olive-pomace oil (COPO), is extracted by organic solvent extraction or centrifugation from olive oil wastes. After the COPO refining step, the refined olive-pomace oil (ROPO) is blended with VOO, obtaining OPO for human consumption. Currently, the growing interest in OPO is due to its biological active minor constituents (Ruiz-Gutiérrez et al., 2009). The concentration of these components in OPO is higher than the concentration in VOO, with the exception of polar phenols (Perez-Camino & Cert, 1999). Today, new processes for COPO refining are studied in order to diminish the loss of minor components (Antonopoulos et al., 2006). Some of these components are recovered in the refining steps.

Alperujo is treated by the OPO extractors for crude olive-pomace oil extraction (**Figure 1**). First, the major part of the stone present is removed, with the initial stone concentration of about 45% and, after the stone extraction, less than 15%. The stone is easily commercialised for numerous uses, such as in combustion materials, activated carbon, liquid and gas production from stone pyrolysis, an abrasive for surface preparation or for cosmetics, in

addition to others (Rodríguez et al., 2008). The pitted alperujo is frequently centrifuged in the OPO extractor because the new decanter technology allows treating low-fat material for oil extraction, through which crude olive-pomace oil is obtained. After this mechanical extraction, a partially defatted and pitted alperujo is obtained, with a humidity close to 50%. This material is dried to no more than 10% humidity for both solvent extraction and combustion. Drying consumes much energy, therefore attempts are continuously to reduce energy costs and to avoid the appearance of undesirable compounds in pomace-olive oil formed by the high temperatures (up to 500 ºC) such as polycyclic aromatic hydrocarbons (PAHs) (León -Camacho et al., 2003) or oxidised compounds (Gomes and Caponio, 1997).

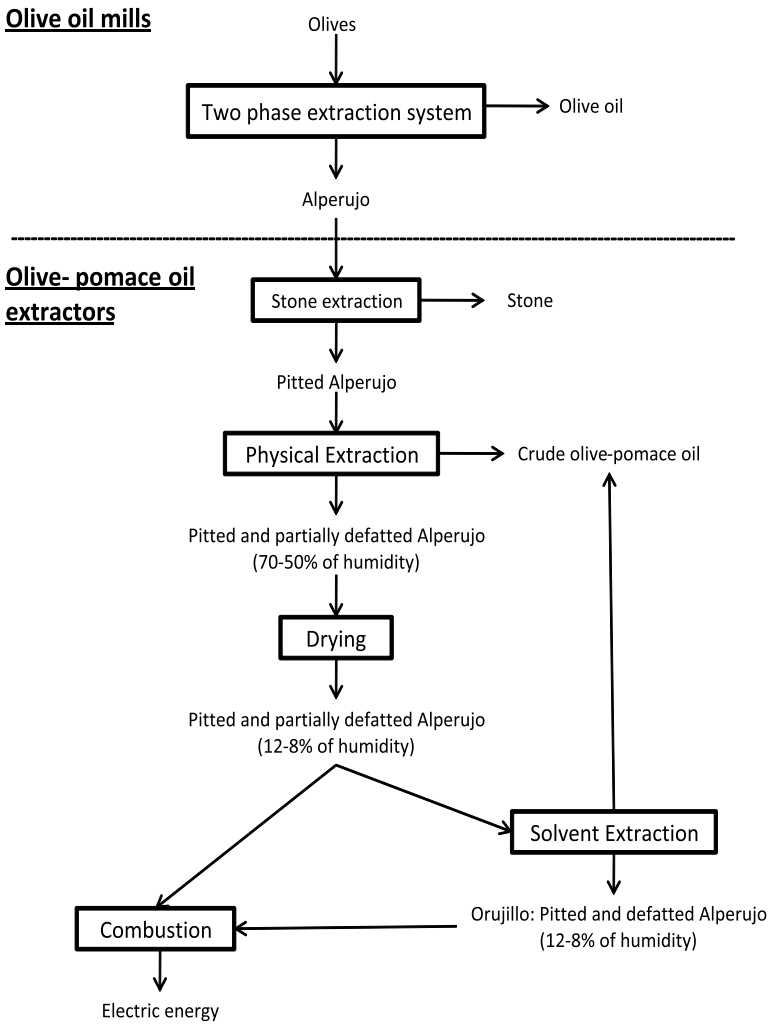

Fig. 1. General scheme of industrial olive oil and olive-pomace oil extraction and by-product processing.

The drying process is usually carried out in rotary heat dryers (Espínola-Lozano, 2003) in which alperujo and hot gases obtained from orujillo, olive stones or exhausted gases from co-generation systems (Sánchez & Ruiz, 2006) are introduced at 400 to 800 ºC. The high temperatures have negative effects on the final composition of COPO. After drying, the pitted and partially de-fatted alperujo, with humidity close to 10%, is extracted with organic solvents. After the extraction, the organic solvent is removed for COPO production. The COPO obtained by physical or chemical methods has to be refined for human consumption. The final solid, called orujillo, is commonly used as a biomass for energy production.

The apparition of alperujo was supposed to be a great advantage for olive oil mills because the liquid waste (alpechin) was removed, but it was a serious inconvenience for COPO extractions with regard to the high humidity of the new semi-solid waste, or alperujo. Previous to the two-phase extraction system, the solid waste, or orujo, from the three-phase extraction system was treated with lower humidity (50%) than the alperujo (70%). Nowadays, despite the use of the final solid as biomass, the extraction of olive-pomace oil does not, in many cases, have economic advantages. In addition, the olive oil mills are improving the centrifugation systems in order to increase the quantity of olive oil, producing alperujo with lower oil concentrations. Consequently, the higher humidity in addition to the high organic content of alperujo complicate the COPO extraction, higher temperatures in heat dryers or alperujo with lower oil content. Therefore, pre-treatment alternatives are necessary to properly process the alperujo in the OPO extractor and improve the oil extraction balance and quality, while at the same time obtain new components and add value to the product.

1.4 Minor components in OPO

Interest in olive-pomace oil is growing due to its economic advantages. It is cheaper than olive oil, and contains many minor components with bioactivities. OPO contains all of the functional compounds found in virgin olive oil, except for the polyphenols, in addition to other biologically active components (De la Puerta et al., 2009; Ruiz-Gutiérrez et al., 2009) that could be solubilised from leaves, skin or seeds of olives, depending on the extraction systems.

Phytosterols, tocopherols, aliphatic alcohols, squalene and triterpenic acid are some of the most important compounds that make the minor components an interesting fraction from the point of view of bioactive compounds that are agents for disease prevention.

The phytosterol´s structure is similar to cholesterol, and they are a powerful agent in the cholesterol-lowering effects in human blood (Jiménez-Escrig et al., 2006) and as a cytostatic agent in inflammatory and tumoral diseases (Sáenz et al., 1998).

Tocopherols (α-, β-, and γ-form) are present in high concentrations in OPO. α-tocopherol is an essential micronutrient involved in several oxidative stress processes related to atherosclerosis, Alzheimer's disease, accelerated aging and cancer (Mardones & Rigotti, 2004). Recently, biological activities against diseases like cancer in animal models have been also attributed to γ-form (Ju et al., 2010).

There is also squalene in olive-pomace oil. This compound has a beneficial effect on atherosclerotic lesions (Guillén et al., 2008, Bullon et al., 2009), dermatitis (Kelly et al., 1999)

and cellular proliferation and apoptosis in skin and intestinal cancers (Rao, 1998). After being absorbed by the human skin surface, squalene acts as a defence against oxidative stress due to the exposure to ultraviolet (UV) radiation from sunlight.

Aliphatic alcohols with long-chain fatty alcohols (C26 or hexacosanol, C28 or octacosanol and C30 or triacontanol) obtained from OPO have shown activity in reducing the release of different inflammatory mediators (Fernández-Arche et al., 2009), reducing platelet aggregation and lowering cholesterol (Taylor et al., 2003, Singh et al., 2006).

Uvaol and erythrodiol are the triterpenic alcohol fraction present in OPO. They are active antioxidant agents in the microsomal membranes of rat liver (Perona et al., 2005), with positive effects on the inflammatory process (Márquez-Martín et al., 2006), or in the prevention and treatment of brain tumours and other cancers (Martín et al., 2009).

1.5 Thermal pre-treatments

Alperujo is a high humidity solid that needs special pre-treatments to obtain a viable utilisation of all its phases. Only a few pre-treatments have been proposed for the total utilisation of alperujo, extracting the main interesting fractions. One of the more attractive processes is based on thermal pre-treatments that allow the recovery of all of the bioactive compounds and valuable fractions, making possible the utilisation of alperujo (Fernández-Bolaños et al., 2004). Thermal treatments produce the solubilisation of bioactive compounds to the liquid phase, leaving a final solid enriched in oil, cellulose and proteins. From the liquid, it is possible to extract and purify the bioactive compounds that confer healthy properties to olive oil, mainly phenols such as hydroxytyrosol (HT). HT is one of the more important phenols in the olive oil and fruit because it has excellent activities as a pharmacological and antioxidant agent (Fernández-Bolaños et al., 2002). HT has been recently commercialised by a patented system (Fernández-Bolaños et al., 2005). In addition to other important compounds, a novel phenol has been isolated and purified for the first time: 3,4-dihydroxyphenylglycol (DHPG). DHPG has never been studied as a natural antioxidant or functional compound with a higher antiradical activity and reducing power than the potent HT (Rodríguez et al., 2007b). After the thermal treatment and the solid-liquid separation, a solid that is rich in cellulose and oil is obtained. The cellulose is easy to extract and use as a source of fermentable sugar, animal feed or fertiliser (Rodríguez et al., 2007a). The thermal reaction improves the concentration in oil of minor components with functional activities. In addition, phenols increase in the liquid due to the solubilisation, with this fraction a rich source of interesting phenols, sugar and oligosaccharides, all of them with a potential use in the food or nutraceutical industry.

This alternative pre-treatment not only increases the concentration of oil in the final solid, but also the content of minor components in COPO prior to the refining process. The thermal treatment improves the functional profile, enhancing the quality and healthy properties of this oil (Lama-Muñoz et al., 2011). The application of thermal pre-treatment to alperujo makes the extraction of olive-pomace oil easier, improving its functional composition. On the other hand, it is important to note that all chemical changes of fats and oils at elevated temperatures result in oxidation, hydrolysis, polymerisation, isomerisation or cyclisation reactions (Quiles et al., 2002, Valavanidis et al., 2004). All of these reactions may be promoted by oxygen, moisture, traces of metal and free radicals (Quiles et al., 2002). Several factors, such as contact with the air, the temperature and the length of heating, the

type of vessel, the degree of oil unsaturation and the presence of pro-oxidants or antioxidants, affect the overall performance of oil (Andrikopoulos et al., 2002). In this work, the effect of two different thermal pre-treatments on COPO composition has been individually studied to balance the positive and negative factors in the final COPO.

1.5.1 Steam explosion system (SES)

The SES is commonly used as a hydrolytic process for lignocellulosic material utilisation (McMillan, 1994). This process (**Figure 2**) combines chemical and physical effects on lignocellulosic materials. The material is treated with high-pressure saturated steam for a few minutes and then the pressure is swiftly reduced, causing the materials to undergo an explosive decompression. The process causes hemicellulose degradation and lignin transformation due to high temperature, increasing the solubilisation of interesting compounds not only into the aqueous phase but also into the oil fraction. It is used mainly for the treatment of bagasse, such as wheat or rice straw, sugar cane, etc. The pre-treatment can enhance the bio-digestibility of the wastes for bioprocess applications to obtain, for instance, ethanol or biogas, and to increase the accessibility of the enzymes to the materials (De Bari et al., 2004; Palmarola-Adrados et al., 2004; Kurabi et al., 2005). High pressures (10-40 Kg/cm^2) and temperatures (180-240 ^{o}C) are applied with or without the addition of acid in a short period of time, followed by explosive depressurisation. The SES makes it possible to obtain a final solid that is rich in COPO from alperujo. The thermal treatment solubilises a high proportion of solid, leaving behind components such as oil, proteins and cellulose. All these components are concentrated in the final solid.

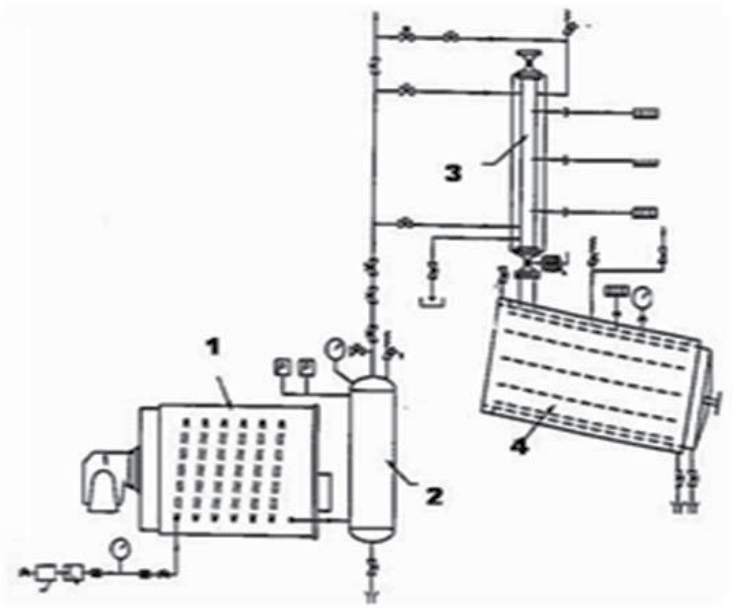

Fig. 2. Steam Explosion System scheme. Laboratory pilot unit designed in the Instituto de la Grasa (Seville, Spain), equipped with: 1) steam generator, 2) steam accumulator, 3) 2 L reactor stainless-steel and 4) expansion chamber.

1.5.2 New steam treatment (ST)

The system scheme is shown in F**igure 3**. A lower range of pressure and temperatures (3-9 Kg/cm^2 and 140-180 ^{o}C) than SES is applied for a longer period of time (15-90 min) in the novel system. The conditions and the contact of steam with the sample have been successfully improved, avoiding the technical complications and the high costs of the SES. This treatment has been recently patented to treat olive oil wastes, and the first tests have been carried out to assess its industrial viability for alperujo utilisation (Fernández-Bolaños et al., 2011).

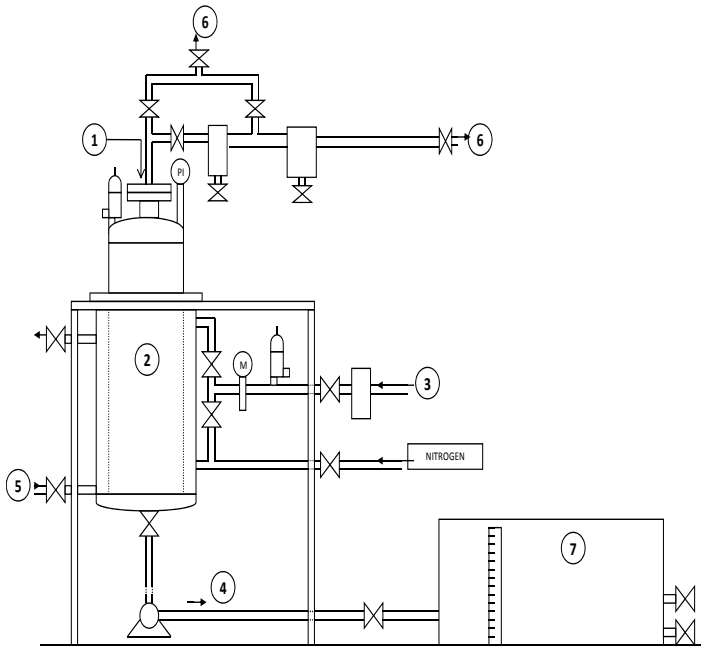

Fig. 3. New steam treatment (ST) reactor scheme designed in the Instituto de la Grasa (Seville, Spain) with: 1) sample entrance, 2) reactor chamber (100 L), 3) water steam, 4) sample exit, 5) cold water for refrigeration system, 6) vacuum and 7) solid-liquid separation.

The sample is introduced into the reaction chamber together with water steam. The sample temperature is increased up to 190^{o}C for 30-60 minutes. After the reaction time, the sample is cooled with indirect water as a refrigerant. The liquid and solid phases of the treated sample are separated and the solid is finally extracted to obtain the crude olive-pomace oil.

The advantages of both systems are based on the important solubilisation of the initial solid to the liquid phase that occurs during the thermal treatment, leaving a final solid in which several components like oil, cellulose and protein are concentrated. The humidity of the final solid is also easier to remove by centrifugation or filtration, simplifying the drying process and the undesirable compounds that are formed at high temperatures.

In addition, the liquid phase is rich in bioactive compounds that are easy to extract. All these factors make possible the total utilisation of olive oil wastes, diminishing their environmental impact (Rodríguez et al., 2007a).

First, the application of SES on alperujo in order to obtain pomace olive oil was studied with or without acid as a catalytic agent. Due to the technical disadvantages of SES and to make the use of thermal pre-treatment in OPO mills easier and more convenient low severities, no depressurisation or acid addition were used in the new system (ST).

2. Experimental procedures

Samples of stored olive pomace or alperujo were collected from the COPO extraction factory Oleícola El Tejar (Córdoba, Spain) with 70% humidity. This by-product is generated as a waste from the two-phase olive oil extraction system.

2.1 Thermal treatments

The thermal treatments were carried out in the Instituto de la Grasa (CSIC) pilot plant by the steam explosion system and a new thermal system:

a. The SES was carried out using a flash hydrolysis laboratory pilot unit with a 2 L reactor. The reactor was equipped with a quick-opening ball valve for the final explosion into the expansion chamber. Alperujo samples of 250 g were treated with saturated steam in a 2 L reactor with a maximum operating pressure of 40 Kg/cm^2. The reactor was equipped with a quick-opening ball valve and an electronic device programmed for the accurate control of steam time and temperature for the final steam explosion. Prior to the treatment, some of the samples were acidified with H_3PO_4. The acid was added to the moist sample so as to reach a final concentration of 2,5 % (v/v). After the treatment, the samples were collected and filtered in vacuo through filter paper using a Buchner funnel.
b. The new ST reactor has recently been patented (Fernández-Bolaños et al., 2011). It has a 100 L capacity stainless steel reservoir that can operate at temperatures between 50 and 190 °C by direct heating and at a maximum pressure of 9 Kg/cm^2. The system allows the appropriate treatment of alperujo without steam explosion or high pressures and temperatures. The wet treated material was filtered by centrifugation at 4700 g (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids.

After solid separation, the solid phase was dried in a stove at 50 °C, and the reduction (%) in the mass of the solid phase was determined.

2.2 Analytical determinations

Oil was extracted from the treated solid obtained by SES and ST with n-hexane using a Soxhlet apparatus. The obtained oils were filtered and stored at -20 °C until analysis. Oil content and fat enrichment (pitted dry matter) were determined and compared with the values for untreated alperujo samples.

Determination of the concentrations of aliphatic alcohols, sterols and triterpenic dialcohols (erythrodiol and uvaol) was performed according to the Commission Regulation (EEC) No 2568/91 for olive oil and pomace oil. After the silylation reaction, 1 mL of heptane was added to the mixture, and 1 µL of the solution was injected into an Agilent 7890A gas chromatograph system (Agilent Technologies, Palo Alto, USA) equipped with an FID detector. The analytical column was an HP-5 (5%-phenyl)-methylpolysiloxane column (30 m x 0,32 mm i.d., 0,25 µm film thickness). The results were expressed as mg/kg of oil.

Tocopherols were evaluated using the IUPAC 2.432 method. Results were expressed as mg/kg of oil.

The wax and squalene compositions were determined according to the European Regulation EEC/183/93, by separation on a silica gel 60 (70-230 mesh ASTM) chromatographic column (Merck KGaA, Darmstadt, Germany) using hexane/ether (98:2) as the eluent with a few drops of Sudan I as a colorant. Dodecyl arachidate (Sigma) and squalane (Fluka) were added as internal standards. The results were expressed as mg/kg of oil.

Polar compounds, triglycerides and their derivatives oxidise and hydrolyse were prepared using solid-phase extraction and size-exclusion chromatography and monostearin as internal standard (Márquez -Ruiz et al., 1996). An aliquot (20 µL) of the final solution was injected into a Hewlett Packard Series 1050 HPLC system equipped with a refractive index detector (LaChrom L-7490 Merck) and a 100-Å PL gel column (5 µm) (Agilent). Elution was performed at 0,6 mL/min, with tetrahydrofuran as the mobile phase.

Determination of fatty acid, free acidity and peroxide value (PV) was carried out according to the Official Methods described in the European Community Regulation EEC/2568/91. The results were expressed as the percentage of oleic acid. The peroxide value was expressed in milliequivalents of active oxygen per kilogram of oil (mequiv O_2/kg oil).

The indexes K_{232}, K_{270} and ΔK were determined using the European Communities official methods (European Union Commission, 1991). Oil samples were diluted in isooctane and placed into a 1 cm quartz cuvette; for values calculation, each solution was analysed at 270 and 232 nm, with isooctane as a blank.

3. Results and discussion

Both systems allow the utilisation of the final solid for crude olive-pomace oil extraction. These COPOs have been characterised to assess the positive and negative effects of both treatments on its composition. The SES was studied as a commonly used method for lignocellulosic materials, and the ST was designed to simplify the first system and to diminish the negative effects of SES on crude olive-pomace oil. The lipid fraction of POO extracted from solids treated with either treatment was evaluated, and the minor components were also characterised, in the case of the ST.

3.1 Steam explosion system (SES)

An average temperature of 200 ^{o}C and a time of 5 minutes were used with or without acid impregnation of alperujo. The acid increases the severity of the treatment, enhancing the oxidation of the samples. A vacuum was applied to one of the treatments, with acid addition in order to diminish the possible oxidative effect of oxygen at high temperatures and pressures. The results showed (**Table 1**) an important solubilisation of the solid in all treatment. In addition, the oil was concentrated in the final solid from 8,3 up to 19,9 % with respect to the dry final solid. Despite the high level of acidity in the initial sample after the treatment, these values decreased.

K_{232} and K_{270} are simple and useful parameters for assessing the state of oxidation of olive oil. The coefficient of specific extinction at 232 nm is related to the presence of products of

the primary stage of oxidation (hydroperoxides) and conjugated dienes, which are formed by a shift in one of the double bonds. The extinction coefficient at 270 nm is related to the presence of products of secondary oxidation (carbonylic compounds) and conjugated trienes (the primary oxidation products of linolenic acid).

The K_{232} values of all treated samples were lower than the untreated alperujo, unlike the K_{270} values in which only the sample treated with vacuum and without acid presented a similar absorbance at 270 nm. Only when vacuum and acid were applied to the SES did the value of K_{270} exceed the maximum concentration in ROPO (2,0), with all the ΔK values lower than the maximum in ROPO (0,2). All these oxidised compounds diminished after the refining process.

The polar compound values that show the alteration level by the non-volatile compounds of oil are practically the same in all treatments, except when the acid and the vacuum are used simultaneously. Polar compounds provide an idea not only of oxidative reactions, but also of hydrolytic degradation, because they are partial constituents of FFA and glycerides.

Curiously, the concentration of oxidised triglycerides and polymers are lower after the SES treatments. This result could be explained by their partial solubilisation during the thermal treatment in the liquid phases that are previously separated by the oil extraction.

	Untreated sample	SES (200° C, 5 min)	SES (200° C, 5 min, 2,5% H_3PO_4)	SES (200° C, 5 min, vacuum)	SES (200° C, 5 min, 2,5% H_3PO_4, vacuum)
% of solid reduction	-	51,7	54,6	52,1	58,5
% of oil in final solid	8,3	17,2	18,0	16,9	19,9
Acidity (% oleic acid)	6,76	4,97	4,44	5,08	5,48
K_{232}	5,72	4,57	3,23	5,18	5,21
K_{270}	1,27	1,74	1,44	1,24	2,37
ΔK	-0,03	0,00	0,04	0,06	0,07
Polar compounds mg/g	114,3	116,59	112,81	111,37	129,87
Oxidised Triglycerides [a]	1,61	1,25	1,34	1,41	1,36
Diglycerides[a]	3,10	4,47	4,83	4,37	5,13
Monoglycerides[a]	0,42	0,53	0,40	0,49	0,56
FFA (% as oleic acid)	6,10	5,28	4,49	4,66	5,73
Polymers[a]	0,23	0,14	0,23	0,21	0,21

[a] % with regard to the oil sample.

Table 1. Solid reduction, oil concentration in final solid and chemical characteristics of crude olive-pomace oil treated or untreated by SES in several conditions.

The quantity of triglycerides decreased after the SES treatment, with an increased presence of diglycerides and monoglycerides as an unmistakable sign of hydrolytic degradation.

The oxidative effects do not seem to be the main cause of the COPO alteration during the SES treatment, while hydrolysis seems to be an important effect on the triglyceride loss.

The composition of triglycerides was determined and the results are shown in the **Table 2**. The main triglyceride peaks in all samples were oleic-oleic-oleic (OOO), oleic-oleic-palmitic (OOP) and linoleic-oleic-oleic (LOO).

Despite the low quality of the initial oil, the relation of triglycerides was not altered by SES. As expected, in the crude olive-pomace oil obtained after SES treatment, the total content of triglycerides decreased up to 22% compared to the oil obtained from the untreated alperujo. Despite the high severity, only 22% of triglyceride composition was lost, with the rest susceptible for refining. The oxidative conditions of SES treatment were minimised using a vacuum or avoiding the acid addition.

The great advantages of the SES application on alperujo are based, in addition to other reasons, on the solid reduction (up to 58%) and oil concentration in the final solid (up to 20%). Because the triglycerides (TG) are concentrated, their loss is not a significant or negative factor to limit the use of this system. These reasons make technically possible the extraction of the crude olive-pomace oil from one sample of alperujo treated by SES. Therefore, the application of SES to this kind of alperujo allows for the obtaining of a final solid rich in oil in a high concentration that is susceptible for further refining processes for olive-pomace oil production. However, the technical inconveniences of SES such as high temperatures and pressures or the explosive decompression make it an inadequate system for olive-pomace oil extractors.

Triglycerides	Untreated sample		SES (200° C, 5 min) treated sample	
	Peak area	% of total glycerides Mean ± SD	Peak area	% of total glycerides Mean ± SD
LLL	18039	0,7 ± 0,05	11076	0,6 ± 0,02
LnLO	15271	0,6 ± 0,03	5886	0,3 ± 0,01
OLL+PoLO	99759	4,0 ± 0,10	75243	3,9 ± 0,77
PLL+LnOO	62860	2,5 ± 0,15	32818	1,7 ± 0,26
POLn	23935	1,0 ± 0,07	0	0,0 ± 0,00
LOO	404446	16,1 ± 1,02	321011	16,6 ± 1,23
LOP	154422	6,2 ± 0,81	126669	6,5 ± 0,87
LPP	4000	0,2 ± 0,01	6064	0,3 ± 0,02
OOO	959632	38,3 ± 1,67	788016	40,7 ± 2,03
OOP	448959	17,9 ± 1,11	364371	18,8 ± 1,30
POP	57959	2,3 ± 0,90	39691	2,0 ± 0,11
SOO	157822	6,3 ± 1,43	108877	5,6 ± 0,95
POS	28350	1,1 ± 0,03	21116	1,1 ± 0,05
AOO	49158	2,0 ± 0,16	26398	1,4 ± 0,06
Área total	2504443		1938316	

P, palmitic, Po, palmitoleic, M, margaric, S, stearic, O, oleic, L, linoleic, Ln, linolenic, and A, arachidic acids

Table 2. Triglycerides composition of crude olive-pomace oil obtained from alperujo untreated and treated by SES.

3.2 Effects of the new steam treatment

The ST effect on POO composition was determined by characterisation of the fatty acid fraction. After the thermal treatment in the range of 150-170ºC for 60 min (**Table 3**), the final treated solid had an increase in oil yield up to 97%, with a reduction in solids up to 35,6-47,6% by solubilisation. The oxidative damage was lower in the new treatment. The analysis of the polar fraction showed that oxidised triglycerides and peroxide values increased slightly and that no polymerisation reactions occurred. The hydrolytic process is shown in the diglycerides increasing from 2,5 to 6,6%, with the FFA and the unsaponifiable matter for all treatments remaining constant.

	Untreated sample	ST (150° C, 60 min)	ST (160° C, 60 min)	ST (170° C, 60 min)
% of solid reduction	-	35,6	47,1	47,6
% of oil in final solid	8,1	11,8	14,3	16,0
Acidity (% oleic acid)	3,6	4,7	4,9	5,1
Peroxide Values (meq/Kg)	8,7	9,4	10,9	12,3
Oxidised Triglycerides [a]	0,7	1,1	1,1	1,6
Diglycerides[a]	2,5	5,2	6,6	6,6
Monoglycerides[a]				
FFA (% as oleic acid)	3,3	2,8	2,9	2,8
Unsaponifiable matter (%)	2,53	3,02	2,50	2,54

Table 3. Solid reduction, oil concentration in final solid and chemical characteristics of crude olive-pomace oil treated or untreated by ST at 150, 160 and 170ºC for one hour. [a] % with regard to the oil sample.

The concentration of minor components (**Table 4**) was significantly increased by ST. Sterols, aliphatic alcohols, triterpenic alcohols, and squalene increased up to 33%, 57%, 23% and 43%, respectively. In addition, the content of tocopherols increased up to 57% compared to untreated POO. This increase is due to solubilisation during the thermal treatment. The waxes level is also increased because of the high solubilisation from the external cuticle of the olive fruit and the leaves. Waxes are easily removed by the refining of COPO.

The samples of alperujo had been stored for a long time and the oil was partially extracted by centrifugation in OPO extractors just before pitting. The alperujo was chosen because its low fat concentration makes the COPO extraction economically unviable. In this condition, the initial oil has a very low quality, as previously shown in the tables. The analysed oils showed, despite the low quality of initial oil present in the alperujo studied, that the effect of thermal treatment increases slightly the values of oxidised components and hydrolytic degradation. All COPOs obtained after the thermal treatments are susceptible for a posterior refining process for OPO obtention.

Components	Untreated sample	ST (150°C, 60 min)	ST (160°C, 60 min)	ST (170°C, 60 min)
Sterols	4927 (104)	5687 (291)	6546 (216)	6555 (298)
Aliphatic alcohols	4065 (77)	5532 (603)	5880 (283)	6389 (68)
Triterpenic alcohols	992 (58)	1054 (34)	1220 (124)	1189 (107)
Waxes	1535 (3)	2971 (5)	3124 (94)	3461 (110)
Squalene	2404 (36)	2472 (11)	2729 (109)	3439 (171)
Tocopherols	425 (33)	460 (6)	668 (14)	533 (20)

Table 4. Total minor component composition (mg/kg ± SD) of oils from steam-treated and untreated alperujo. Numbers between parentheses indicate the standard deviation of three replicates.

For human consumption, the refining process of OPO is necessary. The refining (physical or chemical) process eliminates undesirable compounds (peroxides, degradation products, volatile compounds responsible for off-flavours, free fatty acids, etc.) but also results in the loss of valuable bioactive compounds and natural antioxidants (Ruiz-Méndez et al., 2008). The new trends of refining systems involve losing as few minor components as possible to obtain a final OPO that is rich in the minor components. The thermal treatments increase the minor component of COPO that help to obtain a final olive-pomace oil rich in interesting compounds, whose concentrations might be higher after the refining process, mainly using the new physical systems. Moreover, some of these minor components are recuperated during the refining, such as squalene, the concentration of which is increased up to 43 % after the pre-treatment. After extraction, the defatted solid is lacking in phenols and then in phytoxic compounds for further bio-utilisation and rich in components like cellulose and protein.

Figure 4 shows the main aspects of both thermal systems. The high difference between temperatures and pressures together with the absence of explosive decompression makes ST more appropriate and economically viable for industrial applications. A longer period of reaction time is necessary to treat with ST, but is easily applicable in an industrial continuous reactor. The percentage of solid reduction and, consequently, the final oil concentration show that despite the high severity difference between both treatments, there is not a significant difference in the results. Then, the new ST provides the major advantages of SES without technical complications.

Thus, the positive effect of a novel thermal treatment for the extraction of crude olive-pomace oil that could improve the commercial value of OPO and its bioactivities by increasing the concentrations of minor components concentration has been demonstrated. This treatment also significantly reduces the cost of oil extraction by centrifugation or solvent extraction because the starting solid is more concentrated in oil and is drier than untreated alperujo.

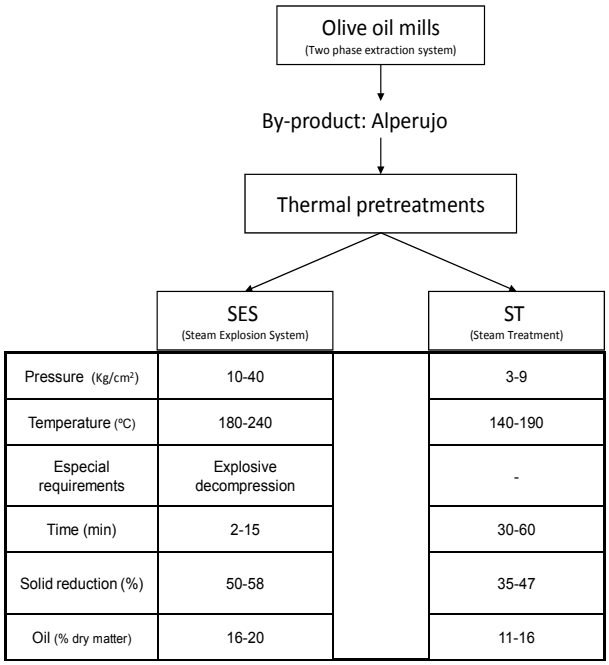

Fig. 4. Comparative scheme of two thermal pre-treatments used for alperujo utilisation.

4. Conclusion

The new treatment ST not only maintains the advantages of the SES with regard to the concentration of oil in the final solid and phase separation, but also diminishes the oxidation and significantly improves the concentration of the most interesting components of the minor fractions of POO. Thus, the application of ST enhances the functional properties of this new POO, increasing the oil extraction yield and the total recovery of bioactive compounds from the refining process.

The steam treatment offers not only serious advantages in terms of the oil but also in terms of the total recovery of alperujo as described above. The application of ST to treat olive oil wastes allows the phase separation and the concentration of interesting compounds and components in each phase. In the liquid phase, bioactive compounds like phenols and oligosaccharides are solubilised and are easy to extract. In the solid fraction, the oil and cellulose are concentrated. After the oil extraction, the solid has a low content of phytotoxics that are in the liquid phase, and therefore, it is susceptible for bio-treatment for the total utilisation of this fraction.

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Genetic Improvement of Olives, Enzymatic Extraction and Interesterification of Olive Oil

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1. Introduction

Extra virgin olive oil (EVOO) contains a wide range of bioactive compounds which give it its particular aroma and taste. It is a well-known key component in the traditional Mediterranean diet and due to its high levels of phenolics and unsaturated fatty acids, it is believed to be associated with good health and a relatively long life (De Faveri et al., 2008). The phenolic compounds have the ability to reduce the oxidative modification of low-density lipoproteins (Fitó et al., 2005), which play a key role in the development of atherosclerosis and coronary heart disease. Moreover, olive oil is very resistant to peroxidation (Najafian et al., 2009), a fact conferring great oxidative stability to the product (Bendini et al., 2006). The sustainable development of the agriculture and food industry is dependent upon powerful biotechnological tools which meet the demands of the new urbanized population. The improvement in the properties of EVOO is a good example of how useful the application of biotechnology to improve food quality is.

The olive oil extraction process is extremely important for its quality. During this step, the content of some components is significantly altered, depending on the extraction technique employed. A new process for the extraction of olive oil that has been studied is the addition of enzyme preparations during malaxation. This reduces the complexing of hydrophilic phenols with polysaccharides, increasing the concentration of free phenolic compounds in the olive paste and their consequent dissolution in the oil and waste waters during processing (De Faveri et al., 2008). The enzymes most used in the extraction of EVOO are microbial pectinases and cellulases, which hydrolyse the cell wall of the olive fruits, liberating the oil and phenolic compounds. This method has some advantages compared to traditional methods, giving higher oil and phenolic compound extraction yield. It also involves lower energy costs and possibly provides an oil with improved health properties

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due to the liberation of the phenolic compounds. Microbial lipases can also be used to synthesize structured lipids from olive oil triacylglycerols.

This review discusses mainly the genetic improvement of *Olea europaea* to achieve higher quality EVOO, with lower production costs and greater productivity. Additionally, it reports on the use of enzymes to improve the extraction of virgin olive oil from olives and the enzymatic synthesis of lipids based on olive oil triacylglycerols.

2. Genetic modification of olive cultivars: Crossbreeding

Improvement of the cultivars is one of the major targets of olive biotechnology. The recent diffusion of the olive outside its traditional cultivation area, the Mediterranean basin, together with a continuous trend for industrial modernization, has recently increased the demand for improved cultivars. As a result, clonal selection and crossbreeding programmes have been applied in olive growing countries, aiming at selecting genotypes with improved characteristics. The desirable characteristics are early bearing, resistance to pests and to abiotic stress (such as frost and drought), limited alternate bearing and suitability for intensive cultivation and mechanical harvesting. In relation to the product, the search is for high-quality production with respect to the organoleptic characteristics of both the fruits and oils, and finally a high content of bioactive substances that may favourably affect human health (Fabbri et al., 2009). Olive crossbreeding programmes have provided new genotypes with a wide range of variation for all the characteristics, including the oil composition (Belaj et al., 2010). This technique has been used to generate new cultivars from traditional ones, which are used as the genitors. For instance, in Tunisia, an olive breeding programme started in 1989 with a cross between Tunisian and foreign cultivars. This created new cultivars with a quality of oil superior to that of *Chemlali* (the main olive cultivar in the country, characterized by low levels of oleic acid) and characteristics close to the standards of the international market (Baccouri et al., 2007; León et al., 2011; Manai et al., 2007; Manai et al., 2008; Rjiba et al., 2010). The large variability in all the components of olive oil in these advanced selections suggests that diversity in olive oil composition could be obtained in any crossbreed progeny. Therefore, any breeding programme provides diversity of the oils.

In Spain a breeding programme began in 1992 to obtain new olive cultivars with some of the following advantages: early bearing, high productivity and oil content, resistance to peacock eye (*Spilocaea oleagina*, Cast), suitability for mechanical harvesting and high olive oil quality (León et al., 2004a).

León et al. (2011) selected fifteen genotypes from crosses between the cultivars *Arbequina*, *Frantoio* and *Picual* on the basis of their agronomic characteristics. In this work, the main components of the olive oil were characterized and compared with their genitors. A wide range of variation was observed for all the fatty acids and minor components, and for the related characteristics evaluated, with significant differences between the genotypes, except for the β-tocopherol content. The values obtained in the selections have extended the range of variation of the three genitors for all the characteristics evaluated. The selections showed the highest average values for tocopherols, polyphenols and the C18:1 content, respectively. The breeding procedures used to obtain these selections including crossing, the forced growth protocol and an initial seedling evaluation, are all described (León et al., 2004a; León et al., 2004b; Santos-Antunes et al., 2005).

A wide range of variation was observed for all the fatty acids, minor components and related characteristics evaluated by León et al. (2011). The fatty acid C18:1 was the predominant fatty acid in all the selections, with values ranging from 62 to 81%. Together with C16:0 and C18:2, it accounted for more than 94% of the total fatty acid composition, on average. The genotypes producing olive oils with high oleic acid percentages could be of particular interest for planting in low latitude locations, where the oleic acid content tends to be too low (Ripa et al., 2008). Of the minor components, α-tocopherol represented more than 90% of the total tocopherols, whereas the total polyphenol content varied widely from 67 to 1033 mg/kg. A wide range of variation was also obtained for stability, with values ranging from 16 to 195 h.

The statistical analysis showed that genotypic variance was the main contributor to the total variance for all the fatty acids and ratios evaluated, with significant differences between the genotypes in all cases. In fact, the effect was significant for all the fatty acids, except C18:3, all the minor components and related characteristics evaluated, α-tocopherol and stability, but was lower for the other characteristics. Several studies have demonstrated that the quality of olive oil is greatly determined by genetic (cultivars) factors. For instance, in the Germplasm Banks of Catalonia and Cordoba, Tous et al. (2005) and Uceda et al. (2005), respectively, showed that more than 70% of the variation in the fatty acids (except for C18:3) and several minor components, such as the polyphenol content, bitter index (K225) and oil stability, was due to genetic effects. It should be noted that many other factors including pedoclimatic aspects, olive ripeness, olive harvesting methods and the olive extraction system have also been reported as quality indicators of virgin olive oil (Aguilera et al., 2005; Guerfel et al., 2009; León et al., 2011).

Ayton et al. (2007) found a stronger relationship between the polyphenols content and oil stability when individual cultivars were analyzed separately, which suggests that the relationship between induction time and total polyphenol content is different for each cultivar. In another study (León et al., 2011), the ranking of the cultivars was different for the polyphenols content and oil stability, which could suggest that not only the total polyphenol content, but also different polyphenol profiles in the different cultivars could have distinct antioxidant effects. Similar results have been reported for the analysis of the composition and oxidative stability of virgin olive oil from selected wild olives (Baccouri et al., 2008). The correlation between the different fatty acids also agrees with what was previously reported for olive cultivar collections and breeding progenies (León et al., 2004a).

Significant differences between the genotypes obtained for crosses between *Arbequina*, *Frantoio* and *Picual* were observed for the fatty acid composition, minor components and related characteristics. The multivariate analysis allowed for the classification of the genotypes into four groups according to their olive oil compositions. Further work will be required to determine the best selections to adapt to different environmental conditions, as well as the optimal harvesting periods in terms of optimal oil quality (León et al., 2011).

Ripa et al. (2008) evaluated oil quality, in terms of fatty acid composition and content in phenolic compounds, for many new genotypes previously selected in a breeding programme and cultivated in three different locations of central and southern Italy. The availability of data from many genotypes cultivated in all three locations allowed quantitative analyses of the genetic and environmental effects on the oil quality traits studied. The acidic composition varied greatly both with genotype and with environment, and so did the concentration in phenols, though the effect of genotype on phenols was not significant. The fatty acid

composition appeared predominantly under genetic control while the environmental effect explained 0.31 of the total variance. The oil content in phenolic compounds, instead, had lower heritability (0.29) and was more affected by the environment, which explained 0.50 of the total variance. Few genotypes were selected as the best for each location, but none performed best in all locations. This suggests that, in olives, the highest oil quality is difficult to achieve with a single genotype in different environments, due to a strong or even predominant effect of the environment on some quality traits. More likely, combinations of genotypes and territories can produce oils with high and typical quality.

3. The use of enzymes in the extraction of olive oil

The most commonly used method for the extraction of olive oil is the mechanical process, however some of the non-extracted oil remains in the solid residue or cake. The majority of the oil is located in the vacuoles as free oil, but oil dispersed in the cytoplasm is not extractable and is therefore lost in the waste (Najafian et al., 2009). Therefore the cell walls must be destroyed to effectively recover the oil enclosed in the cell. The use of enzymes has been studied for the hydrolysis of the different types of polysaccharides in the cell wall structure (Chiacchierini et al., 2007). The major polysaccharides found in the cell wall of the olive fruit were pectic polysaccharides and the hemicellulosic polysaccharides xyloglucan and xylan (Vierhuis et al., 2003).

Several innovating biotechnological techniques have been studied to obtain high-quality oils and/or improve product outputs. They include the use of microorganisms (Kachouri & Hamdi, 2004, 2006) or enzymes (Vierhuis et al., 2001) during different steps of the oil processing procedure. Several enzyme processing aids have been successfully tested for olives in recent years (De Faveri et al., 2006; García et al., 2001). Different enzymes are naturally present inside the olive fruit, but are strongly deactivated during the pressing phase, most likely due to the formation of oxidized phenols bonding to the enzyme prosthetic group (Vierhuis et al., 2001). In this case, the addition of suitable enzymes to the olive paste during the mixing step was proposed as a tool to replace the deactivated natural ones (Ranalli et al., 2001). Furthermore, the enzyme complexes are water-soluble and after the application, they are found in the olive mill waste waters, indicating that the oil composition is not modified (Chiacchierini et al., 2007).

Ranalli et al. (2003a) estimated the composition of three types of olive oil (Caroleo, Coratina and Leccino) extracted by the application of the Bioliva enzymatic complex. During extraction, the action of the enzymes on the fruit tissues resulted in the release of greater amounts of oil and other constituents, which dissolved in the oily phase (Ranalli et al., 2003b). The enzymatic application resulted in an increase in several key compounds, such as phenols, tocopherols, and flavour compounds, without changing the natural parameters related to product authenticity (waxes, sterols, triterpene alcohols, fatty acids and triacylglycerol composition).

The loss of phenols during processing can be attributed to interactions between the polysaccharides and phenolic compounds present in the olive pastes (Servili et al., 2004). Studies show that the addition of commercial enzyme preparations during the malaxation can reduce the complexation of hydrophilic phenols with polysaccharides. It increases the concentration of free phenols in the olive paste and their consequent release into the oil and waste waters during processing (De Faveri et al., 2008).

A mixture of three enzyme formulations was tested by Aliakbarian et al. (2008) to improve the yield and the quality of the olive oil obtained from the Italian cultivar Coratina. Since no single enzyme is adequate for the efficient maceration and extraction of oil from olives, pectinase, cellulase and hemicellulase were essential for this purpose (Chiacchierini et al., 2007; De Faveri et al., 2008). A homogeneous mixture of the three different enzyme formulations was used at the beginning of the malaxation step in the proportions 33.3:33.3:33.3% (v/v/v). This choice was suggested by the higher efficacy of these enzymes in releasing phenolics into the oil when working as a ternary system (A:B:C), rather than in binary combinations (A:B, A:C, B:C) (De Faveri et al., 2008). In summary, A is a complex formulation containing pectinase plus cellulase and hemicellulase; B shows equilibrated pectinase–hemicellulase activity; C is a pectolytic enzyme. The enzymes selected are naturally present inside the olive fruit, but are strongly deactivated during the critical pressing step, presumably because of the oxidation (Chiacchierini et al., 2007). The highest levels of total polyphenols (874 $\mu g_{CAE}/g_{oil}$), antiradical power (25.1 $\mu g_{DPPH}/\mu L_{extract}$) and o-diphenols ($\mu g_{CAE}/g_{oil}$) were all reached at the highest enzyme concentration (25 mL/kg_{paste}). Moreover, the highest oil extraction yield (17.5 $g_{oil}/100\ g_{paste}$) was reached with the longest malaxation time (t = 150 min), always with the highest enzyme concentration.

4. Enzymatic synthesis of structured lipids

The enzymatic synthesis of structured lipids is relatively new in lipid modification. Although enzymes have been used for several years to modify the structure and composition of foods, they have only recently become available for large-scale use, mainly because of the high cost. Within this context lipases are reported for the enzymatic synthesis of structured lipids. They have the ability to carry out hydrolytic reactions, but the manipulation of the reaction at low water levels permits their use also for the synthesis of triacylglycerols. These enzymes can be successfully used in the production of lipids structured for medical purposes (De Araújo, 2011).

Enzymatic modification of olive oil triacylglycerols has been discussed by Boskou (2006, 2009). The development of techniques for the preparation of oils and fats from enzyme-modified olive oil is an attractive prospect for the food industry, given the high oxidative stability of the product at frying temperatures and the health enhancing properties of this material (Criado et al., 2007).

Nunes et al. (2011) produced structured triacylglycerols containing medium chain fatty acids, by the acidolysis of virgin olive oil (VOO) with caprylic or capric acids in a solvent-free media or in n-hexane, catalyzed by immobilized lipases from *Thermomyces lanuginosa, Rhizomucor miehei* and *Candida antarctica*. The results indicated that the incorporation was always greater for capric than for caprylic acid, but for both acids, higher incorporation was always attained in solvent-free media. All the biocatalysts presented 1,3-regioselectivity. The lipases from *Rhizomucor miehei* and *Candida antarctica* were the biocatalysts presenting the highest operational stability, together with high incorporation levels and low acyl migration in the batch production of structured lipids by the acidolysis of VOO with caprylic or capric acids. Therefore, these biocatalysts seem to be the most adequate for the implementation of a process aimed at the production of triacylglicerols containing medium and long fatty acids (MLM) rich in caprylic and capric acids. The structured triacylglycerol obtained from VOO has oleic acid at the sn-2 position, indicating a better absorption, whilst medium chain fatty acids will mainly be esterified at the external positions of the TAG molecules.

Criado et al. (2007) have also studied the enzymatic interesterification of extra virgin olive oil with a fully hydrogenated palm oil to produce lipids with desirable chemical, physical and functional properties. The sn-1,3 non-specific immobilized lipases from Candida antarctica and two sn-1,3 specific immobilized lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei* were employed as the biocatalysts. The authors concluded that the oxidative stability increased when the percentage of TAG containing multiple fully saturated residues increased. In all the cases studied, the stability of the physical blend was higher than that of the reaction products. The final products were considered as plastic over wider temperature ranges. The large amount of unsaturated residues present in these samples, primarily oleic acid residues, was the factor leading to the extended plasticity range of these interesterified mixtures.

5. Conclusion

The improvement of the properties of extra virgin olive is crucial, considering the extensive number of functional compounds present in this oil. Genetic improvement techniques are the option showing the most promising results. The genetic modification of olive cultivar crossbreeding is focused on solving agronomic and commercial problems, such as the control of fruit ripening and increase in the oil content and quality. Another improvement suggested by different research groups is the use of enzymes added to the paste to improve extraction of the oil and the bioactive compounds it contains. Enzymes, such as microbial lipases, can also be used to synthesize different high-value products from tryacylglicerol of olive oil. It is possible to obtain structured lipids that have functional and technological properties suitable for applications in the food, pharmaceutical and oleochemical industries. This can be considered as a "green" industrial process, taking into account that these compounds are currently synthesized by chemical processes that use catalysts and generate byproducts.

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15

Olive Oil Mill Waste Treatment: Improving the Sustainability of the Olive Oil Industry with Anaerobic Digestion Technology

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1. Introduction

The processes used to treat waste streams are chosen according to technical feasibility, simplicity, economics, societal needs and political priorities. However, the needs and priorities of a sustainable society undergo pressure which means a shift in the focus of wastewater treatment from pollution control to resource exploitation (Angenent et al., 2004). The organic fraction of agro-wastes (e.g. olive oil wastes, sugar beet pulp, potato pulp, potato thick stillage or brewer´s grains) has been recognized as a valuable resource that can be converted into useful products via microbially mediated transformations. Organic waste can be treated in various ways, of which bio-processing strategies resulting in the production of bioenergy (methane, hydrogen, electricity) are promising (Khalid et al., 2011). The aim of the present chapter is to discuss: firstly the quantities, characteristics and current treatments of the solid wastes and wastewaters from the continuous olive oil extraction industry for their exploitation and recovery; secondly, anaerobic digestion processes as an alternative for waste treatment and valuable energy recovery

2. Olive mill extraction wastes

2.1 Olive oil extraction technology

The olive mill elaboration system has evolved over time for economic and environmental reasons. The traditional system or "pressing system" was replaced in the 70s by the three-phase continuous centrifugation system. The centrifugation of the milled and beaten olives to obtain olive oil by the three-phase system produces 20% oil, 50% three-phase olive mill wastewaters (3POMWW) and 30% three-phase olive mill solid wastes (3POMSW). This three-phase system led to an increase in the processing capacity and consequently to an increase in the yield of the mills and the growth of the average mill size. However, large quantities of water needed for carrying out the three-phase process generate a high volume of olive mill wastewaters. The uncontrolled discharge of 3POMWW brought environmental problems. In some countries, technology manufacturers developed the "ecological" two-phase process. This system enables

reduced fresh water consumption in the centrifugation phase. The two-phase process has attracted special interest in countries where water supplies are restricted. The quantity of water required to carry out the two-phase process is much lower than in the three-phase process and a considerable reduction in generated two-phase olive mill wastewaters (2POMWW) is achieved. However, the two-phase process led to a slight increase in solid wastes. The quantities of two-phase olive mill solid wastes (2POMSW) are 60% higher than those generated in the three-phase system (3POMSW).

Over 2.9 million tonnes of virgin olive oil are produced annually worldwide, of which 2.4-2.6 million tonnes are produced in the European Union (IOOC, 2009). Currently, both elaboration systems, three- and two-phase, coexist in the Mediterranean area (Niaounakis & Halvadakis, 2004). Spain, the largest producer of olive oil in the world, currently uses the two-phase system in 98% of its olive mills. Over the past few years, Spain has produced between 1,412,000 tonnes (2003/2004 season) and 1,028,000 tonnes (2008/2009 season) of olive oil, which meant 57.7% and 53% of European production (IOOC, 2009). Croatia uses the two-phase system in 55% of its mills and produces 4,500 tonnes of olive oil (2008/2009 season). In olive oil producing countries such as Cyprus, Portugal and Italy, only around 5% of the mills use the two-phase system (Roig et al., 2006). Other large producers such as Greece or Malta have continued using mainly the three-phase system although the two-phase system is being introduced slowly. The high quantities of wastes produced in both systems makes sustainable treatments necessary.

2.2 Waste quantities and characteristics

3POMSW are produced in a proportion of 500 kg per ton of olives and are basically made up of dry pulp and stones.

3POMWW are the main wastes generated in the three-phase olive mill system (1,200 L ton^{-1} milled olives). The annual 3POMWW production of Mediterranean olive-growing countries is estimated between 7-30 million m^3. The chemical composition of 3POMWW is complex due to the water from the milled olives (vegetation water) and the soft tissues from the olive fruit. Typical composition of three-phase olive mill wastewaters is: pH 5.04, COD 43.0 g L^{-1} (COD: Chemical Oxigen Demand), total sugars 17.4 g L^{-1}, total phenols 2.5 g L^{-1} and lipids 0.75 g L^{-1} (D'Annibale et al., 2006).

The change from the three-phase to the two-phase elaboration system reduces the high generation of wastewaters produced in the three-phase process. The two-phase elaboration process generates 800 kg of 2POMSW per ton of olives processed. 2POMSW have a 60%-70% humidity content, 13%-15% lignin, 18%-20% cellulose and hemicellulose and 2.5%-3% oil (Borja et al., 2002). In a similar way to 3POMWW, the composition of 2POMSW is complex due to the vegetation water. Consequently, 2POMSW and 3POMWW are the main problematic streams.

2POMWW are a mixture between the water used for olive washing before the milling process and the water coming from washing the oil in a vertical centrifuge. Initial studies gave volumes of 2POMWW of around 250 L ton^{-1} of olives in total. However, current studies have suggested a significant reduction in the amount of water to be added to the vertical centrifuge.

3. Olive oil mill wastes - Current treatments

3.1 Three-phase olive mill wastes

3.1.1 Three-phase olive mill solid waste (3POMSW)

The traditional use of 3POMSW is to extract the residual olive oil. 3POMSW have around 4% residual oil on wet basis, which can be extracted by mechanical and/or chemical treatments. There are several extraction methods, the most usual being a first centrifugation where 40%-50% of the residual oil is extracted (Sánchez & Ruiz, 2006), followed by a drying process from 60-70% to 8% moisture (400ºC-800ºC) and extraction with solvents (hexane). Finally, the oil extracted 3POMSW is used for co-generation of heat and electricity in combustion-turbine cycles or a gas-turbine cycle. Oil extraction factories usually use this energy for their own drying process before extraction.

3.1.2 Three-phase olive mill wastewater (3POMWW)

Due to their high organic load and problematic disposal, the depuration of 3POMWW has been the subject of a great number of studies over the years. Initial treatments in the 60s focused on the use of 3POMWW as a soil conditioner if previously neutralized with lime (Albi Romero & Fiestas Ros de Ursinos, 1960). The addition of 3POMWW to the soil seems beneficial, as it produces an increase in nitrogen-fixing organisms (Garcia-Barrionuevo et al., 1992).

3POMWW are a potential source of biophenols, some being studied for potential industrial exploitation (Cardoso et al., 2011). The extraction of polyphenols provides a double opportunity to obtain high added value biomolecules and to reduce the phytotoxicity of the effluent (Bertín et al., 2011). López and Ramos-Cormenzana (1996) showed the possibility of obtaining 4.4 g L^{-1} of Xanthan with 3POMWW diluted to 30%-40%. The 3POMWW have also been studied as a source of natural pigments (anthocyanins) and different exopolysaccharides, and as a growth medium for algae (Ramos-Cormenzana et al., 1995). 3POMWW have been used as a growth media for the microbial production of extra-cellular lipase (D'Annibale et al., 2006) and for composting with olive leaves (Michailides et al., 2011).

Most of these studies, although very interesting, do not solve the problem because the quantities required for these studies are very small in contrast to the high quantity generated annually. The final destination of these wastewaters is mainly evaporation ponds. In the Mediterranean countries the summers are very hot, the evaporation ponds are large pools built with waterproof materials where the wastewaters can be stored for their evaporation in the summer period. After solar drying, the remaining solids can be used as fertilizer (Rozzi & Malpei, 1996). Although the evaporation ponds are very simple constructions, failure in the insulation of the basin can contaminate the ground water. Another disadvantage of these ponds is the production of putrid odors and insects during the decomposition processes (Khoufi et al., 2006).

3.2 Two-phase olive mill wastes

3.2.1 Two-phase olive mill solid wastes (2POMSW)

2POMSW have around 3.5% residual oil in wet basis. Like 3POMSW, this waste is also used for residual olive oil extraction. However, the humidity of 2POMSW is higher than

3POMSW. In order to obtain 8% humidity before extraction, the intensity and the length of the drying process are higher for 2POMSW than for 3POMSW. Furthermore, the vegetation water fraction of the olives gives 2POMSW a complex composition generating a high number of problems during the drying process. The high concentration in reducing sugars gives 2POMSW a doughy consistency in the continuous rotary dryer. This consistency creates dead areas which cannot be dried properly in the drying place, making residual oil extraction more difficult. Although the extraction process is more expensive and less profitable for 2POMSW than 3POMSW, this residual oil extraction is still applied. The extracted 2POMSW have 30%-45% stones, 15%-30% olive skin and 30%-50% pulp (Cruz et al., 2006). They are used for the co-generation of heat and electricity in combustion-turbine cycles or a gas-turbine cycle in the same way as 3POMSW. The oil extraction factory usually uses this type of energy for its own drying process before extraction.

Composts of 2POMSW is another alternative. The initial 2POMSW is phytotoxic, but Alburquerque et al. (2006) found the mixture with grape stalk and olive leaves as bulking agents free of phytotoxicity and suitable as soil conditioners.

Currently there are several experimental treatments for 2POMSW using it as a source of pharmaceutical compounds. A new process based on the hydrothermal treatment of 2POMSW led to a final solid enriched in minor components with functional activities (Lama-Muñoz et al., 2011). Other studies have been carried out using the bacteria *Penibacillus Jamila* for the production of exo-polysaccharides with 2POMSW as growth media (Ramos-Cormenzana & Monteoliva-Sánchez, 2000). There are two patented products extracted from 2POMSW: oleanoic acid and maslinic acid. Maslinic acid is being used for a treatment against the human immunodeficiency virus (HIV-1) (Parra et al., 2009). The walls of the olives are rich in polysaccharides such as L-arabinose. These polysaccharides are part of 2POMSW and can also be extracted and exploited (Cardoso et al., 2003).

2POMSW have also been used as feeding for animals. There are several studies about the digestibility of the protein content in 2POMSW used as sheep and goat feed (Martín et al., 2003; Molina Alcaide et al., 2003). Maslinic acid obtained from 2POMSW added to the diet of rainbow trout increased growth and protein-turnover rates (Fernández-Navarro et al., 2008).

The application of 2POMSW as a fertilizer has also been considered. Although the vegetation water gives a phytotoxic effect similar to 3POMWW, it has been observed that the fertilizer effect prevails over the phytotoxic effect when the dosage is not very high (Sierra et al., 2000).

An extremely low quantity of 2POMSW is used in these treatments, so none could be used as an integral treatment for this problematic waste.

3.2.2 Two-phase olive mill wastewaters (2POMWW)

Different options have been studied for the treatment of the wastewaters generated during the purification of olive oil. The use of oxidative methods for the treatment of 2POMWW has been reported in literature (Martínez-Nieto et al., 2011). These methods are based on the use of chemical oxidants such as permanganate, hydrogen peroxide (H_2O_2) or Fenton-like reaction. Aerobic treatment using a completely mixed activated sludge reactor was also reported (Borja et al., 1995a). The results obtained with the aerobic treatment indicated that more than 93% of the input COD concentration can be removed. The most commonly used treatment of both 2POMWW and 3POMWW is storage in evaporation ponds (section 3.1.2).

4. Anaerobic treatments

Anaerobic wastewater treatment has evolved into a competitive treatment technology in the past few decades. Many different types of organically polluted wastewaters, even those that were previously believed not to be suitable for anaerobic wastewater treatment, are now treated by anaerobic high-rate conversion processes (Van Lier, 2008).

Similar to anaerobic wastewater treatment, since the introduction of anaerobic digestion of solid waste in the beginning of the 1990s, adoption of the technology has been increasing (De Baere & Mattheeuws, 2010). European energy output from solid waste digestion plants rose to 5.3 Mtoe in 2009, which is 236 ktoe more than in 2008 (EurObserv´ER, 2010).

This section focuses on the principles of bioenergy production through anaerobic processes. Methanogenic anaerobic digestion (methane), biological hydrogen production (hydrogen) and microbial fuel cell technology (electricity) will be explained and discussed.

4.1 Methanogenic anaerobic digestion

The anaerobic digestion process is a biological process carried out by three different groups of microorganisms (hydrolytics, acetogenics and methanogenics) (Gujer & Zehnder, 1983)) which transform organic matter to obtain 90% biogas [a mixture CH_4/CO_2 (≈65%-35%)] and only 10% excess sludge. Biogas has a high calorific value (5000-6000 kcal m^{-3}) and can be used for electricity or heat.

The main advantages of the anaerobic process compared with other types of treatment are (Van Lier, 2007):

- High applicable chemical oxygen demand (COD).
- No use of fossil fuels for treatment
- No use of or very little use of chemicals; simple technology (Figure 1) with high treatment efficiencies.
- Anaerobic sludge can be stored unfed, so the reactors can be operated during the harvesting seasons only (e.g. 4 months per year in the olive oil mill industry).
- Effective pathogen removal.
- High degree of compliance with many national waste strategies implemented to reduce the amount of biodegradable waste entering landfills.
- The slurry produced (digestate) is an improved fertiliser.

Methanogenic anaerobic digestion of organic material has been performed for about a century. Therefore, the food web of anaerobic digestion is reasonably well understood (Figure 2).

Anaerobic digestion of biodegradable wastes involves a large spectrum of bacteria of which three main groups can be distinguished. The first group comprises fermenting bacteria which perform **hydrolysis and acidogenesis** (e.g. *Clostridium butyricum, Propionibacterium*). This involves the action of exo-enzymes to hydrolyze matter such as proteins, fats and carbohydrates into smaller units which can then enter the cells to undergo an oxidation-reduction process resulting in the formation of volatile fatty acids (VFA) and some carbon dioxide and hydrogen. The fermenting bacteria are usually designated as an acidifying or acidogenic population because they produce VFA.

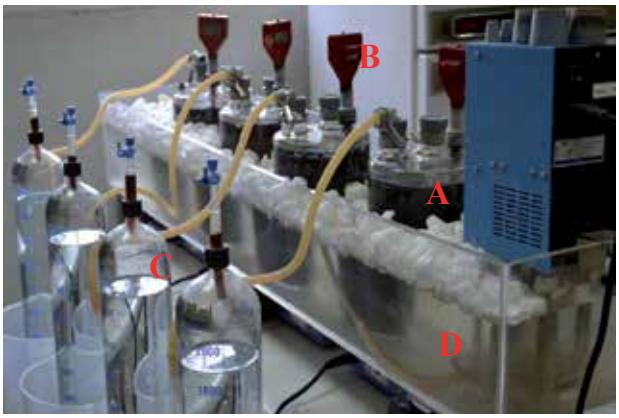

Fig. 1. Experimental laboratory scale anaerobic reactors (A: reactor, B: pH-meter, C: gasometer with NaOH for methane measurement, D: water bath for temperature control).

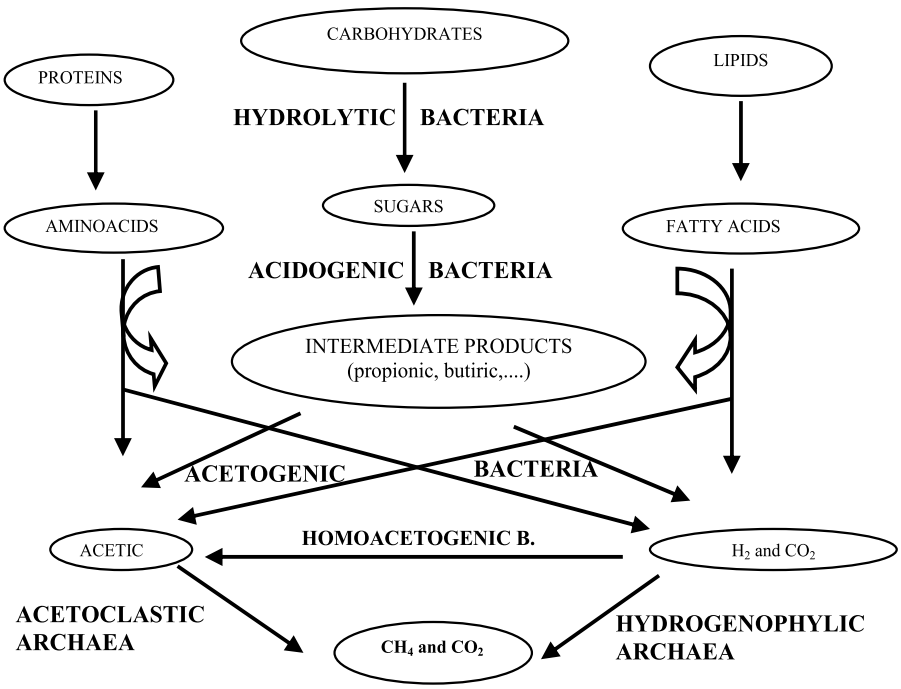

Fig. 2. Diagram of the different steps of anaerobic digestion

Acetogenic bacteria (e.g. *Clostridium aceticum, Acetobacterium woodii)* constitute the second group and are responsible for breaking down the products of the acidification step to acetic acid. In addition, hydrogen and carbon dioxide are also produced during acetogenesis.

The third group involves methanogenic Archaea (e.g. *Methanobrevibacter smithii, Methanobacterium thermoautotrophicum, Methanosarcina barkerii, Methanotrix soebugenii)* convert acetic acid or carbon dioxide and hydrogen into methane. Other possible methanogenic substrates such as formic acid, methanol, carbon monoxide, and

methylamines are of minor importance in most anaerobic digestion processes. In addition to these three main groups, hydrogen consuming acetogenic bacteria are always present in small numbers in an anaerobic digester. They produce acetic acid from carbon dioxide and hydrogen and, therefore, compete for hydrogen with the methanogenic archaea.

The synthesis of propionic acid from acetic acid, as well as the production of longer chain VFA, occur to a limited extent in anaerobic digestion. Competition for hydrogen can also be expected from **sulfate reducing bacteria.**

In conventional applications, the acid- and methane-forming microorganisms are kept together inside the reactor system with a delicate balance between these two groups of organisms, because they differ greatly in terms of physiology, nutritional needs, growth kinetics and their sensitivity to environmental conditions. Problems encountered with stability and control in conventional design applications have led researchers to new solutions such as the physical separation of acid-formers and methane-formers in two separate reactors. Optimum environmental conditions for each group of organism is provided separately to enhance the overall process stability and control (Cha & Noike, 1997).

4.2 Biological hydrogen production

Hydrogen is a clean, recyclable, and efficient energy carrier. The possibility of converting hydrogen into electricity via fuel cells makes the application of hydrogen energy very promising (Chang et al., 2002).

Hydrogen production via dark fermentation is a special type of anaerobic digestion consisting of only hydrolysis and acidogenesis. It leads to the production of hydrogen, carbon dioxide and some simple organic compounds [VFA and alcohols]. These readily degradable organic compounds can be used for further methane production. (Bartacek et al., 2007)

Much interest has recently been expressed in the biological production of hydrogen from waste streams by dark fermentation. Biological hydrogen production shares many common features with methanogenic anaerobic digestion, especially the relative ease with which the two gaseous products can be separated from the treated waste.

From hydrogen-producing mixed cultures, a wide range of species have been isolated, more specifically from the genera Clostridium (*Clostridium pasteurianum, Clostridium saccharobutylicum, C. butyricum*), Enterobacter (*E. aerogenes*) and Bacillus under mesophilic conditions; and from the genera Thermoanaerobacterium (*Thermoanaerobacterium thermosaccharolyticum*), and Caldicellulosiruptor (*Caldicellulosiruptor saccharolyticus*, *C. thermocellum, Bacillus thermozeamaize*) under thermophilic or extremophilic conditions.

However, the low efficiency of the hydrogen production process remains the main limiting factor. Much research will be needed to be carried out to reach hydrogen yields comparable with the theoretical efficiency maximum. Although a relatively high efficiency has been reached using pure substrates, the low hydrogen yield with complex (real) substrates remains a great challenge.

4.3 Microbial fuel cell technology

The microbial fuel cell (MFC) is a new energy technology in which microorganisms produce electricity directly from renewable biodegradable materials (Logan et al., 2006). During microbial oxidation of biodegradable matter, not only are protons and oxidized products formed but electrons are remarkably transferred from the bacteria towards a solid electrode. The electrons flow through an electrical circuit towards the cathode where a final electron acceptor is reduced resulting in generation of electrical power (Figure 3).

Although interest in microbial fuel cells was relatively high in the 1960s, research has been limited as the cost of other energy sources remained low and the available microbial fuel cells lacked efficiency and long term stability. However, in the past seven to eight years there has been a resurgence in microbial fuel cell research. In fact, the efficiency of this energy conversion is potentially higher than the actual waste treatment technology for energy recovery, such as anaerobic digestion or incineration (Logan et al., 2006).

Microbial fuel cells have been validated at lab-scale with simple organic substrates, pure culture and highly controlled experimental conditions. Organic substrates as volatile fatty acids and more recently wastewater have generated high energy production (Catal et al., 2008; Clauwaert et al., 2007; Clauwaert et al., 2008; Rabaey et al., 2003; Rabaey & Verstraete, 2005). Over the last 10 years, the improvement in the design of microbial fuel cells has increased electrical generation 10,000 times (Debabov, 2008). However, full scale application has not yet been developed.

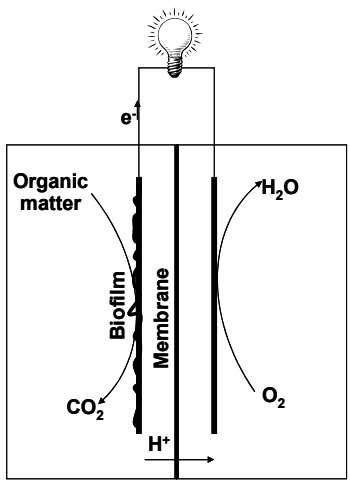

Fig. 3. Microbial Fuel Cell (MFC) set up

5. Anaerobic treatment of olive oil mill wastes

5.1 Three-phase olive mill wastes

5.1.1 Three-phase olive mill solid wastes (3POMSW)

The heterogeneous cellulosic and lignocellulosic structures of the husk make the anaerobic digestion of this waste impossible because the microorganisms are unable to attack these

complex structures. Therefore, anaerobic digestion is not a suitable technology for the treatment of 3POMSW.

5.1.2 Three-phase olive mill wastewaters (3POMWW)

Anaerobic digestion is a promising alternative for the treatment of 3POMWW. It allows for the disposal of these wastewaters achieving considerable organic material removals and producing renewable energy in the form of biogas, which could be used as an energy source in the olive oil mill itself.

Certain components of 3POMWW such as poly-phenols, pH, oil, etc. may inhibit the AD process. Martín et al., (1991) obtained methane yields of 260 mL CH_4 g^{-1} COD for 3POMWW. Borja et al. (1995b) improved the methane production using a pre-treatment stage with *Geotrichum candidum*, *Azotobacter Chroococcum* and *Aspergillus terreus*. The latest study reported methane yield coefficients of 300 (*Geotrichum*-pretreated 3POMWW), 315 (*Azotobacter*-pretreated 3POMWW) and 350 (*Aspergillus*-pretreated 3POMWW) mL CH_4 g^{-1} COD against the 260 mL CH_4 g^{-1} COD obtained for the untreated 3POMWW.

3POMWW have a low nitrogen content which limits the AD process due to the fact that the microorganisms need this element for their metabolism. In this way, co-digestion with rich nitrogen substrates may improve the biodegradability of the mixture. Azbar et al., (2008) studied the co-digestion of 3POMWW with laying hen litter obtaining a significant improvement in the biodegradability of 3POMWW. Co-digestion with liquid cow manure [20% 3POMWW, 80% liquid cow manure (v:v)] also showed good results in terms of COD and volatile solids removal (Dareioti et al., 2010).

Another option is the combination of catalytically oxidized olive mill wastewaters (by Fenton's reagent) plus anaerobic digestion. El-Gohary et al. (2009) found that the digestion of catalytically oxidized 3POMWW followed by a classical upflow anaerobic sludge blanket reactor (UASB) and a hybrid UASB as a post-treatment step is a promising alternative.

Other treatments envisage the combination of an initial liquid-liquid extraction with ethyl acetate for exploitation of the phenol content, followed by aerobic or anaerobic digestion of the phenolic extracted 3POMWW (Khoufi et al., 2006).

The use of sand filtration and subsequent treatment with powered activated carbon in batch systems has also been studied as a pre-treatment. This pre-treatment allowed COD removal efficiencies of 80%-85% for an HRT of 5 days and at an OLR of 8 g COD L^{-1} d^{-1}. A methane yield of 300 mL biogas g^{-1} COD removed was achieved (Sabbah et al., 2004).

The separation of the digestion phases, hydrolytic-acidogenic reactor and methanogenic reactor, in two completely independent reactors can also be considered as a way to improve the AD digestion of these wastes. Bertín et al. (2010) studied different acidogenic configurations of biofilm reactors using ceramic filters or granular activated carbon with good results.

The latest research studies report AD as a promising technology for the treatment of 3POMWW, leading to sustainable waste treatment and an environmentally friendly solution.

5.2 Two-phase olive mill wastes

5.2.1 Two-phase olive mill solid wastes (2POMSW)

Borja et al. (2002) carried out an initial anaerobic digestibility study with four different dilutions of 2POMSW (20%, 40%, 60% and 80 %). The main findings showed that the performance of the reactor [in terms of COD removal (%)] is practically independent of the feed COD concentration. Studies with no-diluted 2POMSW were carried out by the same authors with similar results (Rincón et al., 2007). The methane yields obtained in these studies ranged between 200-300 mL CH_4 g^{-1} COD removed. The 2POMSW is easily biodegradable by mesophilic anaerobic digestion and COD removal efficiencies up to 97 % may be achieved (Rincón et al., 2007).

Rincón et al. (2006, 2008a) studied the different microorganisms participating in the 2POMSW anaerobic digestion. For the determination of the microorganism population polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), cloning and sequencing techniques were employed. The results showed differences in the microbial communities, both bacterial and archaeal, with varying OLRs. Analysis of the microbial communities may be decisive in understanding the microbial processes taking place during 2POMSW decomposition in anaerobic reactors and optimizing their performance. During this experimental study the most frequently encountered microbial group were the Firmicutes (53.3% of analyzed sequences), represented mostly by members of the Clostridiales (Figure 4). Chloroflexi also represented an important bacterial group in the study (23.4% of sequences) and has been reported as a major constituent in anaerobic systems (Rincón, 2006). The Gamma-Proteobacteria (8.5% sequences, represented mainly by the genus *Pseudomonas*), Actinobacteria (6.4%) and Bacteroidetes (4.3%) are also significant components of the microbial communities during the anaerobic decomposition of 2POMSW (Figure 4). The major archaeal component detected for the 2POMSW anaerobic digestion was *Methanosaeta concilii* (formerly *Methanothrix soehngenii*) (Figure 5). Furthermore, results showed the existence of molecular diversity within the genus *Methanosaeta* in the anaerobic process under study (Rincón et al., 2006).

As has been explained in section 4.1, the anaerobic digestion process could be more stable if the hydrolytic-acidogenic and the methanogenic stages were physically separated. The microorganisms participating in this kind of biological treatment (bacteria and methanogenic archaea) have different requirements in terms of growing kinetic, optimal working conditions and sensitivity to environmental conditions. Studies in two stages allow for the enrichment of the different populations of microorganisms (Cha & Noike, 1997). The separation in the hydrolytic-acidogenic and the methanogenic steps showed improved results as compared to one simple AD stage. The acidification of the 2POMSW in an initial hydrolytic-acidogenic step achieved a high concentration of total volatile fatty acids 14.5 g L^{-1} (expressed as acetic acid) at an OLR as high as 12.9 g COD L^{-1} d^{-1} (Rincón et al., 2008b). After this initial acidification, the OLRs achieved in the methanogenic reactor were in the order of 22.0 g COD L^{-1} d^{-1} with COD and volatile solid removals of 94.3%-61.3% and 92.8%-56.1%, respectively, for OLRs between 0.8 and 20.0 g COD $L^{-1}d^{-1}$. Methane yields of 268 mL CH_4 g^{-1} COD removed were achieved (Rincón et al., 2009, 2010).

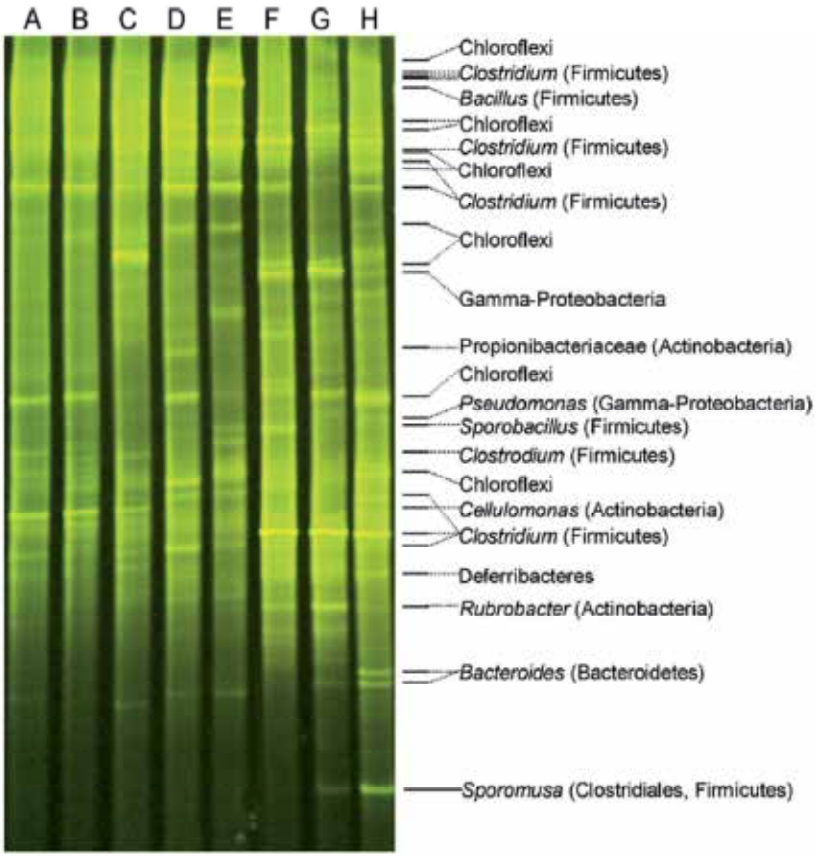

Fig. 4. DGGE analysis of the diversity of bacterial communities at different OLRs studied in one stage anaerobic digestion of 2POMSW (Rincón et al., 2008a). The position of the major electrophoretic bands corresponding to the 16S rRNA gene of the identified bacteria are indicated. A, B, C, D, E, F, G and H are the increasing OLRs studied in g COD L^{-1} d^{-1}: 2.3 (A), 3.0 (B), 4.5 (C), 5.8 (D), 6.8 (E), 8.3 (F), 9.2 (G) and 11.0 (H).

Other studies in two-stages at thermophilic scale reported 2POMSW as an ideal substrate for biohydrogen and methane production. These studies used diluted 2POMSW (1:4) with tap water achieving 18.5 ± 0.4 mmol CH_4 g^{-1} total solid added (TS). Experiments for biohydrogen production followed by methane production, generated 1.6 mmol H_2 g^{-1} TS added and 19.0 mmol CH_4 g^{-1} TS in the methanogenic stage (Gavala et al., 2005). Mesophilic bio-hydrogen production from 3POMSW has shown to be feasible at mesophilic temperature resulting in 2.8-4.5 mmol H_2 per gram of carbohydrates consumed in the reactor (Koutrouli et al., 2006). Methane production in these assays achieved a maximum value of 1.13 L CH_4 L^{-1} d^{-1} at 10 days of HRT. Hydrogen is a renewable energy source and one of the most attractive applications is the conversion of hydrogen to electricity via fuel cells (Koutrouli et al., 2006)

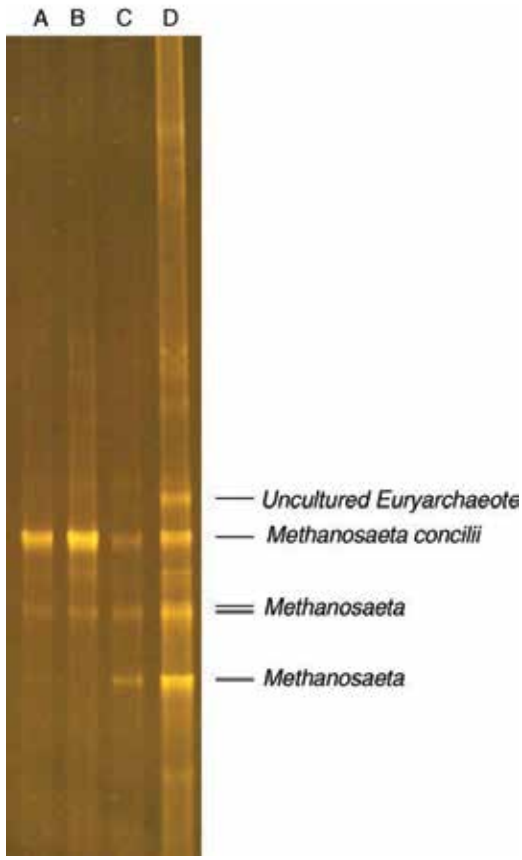

Fig. 5. DGGE analysis of the diversity of archaeal communities at different OLRs studied in one stage anaerobic digestion of 2POMSW (Rincón et al., 2006). The position of the major electrophoretic bands corresponding to the 16S rRNA gene of the identified archaea are indicated. A, B, C and D are the increasing OLRs studied in g COD L^{-1} d^{-1}): 0.75 (A), 1.5 (B), 2.25 (C) and 3.0 (D).

5.2.2 Two-phase olive mill wastewaters

Anaerobic treatment of this wastewater is very promising and beneficial. The production of biogas enables the process to generate or recover energy instead of just saving energy. This reduces operational costs as compared with other processes such as the physical, physicochemical or biological aerobic treatments previously mentioned (Wheatley, 1990). A kinetic study of the anaerobic digestion of wastewaters derived from the washing of virgin olive oil was previously reported (Borja et al., 1993). The study was carried out in a completely mixed reactor with biomass immobilized on sepiolite at a concentration of 10.8 g VSS L^{-1} operating at 35°C. COD removal efficiencies of more than 89% were achieved. Olive oil mills are usually small enterprises. Therefore, complex waste treatment systems are usually difficult to implement. Energy recovery from the generated wastewater with a

single unit like the previously explained MFC (section 4.3) is very promising. Preliminary studies of the treatment of 2POMWW have been reported by Fermoso et al. (2011).

6. Conclusion

Three-phase olive mill wastewaters (3POMWW) and two-phase olive mill solid wastes (2POMSW) are the main wastes generated in the olive mill industry (1,200 L of 3POMWW per ton of milled olives and 800 kg of 2POMSW per ton of milled olives, respectively). The composition of 3POMWW and 2POMSW is very complex due to the vegetation water. Currently, the final destination of 3POMWW is mainly evaporation ponds and the final destination of 2POMSW is evaporation ponds and co-generation. Although the evaporation ponds are very simple constructions, failure in the insulation of the basin can contaminate the ground water and they generate putrid odors and insects during the decomposition processes. The co-generation processes have a high number of environmental disadvantages: nitrogen oxides production, emission of suspended ashes, etc.

Anaerobic digestion is already successfully used for many agro-industrial residues, such as sugar beet pulp, potato pulp, potato thick stillage or brewer´s grains. This technology allows an efficient solids stabilisation and energy recovery. Both 2POMSW and 3POMWW have been shown to be promising substrates for anaerobic digestion, however full scale application is not a reality yet.

7. Acknowledgment

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16

Potential Applications of Green Technologies in Olive Oil Industry

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1. Introduction

Conventional olive oil production methods create large amounts of waste and by-products. Most production plants do not invest in purification and utilization of those by-products. Purification or conversion methods may add value to those by-products and prevent the environmental pollution.

Global trends show that "green" products and technologies are needed. Increasing environmental concerns, government measures and population drive the search for green processes to replace the conventional ones. This search is essential to achieve sustainable processing and to reduce commercial energy use (Clark, 2011). There are several applications for green technology in the olive oil industry.

This chapter reviews the potential applications of major green processes such as supercritical fluid extraction, membrane technology, bioconversions and molecular distillation in the olive oil industry.

2. Supercritical fluid technology

Supercritical Fluid Technology (SFT) has received growing interest as a green technology, with extraction being the main application in the food industry. Fluids become supercritical by increasing pressure and temperature above the critical point. Supercritical fluids have liquid-like solvent power and gas-like diffusivity. These physical properties make them ideal clean solvents for extraction of lipids.

Carbon dioxide (CO_2) is the most widely used supercritical fluid due to a lack of toxicity and flammability, low cost, wide availability, tunable solvent properties, and moderate critical temperature and pressure (31.1°C and 7.38 MPa) (Black, 1996). Because of the relatively low viscosity, high molecular diffusivity and low surface tension of the system, mass transfer is improved in supercritical CO_2 (SC-CO_2) in comparison to liquid organic solvents (Oliveira & Oliveira, 2000). Moreover, separation of CO_2 from the product can easily be achieved by reduction of pressure, because the products do not dissolve in CO_2 at atmospheric pressure.

Another unique property of supercritical fluids is their selectivity. The density of a supercritical fluid is higher than that of a gas, making them better solvents. Extraction selectivity of supercritical fluids can be changed altering density which is done by adjusting

pressure and temperature. Selectivity can also be changed by the addition of a co-solvent such as ethanol, methanol, hexane, acetone, chloroform and water to increase or decrease the polarity. Ethanol is the most preferred co-solvent because it is non-toxic and meets green technology criteria (GRAS status) (Dunford, 2004).

SC-CO_2 processing adds value because products obtained may be considered as natural. Although SFT is used for extraction of plants and vegetables of different sources (Table 1), applications in the olive oil industry have been limited. SFT can be used in olive oil processing for extraction and deacidification, as well as separation, purification or concentration of minor components.

Sample	Analyte	Reference
Carrot	Carotenes	Vega et al. (1996)
Tomato skin	Lycopene	Ollanketo et al. (2001)
Mushrooms	Oleoresins	del Valle & Aguilera (1989)
Tea	Caffeine	Calabuig Aracil (1998)
Grape skin	Anthocyanins	Blasco et al. (1999)
Cottonseed	Lipids	Bhattacharjee et al. (2007)
Hops	Humulone, lupulone and essential oils	Langezaal et al. (1990)
Rosemary	Oil	Bensebia et al. (2009)

Table 1. Supercritical fluid extraction of different plants and vegetables.

2.1 Extraction

SC-CO_2 has been used to replace hexane in the olive oil industry and meets the growing demand for natural products (Temelli, 2009). The most common applications are extraction of total lipids from olive husk or minor lipid components from olive oil. Extraction of high value minor components without degradation led industry and researchers to focus on SC-CO_2 extraction. Fig. 1 represents a typical lab scale SC-CO_2 system used for extraction of lipids.

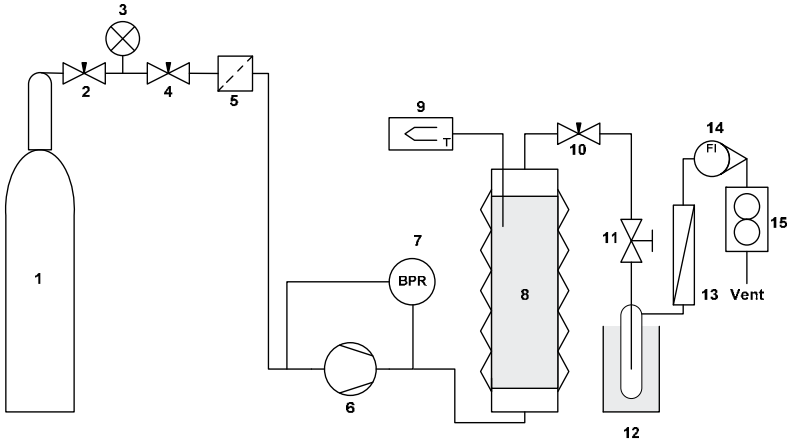

Fig. 1. Schematic diagram of a lab scale SC-CO_2 extraction system: 1, CO_2 tank; 2,4,10, shut-off valves; 3, pressure gauge; 5, filter; 6, compressor; 7, back pressure regulator; 8, extraction vessel; 9, thermocouple; 11, micrometering valve; 12; sample collector; 13, oil and moisture trap; 14, flowmeter; 15, gas meter.

SC-CO_2 extraction of olive husk oil is superior compared to conventional hexane extraction because the oil is also deacidifed and deodorised during the extraction process, and the resulting extract is free of residual solvent (Esquível & Bernardo-Gil, 1993). Esquivel and Bernardo-Gil (1993) extracted olive husk oil using SC-CO_2 under pressures of 12 to 18 MPa and temperatures of 35 to 45 °C.

2.2 Deacidification

Crude olive oil contains free fatty acids (FFA) and other impurities which must be removed, yielding a triacylglycerol (TAG) rich fraction. A high FFA content decreases the oxidative stability of the oil and leads to rancidity. A reduction in FFA content in virgin olive oil results in an increase in commercial value (Vázquez et al., 2009).

Supercritical fluid extraction has been proposed as an alternative technology for deacidification of oils and has been used for deacidification of olive pomace oil, an important by-product of olive oil industry. Crude olive pomace oil is often very acidic, darkly colored and highly oxidized. Intensive refining is thus required to make it suitable for human consumption. Neutralization is currently applied, but there are drawbacks to this process. Product yield is very low and neutralization increases the cost per unit. Therefore, it is necessary to reduce the FFA content before refining (Fadiloglu et al., 2003). Supercritical deacidification is actually a selective supercritical fluid extraction process. During the process, FFAs preferentially extracted with minimum neutral oil (TAGs, tocopherols, phytosterols) loss (Vázquez et al., 2009). A schematic diagram of a supercritical fluid extraction system for deacidification of oils is shown in Fig. 2. The oil is fed to the extraction column by a pump. The extraction column consists of two sections: an enriching (above of the oil feeding point) section, and a stripping section (below the oil feeding point). Raffinate is first separated from the extract and sent to the stripping section. Then, in the stripping section, the extract is separated from raffinate and transported to the enriching section. Extract rich in minor lipid compounds and CO_2 is separated in the separator. A specified amount of the extract is transferred to the top of the column as reflux (Brunner, 2009). CO_2 can be purified and recycled into the system. Raffinate is collected at the bottom of the column.

Deacidification of different oil sources using supercritical fluids have been performed at laboratory scale by several researchers. Turkay et al. (1996) achieved a selective and quantitative (90%) FFA extraction for deacidification of high acidic black cumin seed oil using SC-CO_2 at relatively low pressure (15 MPa) and relatively high (60 °C) temperature. Ooi et al. (1996) decreased the FFA content of palm oil to 0.1% in a continuous SC-CO_2 extractor.

Brunetti et al. (1989) obtained deacidification of high acidic olive oil with SC-CO_2 at pressures of 20 and 30 MPa, and temperatures of 40 and 60 °C. They reported that the selectivity for FFAs was highest at 20 MPa and 60 °C. Bondioli et al. (1992) studied the supercritical fluid deacidification of olive oil in the pressure range of 9–15 MPa and 40–50 °C. The acidity was reduced from 6.3% to values less than 1% at 40 °C and 13 MPa. In another application, Vázquez et al. (2009) used SC-CO_2 as an extraction solvent to remove FFAs from cold-pressed olive oil in a packed column. The acidity was reduced from 4 to 1.43% at 25 MPa and 40 °C.

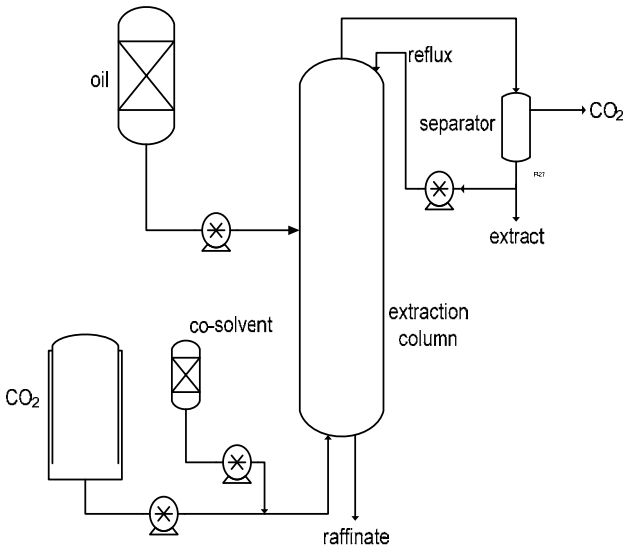

Fig. 2. Schematic diagram of supercritical fluid extraction pilot plant used for deacidification, separation, concentration and purification of oils.

2.3 Separation, concentration and purification of minor lipid compounds

Extraction of high value minor components from natural products is of great interest to food industry. SFT has been applied for purification, separation or concentration of several compounds from vegetable oils, essential oils and deodorizer distillates. These applications include purification of monoacylglycerols (MAGs) and lecithin, removal of cholesterol and limonene, and separation of squalene, tocopherols and fatty acid esters (Brunner, 2009).

Products of the olive oil industry are important sources of high value components such as tocopherols, phytosterols, squalene and fatty acids. The system used for separation of minor lipid compounds is the same as shown in Fig. 1. Fornari et al. (2008) purified squalene from a by-product obtained after distillation, esterification and transesterification of olive oil deodorizer distillates. They obtained 89.4% squalene purity and 64.2% yield at 70 °C and 18 MPa, and obtained a raffinate concentrated in TAGs and sterol compounds.

Dauksas et al. (2002) extracted tocopherols from olive tree leaves using SC-CO_2. They obtained a high value extract of 97.1% (w/w) tocopherol at 25 MPa and 40 °C after 1 h of extraction, and 74.48 % at the same pressure and temperature after 2 h. Le Floch et al. (1998) used supercritical fluid extraction for isolation of phenols from olive leave samples using SC-CO_2 modified with 10% methanol at 33.4 MPa and 100 °C.

2.4 Use of supercritical fluids as reaction media for enzymatic modification of lipids

Enzymatic interesterification in organic solvents leads to very important modifications of lipids. However, the use of organic solvents in these reactions is a disadvantage. Therefore, biosynthesis in supercritical fluids is attracting much attention. Replacement of organic solvents by supercritical fluids makes the process green and eliminates the need of solvent separation. The lower viscosity and the higher diffusivity of supercritical fluids allow easier

transport of substrates to the catalyst and, in the case of enzyme within the pores of enzyme support, this results in an easier access to the enzyme sites leading to higher reaction rates. In addition to the previously mentioned advantages of supercritical fluids, the finding that enzymes can retain their biocatalytic activity at high pressures has also encouraged the use of enzymes under supercritical condition (Rezaei et al., 2007b; Rezaei et al., 2007a).

In general, expansion of the substrates in CO_2 seems to be the main advantage of enzymatic lipid reactions in SC-CO_2. Expanded substrates have better diffusivity, low surface tension and low viscosity. In addition, a lesser amount of substrate available per unit amount of enzyme per unit time will increase the reaction rate (Ciftci & Temelli, 2011). However, at very high pressures, mass transfer properties of the substrates may be affected negatively. High CO_2 densities at high pressures lead to a decrease in enzymatic conversions. It has been reported that diffusion coefficients of fatty acids, fatty acid esters and glycerides in SC-CO_2 may also decrease at high pressures due to increase in the density of CO_2 (Rezaei & Temelli, 2000). Therefore, optimization of the process in terms of pressure and temperatures is crucial. Esmelindro et al. (2008) produced MAGs from olive oil in compressed propane. Their results showed that lipase-catalyzed glycerolysis in compressed propane might be a potential replacement for conventional methods, as high contents of reaction products, MAG and diacylglycerol (DAG), were achieved at mild temperature and pressure conditions (30 °C and 3 MPa) with a low solvent to substrates mass ratio (4:1) in short-reaction times (1 h). Lee et al. (2009) produced biodiesel from various oils, namely, olive, soybean, rapeseed, sunflower and palm oil, using lipase in SC-CO_2. The highest yield (65.18%) was obtained from olive oil at 13 MPa, 45 °C and 20% of lipase concentration (based on weight of oil).

3. Membrane technology

Membrane technology is becoming increasingly important as a green processing and separation method in food processing and waste water treatment Membranes are used as filters in separation processes and have a wide variety of applications Membrane technology is now competitive compared to conventional techniques such as adsorption, ion exchangers and sand filters.

The main advantage of membrane processing is that it avoids the use of any chemicals that have to be discharged. It works with relatively high efficiency and low energy consumption (Mulder, 1996). It also has the advantage of operating at ambient temperature, resulting in preservation of heat-sensitive components and nutritional value of food products (Dewettinck & Le, 2011).

Membrane separation processes differ greatly in the type of membranes and driving forces used for separation, the process design, and the area of application. There are many different membrane processes, including reverse osmosis, micro-, ultra- and nanofiltration, dialysis, electrodialysis, Donnan dialysis, pervaporation, gas seperation, membrane contactors, membrane distillation, membrane based solvent extraction, membrane reactors, etc. Among them, the innovative methods preferred by the food industry are pressure driven separation processes such as reverse osmosis, nanofiltration, ultrafiltration and microfiltration. These preferred methods facilitate the separation of components with a large range of particle sizes. The obtained products are generally of high quality and less post-treatment procedures are required (Baker, 2004).

3.1 Applications of membrane technology in the olive oil industry

Membrane technology has been used in the edible oil industry for degumming, deacidification, waste water treatment, recovery of solvent from micelles, condensate return, catalyst recovery and hydrolysis or synthesis of structured lipids with two-phase membrane reactors, involving pigment removal, separation and concentration of minor compounds in the oil. Despite its use in other sectors of the edible oil industry, this technology has not been broadly extended to olive oil processing.

3.1.1 Deacidification

Conventional chemical and physical deacidification methods have some drawbacks such as use of large amount of water and chemicals, and loss of neutral oil (Kale et al., 1999). Membrane technology may be proposed as a new alternative deacidification process for edible oils (Bhosle & Subramanian, 2005).

A membrane-based process for deacidification of lampante olive oil was undertaken by Hafidi et al. (2005a). Their objective was to deacidify, while also preserving the sensitive and bioactive components in the oil by operating at ambient temperature. The results showed that oils were obtained almost FFA- and soap-free in a single step. In another study, the impact of this process on some minor components and on the organoleptic characteristics of the purified olive oils was investigated (Hafidi et al., 2005b). It was reported that, while a complete deacidification was achieved, some desirable components, mainly phenolics, were eliminated during the filtering process. Thus, it was suggested to focus on reducing the elimination of phenolic compounds and the improvement of the organoleptic characteristics of the filtered oils.

3.1.2 Wastewater treatment

Olive mill wastewater (OMW), a by-product of olive oil extraction, is one of the most contaminated effluents. The polluting load is due to organic substances such as sugars, tannins, polyphenols, polyalcohols, pectins, lipids, proteins and organic acids, (Cassano et al., 2011). Phenolic compounds can act as phytotoxic components, inhibiting microbial growth as well as plant germination and vegetative growth (Morillo et al., 2009).

Biochemical oxygen demand (BOD5) and chemical oxygen demand (COD) of OMW may be as high as 100 and 200 g L^{-1}, respectively (de Morais Coutinho et al., 2009). Besides, OMWs are considered as a potential source for the recovery of antioxidant, antiatherogenic and anti-inflammatory biophenols (Obied et al., 2005). Detoxification and recovery of valuable components from wastewater are among the most useful treatments based on membrane technology.

In the study of Paraskeva et al. (2007), combinations of different membrane processes were used for the fractionation of OMW. Ultrafiltration in combination with nanofiltration and/or reverse osmosis were found to be very efficient for this process. It was shown that better efficiency of the OMW treatment was achieved by applying reverse osmosis after ultrafiltration. The ultrafiltration concentrate was found to contain the largest portion of fats, lipids, solids, etc. Further processing with nanofiltrationmay be employed for the separation of a greater part of phenols.

In another study, OMW was used to investigate the variation of COD and total organic carbon (TOC) removal efficiencies together with permeate fluxes for ultrafiltration process (Akdemir & Ozer, 2009). Two types of ultrafiltration membranes which are JW (polyvinylidine-difluoride) and MW (ultrafilic) gave close removal efficiencies. Ultrafiltration membranes with bigger molecular weight cut-offs for OMW were suggested to increase flux value and decrease efficiency loss. In their previous work, observed COD removal efficiency by ultrafiltration without pretreatment was found higher than 80% by promising value for OMW (Akdemir & Ozer, 2008). El-Abbassi et al. (2009) studied the treatment of OMW to obtain high value-added compounds such as sugar and polyphenols, by membrane distillation. Two types of commercial membranes, polytetrafluoroethylene (TF200) and polyvinilydene fluoride (GVHP), were compared and the effects of membrane parameters on direct contact membrane distillation (DCMD) performance (i.e. permeate flux and polyphenols retention) were investigated. Their results demonstrated that TF200 had a better separation coefficient (99%) after 9 h of DCMD operation than that of GVHP (89%). OMW concentration factor was found to be 1.72 for TF200, whereas it was only 1.4 for GVHP after 9 h.

Another OWM treatment was tested by Dhaouadi and Marrot (2008). Diluted solutions of OMW were treated in a ceramic membrane bioreactor with biomass specially acclimated to phenol. It gave stabilized permeate flux with zero suspended solid and no phenolic compounds. No fouling problems occurred during the experiments. OMW treatment in a membrane bioreactor can be used as a pre-treatment stage for the removal of phenolic compounds before a conventional biological process.

Recently, Coskun et al. (2010) studied the treatment of OWM using nanofiltration and reverse osmosis membranes. They reported that overall COD removal efficiencies were 97.5%. It was shown that reverse osmosis membranes are capable of producing a higher quality effluent from OMW than nanofiltration membranes. NF270 membranes were found to be most applicable among nanofiltration membranes due to their higher fluxes and higher removal efficiencies. In addition, it was found that centrifugation alone can be used as a promising option for primary treatment of OMWs with nanofiltration process.

In summary, there appears to be a potential for the use of membrane technology in the olive oil industry. Membranes can provide an opportunity to develop alternative environmentally friendly processes for the refining of olive oils and treatment of OWM. Despite promising results, further studies must be done on this new approach, namely, to evaluate the effect of the process on the oil composition, to improve flow rate, to reduce fouling inclusions and to assess economic viability.

4. Bioconversions

Lipids require modification in order to be used for special purposes or production of value added products. Lipids can be modified by hydrogenation, blending, fractionation and chemical or enzymatic reactions such as hydrolysis, direct esterification and interesterification.

4.1 Enzymatic conversions

Interesterification reactions are widely studied to produce margarines and shortenings with zero-*trans* fatty acids, cocoa butter equivalents, structured lipids with specific nutritional properties, partial glycerides and biodiesel. Chemical interesterification uses metal

alcoholate catalysts to incorporate fatty acids randomly. This reaction produces a complete positional randomization of acyl groups in TAGs. In enzymatic interesterification the final structure of TAGs is controlled and a desired acyl group can be guided into TAGs using nonspecific, regiospecific (*sn*-1,3- or 2- specific) and fatty acid specific lipases as catalysts. This results in products with predictable composition. Enzymatic interesterification is becoming a more attractive method to convert cheap oils such as olive pomace oil, soya bean oil, rape seed oil, lard, tallow, etc. to high-value-added products and modified fats (An et al., 2007; Liua et al., 1997; Macrae, 1983; Miller et al., 1991; Pomier et al., 2007; Xu, 2003). Furthermore, enzymatic interesterification has milder reaction conditions and produces less waste than the chemical alternative. In addition, the same immobilized enzyme can be used many times (Akoh et al., 1998; Marangoni & Rousseau, 1995; Willis et al., 1998; Willis & Marangoni, 2002). Therefore, intensive research has aimed at replacing chemical interesterification with enzymatic interesterification.

There are three types of interesterification reactions: acidolysis, which is the reaction between an ester and a fatty acid, alcoholysis, the reaction between an ester and an alcohol, and transesterification, the reaction of an ester with another ester, also called ester-ester exchange (Macrae, 1983; Xu, 2003). Production of structured lipids and biodiesel has been the major topics of enzymatic interesterification studies.

4.1.1 Structured lipids

Structured lipids are novel modified TAGs produced by the incorporation of desirable fatty acids at specific positions or by changing the position of the fatty acids on the glycerol backbone. These processes allow for specific characteristics to be obtained such as melting behavior, functionality, and metabolism. Lipases, especially those which are *sn*-1,3 specific, are used for this purpose because these enzymes can make changes at *sn*-1 and *sn*-3 positions by keeping *sn*-2 ester group position unchanged.

Cocoa butter (CB) has a narrow melting range due to its unique TAG composition. This melting behavior is critical. The steepness of the melting profile (% solid fat as a function of time) has an impact on flavor release and crystallization. The high price of cocoa butter has prompted the industry search for CB alternatives. CB equivalents (CBEs) can be produced from palm oil and exotic fats by means of fractionation. Enzymatic synthesis of CBEs from cheap oils and fats using *sn*-1,3 specific lipases is also an alternative method. CB-like fats could be produced which have even more desirable properties than natural CB. Ciftci et al. (2010) used olive pomace oil for the production of CB-like fat using *sn*-1,3 specific lipase. They interesterified refined olive pomace oil, palmitic acid and stearic acid at a molar ratio of 1:2:6, respectively, at 45°C using a pack bed reactor filled with *sn*-1,3 specific lipase. They reported that the CB-like fat could replace CB up to 30% without significantly changing the physical and chemical properties of the product. Chang et al. (1990) also produced CB-like fat by enzymatic interesterification of fully hydrogenated cotton seed and olive oils. The melting point of their CB-like fat was 39°C; close to 36°C, the melting point of CB.

Any lipid containing medium-chain and long-chain unsaturated fatty acids might be useful for certain applications and functionalities. Nunes et al. (2011) produced structured lipids containing medium-chain fatty acids at *sn*-1,3 position and long-chain unsaturated fatty acids at the *sn*-2 position by acidolysis of virgin olive oil and caprylic or capric acids using

1,3-selective *Rhizopus oryzae* heterologous lipase (rROL) immobilized in Eupergit C and modified sepiolite. These structured lipids are low caloric and and have dietetic properties for controlling obesity and malabsorption. They showed that rROL immobilized in Eupergit C was able to catalyze the incorporation of 21.6 and 34.8 mol% of caprylic or capric acid into virgin olive oil, after 24 h at 40 °C in solvent-free media. Fumoso and Akoh (2002) also used lipase-catalyzed acidolysis of olive oil and caprylic acid to produce structured lipids. They used a *sn*-1,3-specific lipase from *Rhizomucor miehei* in a bench-scale packed bed bioreactor. They studied the effect of solvent, temperature, substrate mol ratio, and flow rate/residence time. The optimal solvent-free production of structured lipid was obtained at a substrate flow rate of 1 ml/min, a residence time 2.7 h, 60 °C, and a mol ratio 1:5 (olive oil/caprylic acid). The structured lipid produced at optimal conditions had 7.2% caprylic acid, 69.6% oleic acid, 21.7% linoleic acid and 1.5% palmitic acid at the *sn*-2 position. Another structured lipid used as a constituent of infant formulas, consisting mainly of UPU triglycerides (U=unsaturated acyl chains, P=palmitic acyl group), can be prepared by lipase catalyzed reactions of fractionated palm oil, rich in tripalmitin, and oleic acid from olive oil (Schmid et al., 1998).

4.1.2 Biodiesel

Biodiesel can be obtained from vegetable oils, animal fats, recycled grease, or algae and can be produced by the reaction of TAGs with methanol (methanolysis). Lipase-catalyzed methanolysis is more attractive than conventional base-catalyzed method since the glycerol produced as a by-product can easily be recovered and the purification process for fatty acid methyl esters (FAMEs) is relatively simple. In the oil and fat industry, conversion of waste edible oil and soapstock (a by-product generated in alkali refining of vegetable oils) to biodiesel has attracted a great deal of attention (Azócar et al., 2010; Safieddin Ardebili et al., 2011; Singaram, 2009). Unlike the conventional chemical routes for synthesis of diesel fuels, biocatalytic routes permit one to carry out the interesterification of a wide variety of oil feedstocks in the presence of excess FFAs.

Olive pomace oil was used by Yucel (2011) for enzymatic production of biodiesel. Yucel (2011) immobilized microbial lipase from *Thermomyces lanuginosus* on olive pomace by covalent binding, and then used this immobilized lipase for the methanolysis of olive pomace oil. Under the optimized conditions for solvent-free reaction, the maximum yield was reported to be 93% at 25 °C after 24 h. Sanchez and Vasudevan (2006) produced biodiesel by transesterification of olive oil triolein with methanol using lipase. They studied the effects of the molar ratio of methanol to triolein, semibatch (stepwise addition of methanol) vs batch operation, enzyme activity, and reaction temperature on overall conversion. Because of the inactivation of the enzyme by insoluble methanol, stepwise methanolysis with a 3:1 methanol to triolein molar ratio and an overall ratio of 8:1 gave the best results.

4.2 Enzymatic deacidification

One method to reduce the FFA content in fats and oils is to convert the FFAs to TAGs. This is carried out by direct esterification of fatty acids with glycerol.

A reported application of enzymatic deacidification of olive pomace oil is the enzymatic glycerolysis of highly acidic (32%) olive pomace oil (Fadiloglu et al., 2003). FFAs of olive pomace oil were esterified with glycerol using a nonspecific immobilized lipase, reducing

the acidity of the oil to 2.36%. In another study, the FFA content of high acidic (31.6%) degummed and dewaxed olive oil was reduced to 3.7%.

4.3 Bioremediation

Bioremediation, generally classified as *in situ* or *ex situ*, is the use of microorganism metabolism to remove pollutants. *In situ* bioremediation involves treating the contaminated material at the site, while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are phytoremediation, bioventing, bioleaching, landfarming, composting, bioaugmentation, rhizofiltration, and biostimulation (Shukla et al., 2010). Besides being cost effective, bioremediation can result in the complete mineralization of the pollutant, considered a permanent solution of the pollution problem. Furthermore, it is a non-invasive technique, leaving the ecosystem intact. Bioremediation can deal with lower concentrations of contaminants where cleanup by physical or chemical methods would not be feasible. Unfortunately, it presents some major drawbacks which still limit the application of these techniques, including long processing times and less predictable results compared to conventional methods (Perelo, 2010).

The disposal of OMW is predominantly carried out via land spreading or by means of evaporation ponds, although a wide number of chemical and biological decontamination and valorisation technologies have been reported. The two-phase centrifugation system, as an alternative ecological approach for olive oil production, drastically reduces the water consumption during the process. This system generates olive oil plus a semi-solid waste, known as the two-phase olive-mill waste (Morillo et al., 2009).

Ramos-Cormenzana et al. (1996) performed aerobic biodegradation on OMW by using bacterium *Bacillus pumilus* to reduce the phenol content. They reported 50% reduction in phenol content using *Bacillus pumilus*. The detoxification of OMW following inoculation with *Azotobacter vinelandii* (strain A) was performed for two successive 5-day-period cycles in an aerobic, biowheel-type reactor, under non-sterile conditions by Ehaliotis et al. (1999). The authors indicated that the phytotoxicity of the processed product was reduced by over 90% at the end of both cycles. However, aerobic bacteria cannot generally biodegrade complex phenolic compounds which are responsible for the dark color of OMW. Fungi, compared to bacteria, are more effective at degrading both simple and complex phenolic compounds presenting in olive mill wastes. This is due to the presence of compounds analogous to lignin monomers, which are more easily degraded by wood-rotting fungi (García García et al., 2000).

Demirer et al. (2000) generated biogas containing about 77% methane by anaerobic bioconversion of OMW (57.5 L methane per liter of wastewater). Ammary (2005) treated OMW using a lab scale anaerobic sequencing batch reactor, achieving more than 80% COD removal at 3 d hydraulic retention time. Anaerobic bioconversion has some advantages compared to aerobic processes: (a) high organic load feeds are used, (b) low nutrient requirements are necessary, (c) small quantities of excess sludge are usually produced, and (d) a combustible biogas is generated. However, the nutrient imbalance of OMW, mainly due to its high C/N ratios, low pH and the presence of biostatic and inhibitory substances, cause a problem. Not quite clear Rephrase An additional problem of two-phase olive-mill waste is its high consistency making its transport, storage and handling difficult (Morillo et al., 2009).

Olive mill wastes can be treated with other methods such as composting to produce fertilizers (Ntoulas et al., 2011); using as a culture medium to produce useful microbial biomass (de la Fuente et al., 2011); using as a low-cost fermentation substrate for producing microbial biopolymers for production of polysaccharides and biodegradable plastics (Ntaikou et al., 2009); and as a base-stock for production of biofuels (Rincon et al., 2010).

5. Molecular distillation

Molecular distillation, also called short path distillation, has become an important alternative for separation of heat sensitive compounds or substances with very high boiling points. Molecular distillation is characterized by a short time exposure of the distilled liquid to elevated temperature and high vacuum, with a small distance between the evaporator and the condenser (Lutišan et al., 2002). The small distance between the evaporator and the condenser and a high vacuum in the distillation gap results in a specific mass transfer mechanism with evaporation outputs as high as 20–40 $gm^{-2}\ s^{-1}$ (Cvengroš et al., 2000). Due to short residence time and low temperature, distillation of heat-sensitive materials is accomplished without thermal decomposition. Another advantage of the process is the absence of solvents. Therefore, molecular distillation is considered as a promising method in the separation, purification and concentration of natural products (Martins et al., 2006).

Vegetable oil deodorization process produces a distillate rich in high value components such as phytosterols, tocopherols, and fatty acids, depending on the oil or fat. Martins et al. (2006) separated FFAs from soybean oil deodorizer distillate to obtain a tocopherol concentrate, which contained only 6.4% of FFA and 18.3% of tocopherols (from a raw material containing 57.8% of FFA and 8.97% of tocopherols.) The specific processing conditions were an evaporator temperature of 160 °C and a feed flow rate of 10.4 $gmin^{-1}$. Under these conditions, they achieved 96% FFA elimination and 81% tocopherol recovery.

Although molecular distillation is a promising separation and purification method, it is not commonly applied in the olive oil industry. One relevant application is the purification of the structured lipids enzymatically produced from olive oil and caprylic acid (Fomuso & Akoh, 2002). If the advantages and efficiency of the system are further considered, it may be used in the olive oil industry for deacidification and separation of nutraceuticals. The cost of the system and possible alterations in the structure of the oil during the process seem to be serious disadvantages. Therefore, optimization of each particular system is necessary for a successful industrialization.

6. Use of by-products of olive oil industry for waste treatment

The use of by-products of the olive oil industry for waste treatment is another green approach. Solid olive wastes were used for water purification by El-Hamouz et al. (2007). The solid olive residue was processed to yield relatively high-surface area active carbon after extraction of the oil from the residue. The resulting carbon was used to reversibly adsorb chromate ions from water, aiming at a purification process with reusable active carbon. In another study, olive pomace was used as reactive dye biosorbent material for the removal of RR198 textile dye from aqueous solutions (Akar et al., 2009).

Vlyssides et al. (2004) developed an integrated pollution prevention method which decreased wasterwater production 50% from the 3-phase olive oil extraction process. The

process included mechanical separation, crushing, mixing, composting, malaxation, 3-phase centrifugation, coagulation flocculation, chemical oxidation, biological treatment, and reed beds steps. Furthermore, a Fenton oxidation process was used to detoxify the wastewater, with the possibility of extracting commercially valuable antioxidant products. They also produced high-quality compost from the solid residues.

7. Conclusions

Current trends show that future oil processing technologies will be based on green processes. Laboratory and pilot scale applications of such processes in the olive oil industry show that they can be used as alternatives to conventional processes. Further optimization studies are necessary for more successful applications. In spite of the high first capital investment, these processes are advantageous considering the market value of the natural products obtained and remediation of environmental pollution.

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Microbial Biotechnology in Olive Oil Industry

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1. Introduction

Microbial biotechnology is defined as any technological application that uses microbiological systems, microbial organisms, or derivatives thereof, to make or modify products or processes for specific use (Okafor 2007). Current agricultural and industrial practices have led to the generation of large amounts of various low-value or negative cost crude wastes, which are difficult to treat and valorize. Production of agro-industrial waste pollutants has become a major problem for many industries. The olive oil industry generates large amounts of olive mill wastes (OMWs) as by-products that are harmful to the environment (Roig et al. 2006).

However, OMWs have simple and complex carbohydrates that represent a possible carbon resource for fermentation processes. In addition, OMWs generally contain variable quantities of residual oil, the amount of which mainly depends on the extraction process (D'Annibale et al. 2006). Therefore, OMWs could be used as substrate for the synthesis of biotechnological high-value metabolites that their utilization in this manner may help solve pollution problems (Mafakher et al. 2010).

The fermentation of fatty low-value renewable carbon sources like OMWs to production of various added-value metabolites such as lipases, organic acids, microbial biopolymers and lipids, single cell oil , single cell proteins and biosurfactants is very interesting in the sector of industrial microbiology and microbial biotechnology (Darvishi et al. 2009). Thus, more research is needed on the development of new bioremediation technologies and strategies of OMWs, as well as the valorisation by microbial biotechnology (Morillo et al. 2009).

Few investigations dealing with the development of value-added products from these low cost materials, especially OMWs have been conducted. This chapter discusses olive oil microbiology, the most significant recent advances in the various types of biological treatment of OMWs and derived added-value microbial products.

2. Olive oil microbiology

In applied microbiology, specific microorganisms employed to remove environmental pollutants or industrial productions have often been isolated from specific sites. For example, when attempting to isolate an organism that can degrade or detoxify a specific target compound like OMW, sites may be sampled that are known to be contaminated by

this material. These environments provide suitable conditions to metabolize this compound by microorganisms.

Recent microbiological research has demonstrated the presence of a rich microflora in the suspended fraction of freshly produced olive oil. The microorganisms found in the oil derive from the olives' carposphere which, during the crushing of the olives, migrate into the oil together with the solid particles of the fruit and micro-drops of vegetation water. Having made their way to the new habitat, some microbic forms succumb in a brief period of time whereas others, depending on the chemical composition of the oil, reproduce in a selective way and the typical microflora of each oil (Zullo et al. 2010).

Newly produced olive oil contains numerous solid particles and micro-drops of olive vegetation water containing, trapped within, a high number of microorganisms that remain during the entire period of olive oil preservation. The microbiological analyses highlighted the presence of yeasts, but not of bacteria and moulds (Ciafardini and Zullo 2002). Several isolated genus of yeasts were identified as *Saccharomyces*, *Candida* and *Williopsis* (Ciafardini et al. 2006).

Some types of newly produced oil are very bitter since they are rich in the bitter-tasting secoiridoid compound known as oleuropein, whereas after a few months preservation, the bitter taste completely disappears following the hydrolysis of the oleuropein. In fact, the taste and the antioxidant capacity of the oil can be improved by the β-glucosidase-producing yeasts, capable of hydrolysing the oleuropein into simpler and less bitter compounds characterized by a high antioxidant activity. Oleuropein present in olive oil can be hydrolysed by β-glucosidase from the yeasts *Saccharomyces cerevisiae* and *Candida wickerhamii*. The absence of lipases in the isolated *S. cerevisiae* and *C. wickerhamii* examined that the yeasts contribute in a positive way to the improvement of the organoleptic characteristics of the oil without altering the composition of the triglycerides (Ciafardini and Zullo 2002).

On the other hand, the presence of some lipase-producing yeast can worsen oil quality through triglycerides hydrolysis. Two lipase-producing yeast strains *Saccharomyces cerevisiae* 1525 and *Williopsis californica* 1639 were found to be able to hydrolyse olive oil triglycerides. The lipase activity in *S. cerevisiae* 1525 was confined to the whole cells as cell-bound lipase, whereas in *W. californica* 1639, it was detected as extracellular lipase. Furthermore, the free fatty acids of olive oil proved to be good inducers of lipase activity in both yeasts. The microbiological analysis carried out on commercial extra virgin olive oil demonstrated that the presence of lipase-producing yeast varied from zero to 56% of the total yeasts detected (Ciafardini et al. 2006).

Some dimorphic species can also be found among the unwanted yeasts present in the olive oil, considered to be opportunistic pathogens to man as they have often been isolated from immunocompromised hospital patients. Recent studies demonstrate that the presence of dimorphic yeast forms in 26% of the commercial extra virgin olive oil originating from different geographical areas, where the dimorphic yeasts are represented by 3-99.5% of the total yeasts. The classified isolates belonged to the opportunistic pathogen species *Candida parapsilosis* and *Candida guilliermondii*, while among the dimorphic yeasts considered not pathogenic to man, the *Candida diddensiae* species (Koidis et al. 2008; Zullo and Ciafardini 2008; Zullo et al. 2010).

Overall, these findings show that yeasts are able to contribute in a positive or negative way to the organoleptic characteristics of the olive oil. Necessary microbiological research carried out so far on olive oil is still needed. From the available scientific data up to now, it is not possible to establish that other species of microorganisms are useful and harmful in stabilizing the oil quality. In particular, it is not known if the yeasts in the freshly produced olive oil can modify some parameters responsible for the quality of virgin olive oil. Further microbiological studies on olive oil proffer to isolation of new microorganisms with biotechnological potential. The OMWs due to their particular characteristics, in addition to fat and triglycerides, sugars, phosphate, polyphenols, polyalcohols, pectins and metals, could provide microorganisms with biotechnological potential and low-cost fermentation substrates. For example, the exopolysaccharideproducing bacterium *Paenibacillus jamilae* (Aguilera et al. 2001) and the obligate alkaliphilic *Alkalibacterium olivoapovliticus* (Ntougias and Russell 2001) were isolated from olive mill wastes.

3. Olive mill waste as renewable low-cost substrates

According to the last report of Food and Agriculture Organisation of the United Nations (FAOSTAT 2009), 2.9 million tons of olive oil are produced annually worldwide, 75.2% of which are produced in Europe, with Spain (41.2%), Italy (20.1%) and Greece (11.4%) being the highest olive oil producers. Other olive oil producers are Asia (12.4%), Africa (11.2%), America (1.0%) and Oceania (0.2%). Olive oil production is a very important economic activity, particularly for Spain, Italy and Greece; worldwide, there has been an increase in production of about 30% in the last 10 years (FAOSTAT 2009).

Multiple methods are used in the production of olive oil, resulting in different waste products. The environmental impact of olive oil production is considerable, due to the large amounts of wastewater (OMWW) mainly from the three-phase systems and solid waste. The three-phase system, introduced in the 1970s to improve extraction yield, produces three streams: pure olive oil, OMWW and a solid cake-like by-product called olive cake or *orujo*. The olive cake, which is composed of a mixture of olive pulp and olive stones, is transferred to central seed oil extraction plants where the residual olive oil can be extracted. The two-phase centrifugation system was introduced in the 1990s in Spain as an ecological approach for olive oil production since it drastically reduces the water consumption during the process. This system generates olive oil plus a semi-solid waste, known as the two-phase olive-mill waste (TPOMW) or *alpeorujo* (Alburquerque et al. 2004; McNamara et al. 2008; Morillo et al. 2009).

The olive oil industry is characterized by its great environmental impact due to the production of a highly polluted wastewater and/or a solid residue, olive skin and stone (olive husk), depending on the olive oil extraction process (Table 1) (Azbar et al. 2004).

Pressure and three-phase centrifugation systems produce substantially more OMWW than two-phase centrifugation, which significantly reduces liquid waste yet produces large amounts of semi-solid or slurry waste commonly referred to as TPOMW. The resulting solid waste is about 800 kg per ton of processed olives. This ''alpeorujo'' still contains 2.5–3.5% residual oil and about 60% water in the two-phase decanter system (Giannoutsou et al. 2004).

Production process	Inputs	Outputs
Traditional process (pressing)	Olives (1 ton)	Oil (~200 kg)
	Wash water (0.1-0.12 m3)	Solid waste (~400 kg)
		Wastewater (~600 kg)
	Energy (40-63 kWh)	-
Three-phase process	Olives (1 ton)	Oil (200 kg)
	Wash water (0.1-0.12 m3)	Solid waste (500-600 kg)
	Fresh water for decanter (0.5-1.0 m3)	Wastewater (800-950 kg)
	Water to polish the impure oil (10 kg)	-
	Energy (90-117 kWh)	-
Two-phase process	Olives (1 ton)	Oil (200 kg)
	Wash water (0.1-0.12 m3)	Solid waste (800 kg)
		Wastewater (250 kg)
	Energy (90-117 kWh)	-

Table 1. Inputs and outputs from olive oil industry (Adapted from Azbar et al. 2004)

The average amount of OMWs produced during the milling process is approximately 1000 kg per ton of olives (Azbar et al. 2004). 19.3 million tons of olive are produced annually worldwide, 15% of them used to produce olive oil (FAOSTAT 2009). As an example of the scale of the environmental impact of OMWW, it should be noted that 10 million m^3 per year of liquid effluent from three-phase systems corresponds to an equivalent load of the wastewater generated from about 20 million people. Furthermore, the fact that most olive oil is produced in countries that are deficient in water and energy resources makes the need for effective treatment and reuse of OMWW (McNamara et al. 2008). Overall, about 30 million tons of OMWs per year are produced in the world that could be used as renewable negative or low-cost substrates.

4. Microbial biotechnology applications in olive oil industry

Microbial biotechnology applications in olive oil industry, mainly attempts to obtain added-value products from OMWs are summarised in Fig. 1. OMWs could be used as renewable low-cost substrate for industrial and agricultural microbial biotechnology as well as for the production of energy.

The chemical oxygen demand (COD) and biological oxygen demand (BOD) reduction of OMWs with a concomitant production of biotechnologically valuable products such as enzymes (lipases, ligninolytic enzymes), organic acids, biopolymers and biodegradable plastics, biofuels (bioethanol, biodiesel, biogas and biohydrogen), biofertilizers and amendments will be review.

4.1 Olive mill wastes biological treatment

Ironically, while olive oil itself provides health during its consumption, its by-products represent a serious environmental threat, especially in the Mediterranean, region that accounts for approximately 95% of worldwide olive oil production.

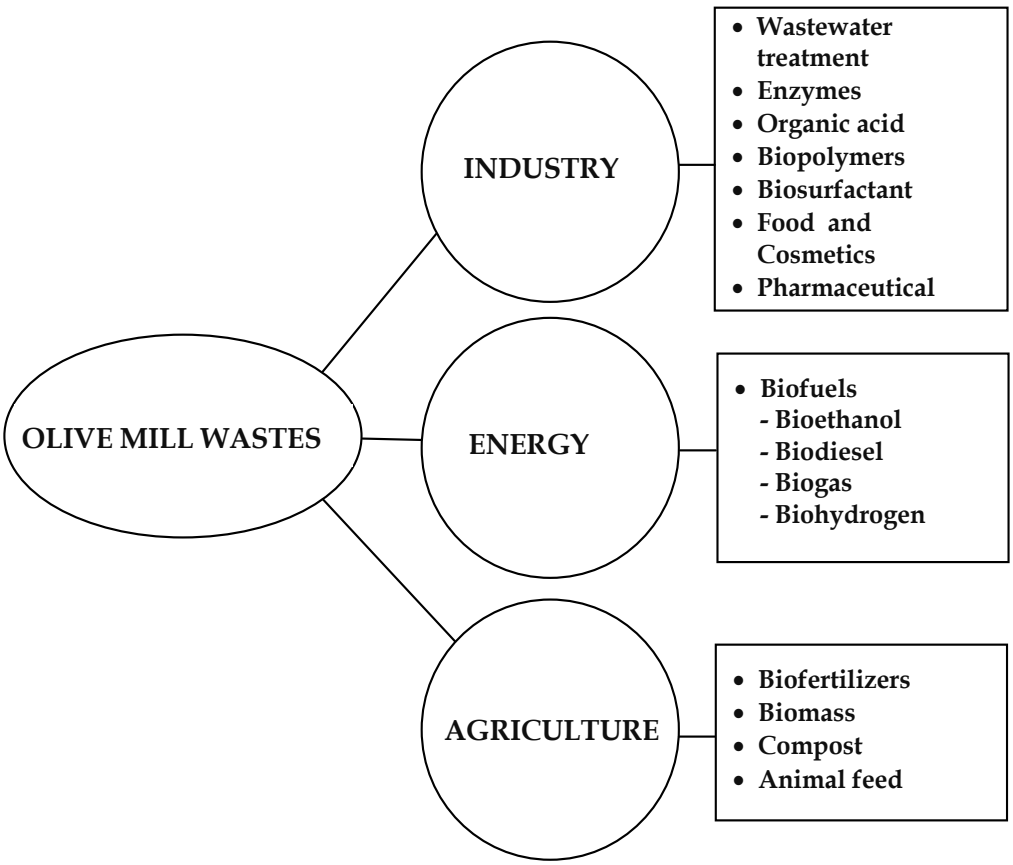

Fig. 1. Potential uses of olive mill wastes in microbial biotechnology.

Moreover, olive oil production is no longer restricted to the Mediterranean basin, and new producers such as Australia, USA and South America will also have to face the environmental problems posed by OMWs. The management of wastes from olive oil extraction is an industrial activity submitted to three main problems: the generation of waste is seasonal, the amount of waste is enormous and there are various types of olive oil waste (Giannoutsou et al. 2004).

OMWs have the following properties: dark brown to black colour, acidic smell, a high organic load and high C/N ratio (chemical oxygen demand or COD) values up to 200 g per litre, a chemical oxygen demand/biological oxygen demand (COD/BOD5) ratio ranging from 2.5 to 5.0, indicating low biodegrability, an acidic pH of between 4 and 6, high concentration of phenolic substances 0.5–25 g per litre with more than 30 different phenolic compounds and high content of solid matter. The organic fraction contains large amounts of proteins (6.7–7.2%), lipids (3.76–18%) and polysaccharides (9.6–19.3%), and also phytotoxic components that inhibit microbial growth as well as the germination and vegetative growth of plants (Roig et al. 2006; McNamara et al. 2008).

OMWs treatment processes tested employ physical, chemical, biological and combined technologies. Several disposal methods have been proposed to treat OMWs, such as traditional decantation, thermal processes (combustion and pyrolysis), agronomic applications (e.g. land spreading), animal-breeding methods (e.g. direct utilisation as animal feed or following protein enrichment), physico-chemical treatments (e.g. precipitation/flocculation, ultrafiltration and reverse osmosis, adsorption, chemical oxidation processes and ion exchange), extraction of valuable compounds (e.g. antioxidants, residual oil, sugars), and biological treatments (Morillo et al. 2009).

Among the different options, biological treatments or bioremediation are considered the most environmentally compatible and the least expensive (Mantzavinos and Kalogerakis 2005). Bioremediation is a treatment process employing naturally microorganisms (bacteria and fungi like yeasts, molds and mushrooms) to break down, or degrade, hazardous substances into less toxic or non-toxic substances. Bioremediation technologies can be classified as *in-situ* (bioaugmentation, bioventing, biosparging) or *ex-situ* (bioreactors, landfarming, composting and biopiles). *In-situ* bioremediation treats the contaminated water or soil where it was found, whereas *ex-situ* bioremediation processes involve removal of the contaminated soil or water to another location prior to treatment (Arvanitoyannis et al. 2008).

Bioremediation occurs either under aerobic or anaerobic conditions. Many aerobic biological processes, technologies and microorganisms have been tested for the treatment of OMWs, aimed at reducing organic load, dark colour and toxicity of the effluents (Table 2). In general, aerobic bacteria appeared to be very effective against some low molecular mass phenolic compounds but are relatively ineffective against the more complex polyphenolics responsible for the dark colouration of OMWs (McNamara et al. 2008). A number of different species of bacteria, yeasts, molds and mushrooms have been tested in aerobic processes to treat OMWs that are listed (Table 2).

A number of studies have utilized bacterial consortia for bioremediation of OMWW. Bioremediation of OMWW using aerobic consortia has been quite successful in these studies, achieving significant reductions in COD (up to 80%) and the concentration of phytotoxic compounds, and complete removal of some simple phenolics (Zouari and Ellouz 1996; Benitez et al. 1997). A combined bacterial–yeast system of *Pseudomonas putida* and *Yarrowia lipolytica* were used to degrade OMWW (De Felice et al. 1997).

Anaerobic bioremediation of OMWs has employed, almost exclusively, uncharacterized microbial consortia derived from municipal and other waste facilities. This technique presents a number of advantages in comparison to the classical aerobic processes: (a) a high degree of purification with high-organic-load feeds can be achieved; (b) low nutrient requirements are necessary; (c) small quantities of excess sludge are usually produced; and (d) a combustible biogas is generated (Dalis et al. 1996; Zouari and Ellouz 1996; Borja et al. 2006). Combined aerobic–anaerobic systems have also been used effectively in the bioremediation of OMWs (Hamdi and Garcia 1991; Borja et al. 1995). Aerobic processes are applied waste streams of OMWs with low organic loads, whereas anaerobic processes are applied waste streams with high organic loads.

Microorganism	Waste type	Method	Results	Reference
Bacteria				
Azotobacter vinelandii	OMWW	Culture in OMWW	70% COD reduction	(Piperidou et al. 2000)
Bacillus pumilus	OMWW	Culture in OMWW	50% phenol reduction	(Ramos-Cormenzana et al. 1996)
Lactobacillus paracasei	OMWW	Culture in OMWW with cheese whey's	47% colour removal 22.7% phenol reduction	(Aouidi et al. 2009)
Lactobacillus plantarum	OMWW	Culture in OMWW	Increase of simple polyphenols content	(Kachouri and Hamdi 2004)
Pseudomonas putida and *Ralstonia* spp.	OMWW	Culture two strains in OMWW	Biodegradation of aromatic compounds	(Di Gioia et al. 2001)
Yeasts				
Candida boidinii	TPOMW	Fed-batch microcosm	57.7% phenol reduction	(Giannoutsou et al. 2004)
Candida cylindracea	OMWW	Culture in OMWW	reduction of phenolic compounds and COD	(Gonçalves et al. 2009)
Candida holstii	OMWW	Culture in OMWW	39% phenol reduction	(Ben Sassi et al. 2008)
Candida oleophila	OMWW	Bioreactor batch culture with OMWW	Tannins content reduction	(Peixoto et al. 2008)
Candida rugosa	OMWW	Culture in OMWW	reduction of phenolic compounds and COD	(Gonçalves et al. 2009)
Candida tropicalis	OMWW	Culture in OMWW	62.8% COD reduction 51.7% phenol reduction	(Fadil et al. 2003)
Geotrichum candidum	OMWW	Culture in bioreactors with OMWW	60% COD reduction	(Asses et al. 2009)
Geotrichum candidum	TPOMW	Fed-batch microcosm	57% phenol reduction	(Giannoutsou et al. 2004)
Saccharomyces spp.	TPOMW	Fed-batch microcosm	61% phenol reduction	(Giannoutsou et al. 2004)
Trichosporon cutaneum	OMWW	Culture in OMWW	removal of mono- and polyphenols	(Chtourou et al. 2004)
Yarrowia lipolytica	OMWW	Culture in OMWW	20-40% COD reduction < 30% phenol reduction	(Lanciotti et al. 2005)
Yarrowia lipolytica W29	OMWW	Culture in OMWW	67-82% COD reduction	(Wu et al. 2009)
Molds				
Aspergillus niger	OMWW	Culture in OMWW	73% COD reduction 76% phenol reduction	(García García et al. 2000)
Aspergillus spp.	OMWW	Culture in OMWW	52.5% COD reduction 44.3% phenol reduction	(Fadil et al. 2003)
Aspergillus terreus	OMWW	Culture in OMWW	63% COD reduction 64% phenol reduction	(García García et al. 2000)
Fusarium oxysporum	DOR	Culture in DOR	16-71% phytotoxicity reduction	(Sampedro et al. 2007a)
Penicillium spp.	OMWW	Culture in OMWW	38% COD reduction 45% phenol reduction	(Robles et al. 2000)

Microorganism	Waste type	Method	Results	Reference
Phanerochaete chrysosporium	OMWW	Culture in bioreactors with OMWW	75% COD reduction 92% phenol reduction	(García García et al. 2000)
Phanerochaete chrysosporium	TPOMW	Culture in TPOMW	9.2% TOC reduction 14.5% phenol reduction	(Sampedro et al. 2007b)
Phanerochaete flavido-alba	OMWW	Culture in bioreactors with OMWW	52% phenol reduction	(Blánquez et al. 2002)
Phanerochaete flavido-alba	TPOMW	Solid-state culture	70% phenol reduction	(Linares et al. 2003)
Mushrooms				
Coriolopsis rigida	TPOMW	Culture in OMWW	9% TOC reduction 89% phenol reduction	(Sampedro et al. 2007b)
Coriolopsis polyzona	OMWW	Culture in OMWW	75% colour removal	(Jaouani et al. 2003)
Coriolus versicolor	OMWW	Culture in OMWW	65% COD reduction 90% phenol reduction	(Yesilada et al. 1997)
Funalia trogii	OMWW	Culture in OMWW	70% COD reduction 93% phenol reduction	(Yesilada et al. 1997)
Lentinula edodes	OMWW	Culture in OMWW	65% COD reduction 88% phenol reduction	(D'Annibale et al. 2004)
Lentinus tigrinus	OMWW	Culture in OMWW	Effective in decolorization	(Jaouani et al. 2003)
Pleurotus eryngii	OMWW	Culture in OMWW	> 90% phenol reduction	(Sanjust et al. 1991)
Pleurotus floridae	OMWW	Culture in OMWW	> 90% phenol reduction	(Sanjust et al. 1991)
Pleurotus ostreatus	OMWW	Culture in OMWW	100% phenol reduction	(Tomati et al. 1991)
Pleurotus ostreatus	OMWW	Culture in bioreactors with OMWW	Phenol reduction nearly complete	(Aggelis et al. 2003)
Pleurotus ostreatus	OMWW	Solid-state culture	67% phenol reduction	(Fountoulakis et al. 2002)
Pleurotus ostreatus	TPOMW	Plastic bag	22% TOC reduction 90% phenol reduction	(Saavedra et al. 2006)
Pleurotus pulmonarius	TPOMW	Culture in TPOMW	9.7% TOC reduction 66.2% phenol reduction	(Sampedro et al. 2007b)
Pleurotus sajor-caju	OMWW	Culture in OMWW	> 90% phenol reduction	(Sanjust et al. 1991)
Pleurotus spp.	OMWW	Culture in OMWW	76% phenol reduction	(Tsioulpas et al. 2002)
Phlebia radiata	TPOMW	Culture in TPOMW	13% TOC reduction 95.7% phenol reduction	(Sampedro et al. 2007b)
Poria subvermispora	TPOMW	Culture in TPOMW	13.2% TOC reduction 72.3% phenol reduction	(Sampedro et al. 2007b)
Pycnoporus cinnabarinus	TPOMW	Culture in TPOMW	7.6% TOC reduction 88.7% phenol reduction	(Sampedro et al. 2007b)
Pycnoporous coccineus	OMWW	Culture in OMWW	Effective in decolorization	(Jaouani et al. 2003)

OMWW: olive oil wastewater, TPOMW: two-phase olive-mill waste, COD: chemical oxygen demand, TOC: Total organic carbon, DOR: olive-mill dry residue.

Table 2. Aerobic treatment of OMWs by microorganisms

In general, available scientific information shows that fungi are more effective than bacteria at degrading both simple phenols and the more complex phenolic compounds present in olive-mill wastes. For example, several species of the genus *Pleurotus* were found to be very effective in the degradation of the phenolic substances present in OMWs (Hattaka 1994). For OMWs biotreatment in large-scale, the use of filamentous fungi have considerable problems because of the formation of fungal pellets and other aggregations. The use of yeast in bioreactors could be a way forward to overcome this limitation.

4.2 Enzymes

In recent years, many researchers have utilized OMWs as growth substrates for microorganisms, obtaining a reduction of the COD level, together with enzyme production. The addition of nutrients can modify the pattern of degrading enzymes production by specific microorganisms from OMWs. (De la Rubia et al. 2008).

Lipases (EC 3.1.1.3) are among the most important classes of industrial enzymes (Darvishi et al. 2009). Many microorganisms are known as potential producers of lipases including bacteria, yeast, and fungi. Several reviews have been published on microbial lipases (Arpigny and Jaeger 1999; Vakhlu and Kour 2006; Treichel et al. 2010).

Lipolytic fungal species, such as *Aspergillus oryzae*, *Aspergillus niger*, *Candida cylindracea*, *Geotrichum candidum*, *Penicillium citrinum*, *Rhizopus arrhizus* and *Rhizopus oryzae* were preliminarily screened for their ability to grow on undiluted OMWW and to produce extracellular lipase. A promising potential for lipase production was found by *C. cylindracea* NRRL Y-17506 on OMWW (D'Annibale et al. 2006).

Among the different yeasts tested, the *Y. lipolytica* most adapted to grow on OMW. the *Y. lipolytica* strains were produced 16-1041 U/L of lipase on OMWs and also reduced 1.5-97% COD, 80% BOD and 0-72% phenolic compounds of OMWs (Fickers et al. 2011). The yeasts *Saccharomyces cerevisiae* and *Candida wickerhamii* produce β-glucosidase enzyme to hydrolyse oleuropein present in olive oil (Ciafardini and Zullo 2002).

Olive oil cake (OOC) used as a substrate for phytase production in solid-state fermentation using three strains of fungus *Rhizopus* spp. OOC of initial moisture 50% was fermented at 30°C for 72 hours and inoculated with *Rhizopus oligosporus* NRRL 5905, *Rhizopus oryzae* NRRL 1891 and *R. oryzae* NRRL 3562. The results indicated that all three *Rhizopus* strains produced very low titers of enzyme on OOC (Ramachandran et al. 2005).

Tannase could be utilized as an inhibitor of foam in tea production, clarifying agent in beer and fruit juices production, in the pharmaceutical industry and for the treatment of tannery effluents. *Aspergillus niger* strain HA37, isolated from OMW, was incubated on a synthetic medium containing tannic acid and on diluted OMW on a rotary shaker at 30°C. On the medium containing tannic acid, tannase production was 0.6, 0.9 and 1.5 U/ml at 0.2%, 0.5% and 1% initial tannic acid concentration, respectively (Aissam et al. 2005).

Extracellular ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) were produced by the white rot fungus *Phanerochaete flavido-alba* with a concomitant decoloration and decrease in phenolic content and toxicity of OMWW. Laccase was the sole ligninolytic enzyme detected in cultures containing monomeric aromatic compounds. Laccase and an acidic manganese-dependent peroxidase (MnPA, pI

62.8) were accumulated in cultures with OMWW or polymeric pigment. Furthermore, modified manganese-dependent peroxidases were observed mainly in OMWW-supplemented cultures. Laccase was more stable to the effect of OMWW toxic components and was accumulated in monomeric aromatic-supplemented cultures, suggesting a more important role than manganese-dependent peroxidases in OMWW detoxification. Alternatively, MnPA accumulated in cultures containing the polymeric pigment seemed to be more essential than laccase for degradation of this recalcitrant macromolecule by *P. flavido-alba*. (Ruiz et al. 2002).

Enzyme laccase, produced by fungus *Pycnoporus coccineus*, is responsible for OMWW decolorization and decrease COD and phenolic compounds. The highest laccase level was 100 000 U/l after 45 incubation-days. The enzyme was stable at pH 7, at room temperature and showed a half-life of 8 and 2 h at 50 and 60°C, respectively (Jaouani et al. 2005). In order to decolourise OMWW efficiently, production and differential induction of ligninolytic enzymes by the white rot *Coriolopsis polyzona*, were studied by varying growth media composition and/or inducer addition (Jaouani et al. 2006). The production of lignin peroxidase (LiP), manganese peroxidase (MnP) and lipases by *Geotrichum candidum* were performed in order to control the decolourisation and biodegradation of OMWW (Asses et al. 2009).

Sequential batch applications starting with adapted *Trametes versicolor* FPRL 28A INI and consecutive treatment with *Funalia trogii*, possible to remove significant amount of total phenolics content and higher decolorization as compared to co-culture applications. Also highest laccase and manganese peroxidase acitivities were obtained with *F. trogii* (Ergul et al. 2010).

4.3 Organic acids

Some *Y. lipolytica* strains are good candidates for the reduction of the pollution potential of OMWW and for the production of enzymes and metabolites such as lipase and citric acid (Lanciotti et al. 2005). *Y. lipolytica* strain ACA-DC 50109 demonstrated efficient growth on media containing mixtures of OMWs and commercial glucose. In nitrogen-limited diluted and enriched with high glucose quantity OMWW, a noticeable amount of total citric acid was produced. The ability of *Y. lipolytica* to grow on relatively high phenolic content OMWs based media and produce in notable quantities citric acid, make this non-conventional yeast worthy for further investigation (Papanikolaou et al. 2008).

The biochemical behavior and simultaneous production of valuable metabolites such as lipase, citric acid (CA), isocitric acid (ICA) and single-cell protein (SCP) were investigate by *Y. lipolytica* DSM 3286 grown on various plant oils as sole carbon source. Among tested plant oils, olive oil proved to be the best medium for lipase and CA production. The *Y. lipolytica* DSM 3286 produced 34.6 ± 0.1 U/ml of lipase and also CA, ICA and SCP as by-product on olive oil medium supplemented with yeast extract. Urea, as organic nitrogen, was the best nitrogen source for CA production. The results of this study suggest that the two biotechnologically valuable products, lipase and CA, could be produced simultaneously by this strain using renewable low-cost substrates such as plant oils in one procedure (Darvishi et al. 2009).

In the other study, a total of 300 yeast isolates were obtained from samples of agro-industrial wastes, and M1 and M2 strains were investigated for their ability to produce lipase and

citric acid. Identification tests showed that these isolates belonged to the species *Y. lipolytica*. M1 and M2 strains produced maximum levels of lipase on olive oil, and high levels of citric acid on citric acid fermentation medium (Mafakher et al. 2010).

The highest oxalic acid quantity (5 g/l) was obtained by the strain *Aspergillus* sp. ATHUM 3482 on waste cooking olive oil medium. For strain *Penicillium expansum* NRRL 973 on this medium sole organic acid detected was citric acid with maximum concentration achieved 3.5 g/l (Papanikolaou et al. 2011).

4.4 Biopolymers and biodegradable plastics

Exopolysaccharides (EPSs) often show clearly identified properties that form the basis for a wide range of applications in food, pharmaceuticals, petroleum, and other industries. The production of these microbial polymers using OMWW as a low-cost fermentation substrate has been proposed (Ramos-Cormenzana et al. 1995). This approach could reduce the cost of polymer production because the substrate is often the first limiting factor. Moreover, OMWW contains free sugars, organic acids, proteins and other compounds such as phenolics that could serve as the carbon source for polymer production, if the chosen microorganism is able to metabolize these compounds (Fiorentino et al. 2004).

Xanthan gum, an extracellular heteropolysaccharide produced by the bacterium *Xanthomonas campestris* has been obtained from OMWW. Growth and xanthan production on dilute OMWW as a sole source of nutrients were obtained. Addition of nitrogen and/or salts led to significantly increased xanthan yields with a maximum of 7.7g/l (Lopez and Ramos-Cormenzana 1996).

The fungus *Botryospheria rhodina* has been used for the production of β-glucan from OMWW with yield of 17.2 g/l and a partial dephenolisation of the substrate (Crognale et al. 2003). A metal-binding EPS produced by *Paenibacillus jamilae* from OMWs. Maximum EPS production (5.1 g/l) was reached in batch culture experiments with a concentration of 80% of OMWW as fermentation substrate (Morillo et al. 2007).

Polyhydroxyalkanoates (PHAs) are reserve polyesters that are accumulated as intracellular granules in a variety of bacteria. Of these polymers, poly-β-hydroxybutyrate (PHB) is the most common. Since the physical properties of PHAs are similar to those of some conventional plastics, the commercial production of PHAs is of interest. However, these biodegradable and biocompatible 'plastics' are not priced competitively at the present, mainly because the sugars (i.e. glucose) used as fermentation feed-stocks are expensive. Finding a less expensive substrate is, therefore, a major need for a wide commercialisation of these products. Large amounts of biopolymers containing β-hydroxybutyrate (PHB) and copolymers containing β-hydroxyvalerate (P[HB-co-HV]) are produced by *Azotobacter chroococcum* in culture media amended with alpechin (wastewater from olive oil mills) as the sole carbon source (Pozo et al. 2002).

4.5 Biosurfactants

Rhamnolipids, typical biosurfactants produced by *Pseudomonas aeruginosa*, consist of either one or two rhamnose molecules, linked to one or two fatty acids of saturated or unsaturated alkyl chain between C8 and C12. The *P. aeruginosa* 47 T2 produced two main rhamnolipid

homologs, (Rha-C10-C10) and (Rha-Rha-C10-C10), when grown in olive oil waste water or in waste frying oils consisting from olive/sunflower (Pantazaki et al. 2010).

4.6 Food and cosmetics

A few edible fungi, especially species of *Pleurotus*, can also be grown using OMWs as the source of nutrients by the application of different strategies. Recently the cultivation of the oyster mushroom *Pleurotus ostreatus* was suggested on OMWW (KalmIs et al. 2008).

Hydroxy fatty acids (HFAs) are known to have special properties such as higher viscosity and reactivity compared to other normal fatty acids. These special properties used in a wide range of applications including resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings. Some HFAs are also reported as antimicrobial agents against plant pathogenic fungi and some of food-borne bacteria. Bacterium *Pseudomonas aeruginosa* PR3 produce several hydroxy fatty acids from different unsaturated fatty acids. Of those hydroxy fatty acids, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD) was efficiently produced from oleic acid by strain PR3. DOD production yield from olive oil was 53.5%. Several important environmental factors were also tested. Galactose and glutamine were optimal carbon and nitrogen sources, and magnesium ion was required for DOD production from olive oil (Suh et al. 2011).

4.7 Pharmaceutical

The enhancing effect of various concentrations of 18 oils and a silicon antifoam agent on erythromycin antibiotic production by *Saccharopolyspora erythraea* was evaluated in a complex medium containing soybean flour and dextrin as the main substrates. The highest titer of erythromycin was produced in medium containing 55 g/l black cherry kernel oil (4.5 g/l). The titers of erythromycin in the other media were also recorded, with this result: black cherry kernel > water melon seed > melon seed > walnut > rapeseed > soybean > (corn = sesame) > (olive = pistachio = lard = sunflower) > (hazelnut = cotton seed) > grape seed > (shark = safflower = coconut). In medium supplement with olive oil, concentration of erythromycin was 2.15±0.03 and 2.75±0.02 g/l before and after optimization, respectively (Hamedi et al. 2004).

4.8 Biofuels

It is widely recognised that clean and sustainable technologies, e.g. biofuels, are only part of the solution to the impending energy crisis. Comparing the heating value of biohydrogen (121 MJ/kg), methane (50.2 MJ/kg) and bioethanol (23.4 MJ/kg), the production of hydrogen will be more attractive. Nevertheless, the use of biohydrogen is still not practical and thus there is a higher demand for methane and bioethanol because they can be used directly as biofuels with the existing technology (Duerr et al. 2007).

Ethanol production as a biofuel from OMWs with high content of organic matter is interesting (Li et al. 2007). The two main components of TPOMW (stones and olive pulp) as substrates were used to production of ethanol by a simultaneous saccharification and fermentation process (Ballesteros et al. 2001). In recent study, an enzymatic hydrolysis and subsequent glucose fermentation by baker's yeast were evaluated for ethanol production

using dry matter of TPOMW. The results showed that yeasts could effectively ferment TPOMW without nutrient addition, resulting in a maximum ethanol production of 11.2 g/l and revealing the tolerance of yeast to TPOMW toxicity (Georgieva and Ahring 2007).

Anaerobic digestion is a biological process in which organic material is broken down by microorganisms. Unlike composting, the process occurs in the absence of air. Anaerobic digestion is a practical alternative for the treatment of TPOMW, which produces biogas. The TPOMW is biodegradable by anaerobic digestion at mesophilic temperatures in stirred tank reactors, with COD removal efficiencies in the range of 72–89% and an average methane yield coefficient of 0.31 dm^3 CH_4 per gramme COD removed. Hydrogen production was coupled with a subsequent step for methane production, giving the potential for production of 1.6 mmol H_2 per gramme of TPOMW (Borja et al. 2006).

The OMW used as a sole substrate for the production of hydrogen gas with *Rhodobacter sphaeroides* O.U.001. The bacterium was grown in diluted OMW media, containing OMW concentrations between 20% and 1% in a glass column photobioreactor at 32°C. The released gas was nearly pure hydrogen, which can be utilized in electricity producing systems, such as fuel cells. The maximum hydrogen yield (145 ml) was obtained with 3% and 4% OMW concentrations. However, as well as hydrogen production, COD, BOD and phenol reduction from OMW were recorded (Eroglu et al. 2004).

Biodiesel, a fuel that can be made from renewable biological sources such as vegetable oils or animals fats, has been recognized recently as an environment friendly alternative fuel for diesel engines. Among liquid biofuels, biodiesel derived from vegetable oils is gaining ground and market share as diesel fuel in Europe and the USA. A mixture of frying olive oil and sunflower oil for the production of methyl esters that can be used as biodiesel (Encinar et al. 2005).

4.9 Biofertilizers

As far as agronomic use of the waste is concerned, the idea of re-using microbially treated OMWW as fertiliser has been also proposed. An acidogenic fungus strain *Aspergillus niger* was grown in either free or immobilised form on OMWW with rock phosphate added in order to solubilise it. It was found that at optimized process conditions (moisture 70%; corn steep liquor as a nitrogen source; inoculum size of 3-4 ml; presence of slow release phosphate), the filamentous fungal culture was able to produce 58 U phytase/g dry substrate and 31 mg soluble phosphate per flask (Vassilev et al. 1997; Vassilev et al. 2007).

4.10 Biomass

Already 50 years ago, the production of yeast biomass using OMWW in a chemostat for use in industrial applications was reported. The microbial biomass produced from OMW fermentations either as an additive to animal feed or to improve its agronomic use. For example, an intense degradation of most polluting substances of OMWW and the production of biomass could be used as an animal feed integrator using a chemical–biological method (Morillo et al. 2009).

Seven strains of *Penicillium* isolated from OMWW disposal ponds were tested for biomass production and biodegradation of undiluted OMWW. Best results were obtained by using

strain P4, which formed 21.50 g (dry weight) of biomass per litre of undiluted wastewater after 20 days of cultivation. This and other strains also carried out an outstanding reduction of the COD and the phenolic content of OMW, as well as a pH raise (Robles et al. 2000). The *Y. lipolytica* strain ATCC 20255 strain has been effective in the treatment of OMWW that yield of the biomass (single-cell protein) was 22.45 g/l (Scioli and Vollaro 1997).

Microalgal biomass is as a potential source of proteins, carbohydrates, pigments, lipids, and hydrocarbons. In addition, the biomass can be used as a low-release fertilizer. This chemical composition has great variation, depending on the species, culture medium, and the operating conditions. Microalga *Scenedesmus obliquus* was used to biomass production from rinse water (RW) from two-phase centrifugation in the olive-oil extraction industry. Maximum specific growth rate, 0.044 per hour was registered in the culture with 5% RW and reduces 67.4% BOD when operating with 25% RW. The greater specific rate of protein synthesis during the exponential phase was 3.7 mg/g h to 50% RW (Hodaifa et al. 2008).

Microbial lipid (single cell oil or SCO) production has been an object of research and industrial interest for more than 60 years. Microorganisms can store triacylglycerol (TAG) as intracellular oil droplets. *Gordonia* sp. DG accumulated more than 50% lipid with most tested wastes, while only 29, 36 and 41% was accumulated in presence of olive mill waste, hydrolyzed barely seeds and wheat bran, respectively (Gouda et al. 2008).

Carbon-limited cultures were performed on waste cooking olive oil, added in the growth medium at 15 g/l, and high biomass quantities were produced up to 18 g/l. Cellular lipids were accumulated in notable quantities in almost all cultures. *Aspergillus* sp. ATHUM 3482 accumulated lipid up to 64% (w/w) in dry fungal mass. In parallel, extracellular lipase activity was quantified, and it was revealed to be strain and fermentation time dependent, with a maximum quantity of 645 U/ml being obtained by *Aspergillus niger* NRRL 363. Storage lipid content significantly decreased at the stationary growth phase (Papanikolaou et al. 2011).

4.11 Compost

Composting is the aerobic processing of biologically degradable organic waste to produce a reasonably stable, granular material and valuable plant nutrients. Composting removes the phytotoxicity of the residues within a few weeks and allows the subsequent enrichment of croplands with nutrients that were originally taken up by olive tree cultivation. Composting of OMWs requires the proper adjustment of pH, temperature, moisture, oxygenation and nutrients, thereby allowing the adequate development of the microbial populations (Arvanitoyannis and Kassaveti 2007).

Among the possible technologies for recycling the TPOMW, composting is gaining interest as a sustainable strategy to recycle this residue for agricultural purposes. Dry olive cake alone or mixed with municipal biosolids vermicomposted for 9 months in order to examine the behaviour of three specific humic substance-enzyme complexes. During the process, β-glucosidase synthesis and release was observed, whereas no significant change in urease and phosphatase activity was recorded. The vermicomposted olive cake, alone or in blends with biosolids, could be effectively used as amendment due to their ability to reactivate the C, P and N-cycles in degraded soils for regeneration purposes (Benitez et al. 2005).

Olive pomace, a wet solid waste from the three-phase decanters and presses, was composted by using a reactor for a period of 50 days in four bioreactors. Urea was added to

adjust C/N ration between 25-30. At the end of 50 days of composting using *Trichoderma harzianum* and *Phanerochaete chrysosporium*, cellulose and lignin were highly degraded. It was found that after 30 days, *P. chrysosporium* and *T. harzianum* degraded approximately 71.9% of the lignin and 59.25% of the cellulose, respectively (Haddadin et al. 2009).

4.12 Animal feed

Treated OMW may find applications as a raw material in various biotechnological processes or as animal food. The appropriate utilization of by-products in animal nutrition can improve the economy and the efficiency of agricultural, industrial and animal production.

The olive pomace was alkali-treated, transferred to culture flasks and inoculated with the above fungi. After inoculation, the fermentation process was carried out at 25°C for 60 days. The results indicated that *Oxysporus* spp. degraded lignin up to 69%, whereas *Phanerochaete chrysosporium* and *Schizophyllum commune* delignified olive pomace 60% and 53%, respectively. However, the potential use of treated olive pomace as a feed for poultry is still under investigation. The fermented olive pomace can be used as a feed for the poultry industry (Haddadin et al. 2002).

5. Conclusion

The olive oil industry generates large amounts of olive mill wastes (OMWs) as by-products that are harmful to the environment. About 30 million tons of OMWs per year are produced in the world. Thus, more research is needed on the development of new bioremediation technologies and strategies of OMWs, as well as the valorisation by microbial biotechnology. The fermentation of fatty low-value renewable carbon sources like OMWs aiming at the production of various added-value metabolites is a noticeable interest in the sector of industrial microbiology and microbial biotechnology.

Microbiological studies show that presence of yeasts, but not of bacteria and moulds in the olive oil. Some of the yeasts are considered useful as they improve the organoleptic characteristics of the oil during preservation, whereas others are considered harmful as they can damage the quality of the oil through the hydrolysis of the triglycerides. Olive oil and its by-products could provide a source of low-cost fermentation substrate and isolation of new microorganisms with biotechnological potentials.

OMWs treatment processes that employ physical, chemical, biological and combined technologies have been tested. Among the different options, biological treatments or bioremediation are considered the most environmentally compatible and the least expensive. Bioremediation occurs either under aerobic or anaerobic conditions. Aerobic processes are applied waste streams of OMWs with low organic loads, whereas anaerobic processes are applied waste streams with high organic loads.

Microbial biotechnology strategies and methods in olive oil industry were used to reduce chemical oxygen demand (COD), biological oxygen demand (BOD) and phenolic compounds of OMWs with a concomitant production of biotechnologically valuable products such as enzymes (lipases, β-glucosidase, phytase, tannase, lignin peroxidase, manganese peroxidise, laccase and pectinases), organic acids (citric, isocitric and oxalic acids), biopolymers and biodegradable plastics (xanthan, β-glucan and polyhydroxyalkanoates), biosurfactants, food

and cosmetics, pharmaceutical, biofuels (bioethanol, biogas, biohydrogen and biodiesel), biofertilizers and amendments, biomass (single cell proteins, single cell oil), compost and animal feed.

What has been discussed in this review indicate that microbial biotechnology can be used for the production of value-added products from olive oil by-products and can facilitate a significant reduction in waste treatment costs.

6. References

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Part 3

Bioavailability and Biological Properties of Olive Oil Constituents

18

Metabolism and Bioavailability of Olive Oil Polyphenols

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1. Introduction

The significance of virgin olive oil (VOO), hinged to its many virtues in both gastronomy and health, is nowadays undeniable. Their protective effects are attributed to its high content of monounsaturated fatty acids and to the presence of some minor components, which add up to 2% of the weight. Among its several minor constituents, polar phenolic compounds, usually characterized as polyphenols, have become the subject of intensive research because of their biological activities, their influence on the organoleptic properties of VOO and their contribution to its oxidative stability (Bendini et al., 2007).

The phenolic fraction of VOO consists of a heterogeneous mixture of compounds belonging to several families with varying chemical structures. A brief description of the main classes of phenolic compounds contained in VOO is given below:

- *Phenolic acids.* There are two main series of these acids, depending on the carbon skeleton: benzoic acids (C6-C1: 3-hydroxybenzoic, *p*-hydroxybenzoic, protocatechuic, gentisic, vanillic, syringic and gallic acids) and cinnamic acids (C6-C3: *o*-coumaric, *p*-coumaric, caffeic, ferulic and sinapic acids).
- *Phenolic alcohols.* The two most important in VOO are hydroxytyrosol (Hyty) and tyrosol (Ty), although two Hyty derivatives, its acetate and its glucoside, can be also found. Hyty and Ty only differ in a hydroxyl group in the *meta* position.
- *Secoiridoids.* They are present exclusively in plants of the Olearaceae family. The olives mainly contain the polar oleuropein (Ol) and ligstroside (Lig) glycosides. Ol is the ester of elenolic acid (EA) with Hyty, and Lig is the ester of EA with Ty. Ol and Lig aglycones (Ol Agl and Lig Agl, respectively) are formed by removal of the glucose moiety from glycosides by endogenous β-glucosidases during ripening, oil extraction and storage.
- *Lignans*. (+)-1-Pinoresinol, (+)-1-hydroxypinoresinol and (+)-1-acetoxypinoresinol are the most reported compounds in olive oil.
- *Flavonoids.* The main flavonoids present in VOO are apigenin and luteolin, which are originated from their corresponding glucosides present in the drupe.

The qualitative and quantitative composition of VOO hydrophilic phenols is strongly affected by the agronomic and technological conditions of production (Servili et al., 2004). Among agronomic parameters, the cultivar, the fruit ripening degree, the agronomic

techniques used and the pedoclimatic conditions are the aspects more extensively studied (Tovar et al., 2001; Uceda et al., 1999). Moreover, by modulating technology, it is possible to some extent to optimize the transfer of some polar minor constituents into the oil or reduce their level (Boskou, 2009). The influence of variety, extraction system, ripening degree and storage in the polyphenolic content of a VOO has been extensively discussed in the literature (Boskou, 2009; Uceda et al., 1999).

Wide ranges of total polar phenols concentration have been reported in olive oils (50-1000 mg/kg), although the most usual value is found between 100-350 mg/kg (Boskou et al., 2006). In general, the most abundant phenolic compounds in VOO are aglycones deriving from secoiridoids. Trying to establish levels of individual phenols, Servili & Montedoro (2002) calculated average values of 7 phenolic compounds from a considerable number of samples of industrial olive oils. They concluded that Hyty and Ty were found only in trace amounts (less than 10 mg/kg oil) and the most abundant phenols were decarboxylated Ol Agl (63-840 mg/kg), Ol Agl (85–310 mg/kg), and decarboxylated Lig Agl (15-33 mg/kg). Brenes et al. (2002) published values ranging from 3-67 mg/kg for 1-acetoxypinoresinol, and from 19-41 mg/kg for pinoresinol in 5 Spanish olive oils, data that can be completed with the researches carried out by Romero et al. (2002) and Tovar et al. (2001). Levels of luteolin have been found to be around 10 mg/kg in some Spanish olive oils (Brenes et al., 1999) or ranging between 0.2-7 mg/kg for Greek oils (Murkovic et al., 2004). Carrasco-Pancorbo et al. (2006) developed a method to quantify 14 individual phenols belonging to different families in 7 Spanish extra-virgin olive oils (EVOOs). They also quantified them, finding the following contents (mg/kg): simple phenols: 6.8-11.5; complex phenols: 70.5-799.5; lignans: 0.81-20.6; and flavonoids: 1.4-8.6.

Intake of olive oil in the Mediterranean countries is estimated to be 30–50 g/day, based on the per capita olive oil consumption of 10–20 kg/year in Greece, Italy and Spain (Boskou, 2000; Food and Agricultural Organization, 2000). A daily consumption of 50 g olive oil with a concentration of 180 mg/kg of phenols would result in an estimated intake of about 9 mg of olive oil phenols per day (de la Torre, 2008; Vissers et al., 2004), of which at least 1 mg is derived from free Hyty and Ty, and 8 mg are related to their elenolic esters and also to Ol Agl and Lig Agl (de la Torre, 2008). Some other estimations have been made. For the Greek population (Dilis & Trichopolou, 2009), the daily per-capita intake is about 17 mg. Vissers et al. (2004) estimated that about 1 mg of the phenol intake per day (6 mmol) is derived from Hyty and Ty, about 8 mg (23 mmol) from the aglycones, and so the total phenol intake would be about 29 mmol.

2. Bioavailability of olive oil polyphenols

Accumulating evidence suggests that VOO may have health benefits; it can be considered as an example of a functional food containing a variety of components that may contribute to its overall therapeutic characteristics (Stark & Madar, 2002; Visioli & Bernardini, 2011). To explore and determine the mechanisms of action of olive oil polyphenols and their role in disease prevention, understanding the factors that constrain their release from the olive oil, their extent of absorption, and their fate in the organism is crucial. These issues can be described under the term *bioavailability,* borrowed from the field of pharmacology, redefined as "that fraction of an oral dose, either parent compound or active metabolite, from a particular preparation that reaches the systemic circulation" (Stahl et al., 2002). To simplify this definition, D'Archivio et al. (2010) explained that it simply means how much of the

ingested amount of polyphenols is able to exert its beneficial effects in the target tissues. It is important to realize that the most abundant phenolic compounds in our diet are not necessarily those that have the best bioavailability profile, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, the metabolites that are found in blood and target organs, resulting from digestive or hepatic activity, may differ from the native compounds in terms of biological activity (Manach et al., 2004).

Although the information concerning the bioavailability of most olive oil polyphenols is limited, intensive research has been carried out in the past decade. This fact is reflected in the number of reviews published since 2002 (Corona et al., 2009; Covas et al., 2009; de la Torre, 2008; Fitó et al., 2007; Tuck & Hayball, 2002; Visioli et al., 2002; Vissers et al., 2004). To address the bioavailability of olive oil phenolic compounds, we have reviewed *in vitro* and *in vivo (*both animal and human) studies on the absorption, transport, metabolism and excretion of olive oil phenolic compounds.

2.1 Absorption and disposition

Direct evidence on bioavailability of olive oil phenolic compounds has been obtained by measuring the concentration of the polyphenols and their metabolites in biological fluids, mostly plasma and urine, after ingestion of pure compounds or of olive oil, either pure or enriched with the phenolics under study. The majority of research regarding the bioavailability of olive oil polyphenols has been focused on three major phenolics: Hyty, Ty and Ol, as can be seen in **Tables 1** and **2**.

After ingestion, olive oil polyphenols can be partially modified in the acidic environment of the stomach. The effect of such environment on aglycone secoiridoids has been examined *in vitro* by incubating the compounds at 37 ^{o}C in simulated gastric pH conditions and during normal physiological time frames (Corona et al., 2006; Pinto et al., 2011). Although hydrolysis takes place releasing free phenolic alcohols, a significant amount remains intact and thus, enters the small intestine unmodified. Ol Agl and its dialdehydic form, however, are likely not absorbed as such in the small intestine; the major metabolites detected using the perfused rat intestine model were the glucuronide conjugates of the reduced form of both compounds (Pinto et al., 2011).

Manna et al. (2000) carried out studies on the transport kinetics of radiolabeled Hyty using differentiated Caco-2 cells. The only metabolite found in the culture medium was the methylated derivative (i.e. homovanillic alcohol - HVAlc). They also demonstrated that Hyty was transported across the membrane of the human enterocytes by a bidirectional passive diffusion mechanism. Caco-2/TC7 cell monolayers have been used to study the metabolism of other olive oil polyphenols, such as Ty, *p*-coumaric acid, pinoresinol, luteolin (Soler et al., 2010) and Hyty acetate (Mateos et al., 2011). Results showed that the methylated conjugates are the main metabolites and that the acetylation of Hyty significantly increases its transport across the small intestinal epithelial cell barrier, enhancing the delivery of Hyty to the enterocytes.

To study the potential hepatic metabolism of olive oil phenols, human hepatoma HepG2 cells were incubated for 2 and 18 h with Ty, Hyty and Hyty acetate (Mateos et al., 2005). Extensive uptake and metabolism of Hyty and Hyty acetate were observed, with scarce metabolism of Ty. Hyty acetate was converted into free Hyty and then metabolized;

Tested Phenol	Model system[a]	Methods	Metabolites Detected	Study Outcome	Ref.
[^{14}C] Hyty	Caco-2 cell monolayers	Transport kinetics: incubation with increasing concentrations (50-500 µM) at 37 and 4 °C for 2 min. Transepithelial transport: incubation with 100 µM Hyty, glucose and mannitol	HVAlc	Hyty transport occurs via a passive diffusion mechanism, bidirectionally and in a dose-dependent manner. Hyty is quantitatively absorbed in the intestine	Manna et al., 2000
Ol glycoside	Isolated rat intestine	In situ intestinal perfusion technique: infusion of aqueous solution (1 mM, 50 µl/min) at 37 °C during 40 min in both iso-osmotic and hypotonic luminal conditions		Ol in aqueous solution can be absorbed, albeit poorly, from isolated perfused rat intestine. The P_{app} of Ol in hypotonic conditions is significantly higher	Edge-combe et al., 2000
Hyty, Ty, Hyty-Ac	Hepatoma HepG2 cells	Cell uptake and metabolism of phenols: incubation with 100 µM at 37 °C for 2 and 18 h	Hyty mono-gluc and methyl-gluc, HVA, Ty gluc, Hyty-Ac mono-gluc	Extensive uptake and hepatic metabolism of Hyty and Hyty-Ac with scarce metabolism of Ty; main derivatives formed: glucuronidated and methylated conjugates	Mateos et al., 2005
Hyty, Ty, Ol	Caco-2 cell monolayers and rat segments of jejunum and ileum		Hyty and Ty gluc, HVAlc, Hyty glutathionylated	Hyty and Ty were transferred across the cell monolayers and rat segments of intestine and were subjected to classic phase I/II biotransformation. No absorption of Ol	Corona et al., 2006
Hyty, Ty, *p*-coumaric acid, pinoresinol, luteolin	Caco-2/TC7 cell monolayers	Phenols metabolism: incubation with 40, 50 and 100 µM at 37 °C for 1, 6 and 24 h. Transport experiments in the AP, cellular and BL compartments: AP loading of phenol at 100 µM	*Hyty:* methyl, sulfate, methyl-sulfate. *Ty:* methyl, sulfate. *p-Coumaric acid:* disulfate, methyl. *Pinoresinol:* gluc, sulfate. *Luteolin:* gluc, methyl, methyl-gluc,	Limited intestinal metabolism. Major metabolites: methylated conjugates. Time-dependent efflux of various free and conjugated forms, showing preferential AP to BL transport after 24 h of incubation	Soler et al., 2010
Hyty, Hyty-Ac	Caco-2/TC7 cell monolayers	Metabolism experiments and transport experiments in the AP and BL compartments: incubation with 50 µM at 37 °C for 1, 2 and 4 h	*Hyty:* HVAlc. *Hyty-Ac:* Hyty, HVAlc, mono-gluc.	Hyty-Ac is better absorbed than free Hyty and serves to enhance delivery of Hyty to the enterocytes for subsequent metabolism and BL efflux)	Mateos et al., 2011
Ol Agl, dialdehydic form of Ol Agl	Human Caco-2 cell monolayers and isolated lumen of rat intestine (jejunum and ileum)	Transport experiments using Caco-2 cells: incubation with 50, 100 and 200 µM at 37 °C for 2 h; AP loading. Transport experiments using rat intestine: perfusion of methanol solution (100 µM) at 37 °C during 80 min	Hyty, HVAlc, Hyty and HVAlc gluc, gluc conjugates of the reduced forms of tested compounds	Caco-2 cells expressed limited metabolic activity. Major metabolites using the perfused rat intestine model: gluc of the reduce forms. Secoiridoids in the parental form were little absorbed in the small intestine	Pinto et al., 2011

[a] Caco-2 cells: model system of the human intestinal epithelium; HepG2 cells: model system of the human liver; TC7 cells: spontaneously differentiating clone derived from the original Caco-2 cell population.
Abbreviations: AP: apical; BL: basolateral; gluc: glucuronide; Hyty: hydroxytyrosol; Hyty-Ac: hydroxytyrosol acetate; HVA: homovanillic acid; HVAlc homovanillic alcohol; Ol: oleuropein; Ol Agl: oleuropein aglycone; P_{app}: apparent permeability coefficient; Ty: tyrosol.

Table 1. *In vitro* studies carried out with olive oil polyphenols.

glucurono- and methyl-, but no sulfo-conjugates, were found. Olive oil phenols are metabolized by the liver as well, as suggested by these results.

The colonic metabolism of olive oil polyphenols is scarcely reported. Corona et al. (2006) demonstrated that secoiridoids, which appear not to be absorbed in the small intestine, suffer bacterial catabolism in the large intestine with Ol undergoing rapid degradation by the colonic microflora producing Hyty as the major end product.

It is essential to establish whether olive oil phenolics are absorbed in the intestine *in vivo* and how they are distributed in the organism. **Table 1** shows the *in vivo* bioavailability studies of olive oil polyphenols carried out so far. For practical reasons, rats are used as the model of choice for *in vivo* studies. Bai et al. (1998) studied the absorption and pharmacokinetics of Hyty in rats, finding that the absorption of Hyty after the ingestion of a single dose is very fast. The metabolic fate of Hyty and Ty in rats has been also evaluated by administration of the radiolabeled polyphenols. Hyty appeared in plasma at maximum levels 5 min after oral administration, although the proportion of free aglycones in some tissues differed to that observed in plasma (D'Angelo et al., 2001). In all of the investigated tissues, Hyty was enzymatically converted in oxidized and/or methylated derivatives, whereas the major urinary products were sulfo-conjugates. Tuck et al. (2001) compared the elimination of Hyty and Ty in rat urine within 24 h after administration, both orally (in oil- and water-based solutions) and intravenously (in saline). When orally administrated, polyphenols will be subjected to first-pass metabolism, so that the contribution of intestinal metabolism will be quite relevant. If the administration is intravenous, only hepatic contribution to its disposition will be seen. Results showed that Hyty and Ty can be absorbed into the systemic circulatory system after oral dosing and that their bioavailability when administered as an olive oil solution is almost complete. Later, urine samples were re-examined and Hyty and five of its metabolites were detected (Tuck et al., 2002). Three were conclusively identified as monosulfate and 3-*O*-glucuronide conjugates of Hyty, and homovanillic acid (HVA), and one was tentatively identified as *O*-glucuronide conjugate of HVA. Although there is no disagreement between studies, a major limitation is that they were done with rats and some researches suggest that comparisons between the model species might not be adequate. Visioli et al. (2003) observed a 25 fold higher basal excretion of Hyty and of its main metabolites in rats than humans.

In a well-designed approach, Vissers et al. (2002) measured the absorption and urinary excretion of olive oil polyphenols in healthy ileostomy subjects and subjects with a colon after the ingestion of increasing doses of extracted phenols. Only a small amount of the ingested compounds was recovered in the urine, supporting the hypothesis that humans absorb a major fraction of the olive oil phenols consumed. Furthermore, the comparison between the absorbed polyphenols in normal and ileostomy subject showed similar results, which implies that the small intestine is the major site for the absorption of those compounds. Free Hyty and Ty and their glucurono-conjugates were the only metabolites detected in the urine samples. Another study carried out in human subjects assessed quantitatively the uptake of phenolics from olive oils containing different amounts of Ty and Hyty (Visioli et al., 2000). It was observed that these compounds were absorbed in a dose-dependent manner, that they were excreted in urine as glucuronide conjugates and that, as the concentration of phenols administered increased, the proportion of conjugation with glucuronic acid also increased. Upon re-examination of samples two more metabolites of Hyty were identified: HVA and HVAlc (Caruso et al., 2001).

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurements	Analysis Methods	Ref.
Synthetic Hyty in 0.5% tragacanth solution	Oral, 1 ml single dose: Hyty 10 mg /ml	Rat plasma	Hyty 0.89-3.26 µg/ml (after 10 min)				GC-MS	Bai et al., 1998
Olive oil enriched with increasing concentrations of phenols	Oral, 50 ml single dose. Phenolic content (mg/l): total phenols 487.5-1950; Hyty 20-84; Ty 36-140	Human urine		Total Hyty 30-60%; Total Ty 20-22%	Hyty and Ty gluc		GC-MS	Visioli et al., 2000
Synthetic Hyty in aqueous solution	Oral, single dose: Hyty: 20 mg/kg	Rat plasma	Hyty 1.91 µg/ml (after 10 min)				HPLC-UV (280 nm)	Ruiz-Gutiérrez et al., 2000
EVOO	Oral (a) Sustained doses for 1 month of 50 g EVOO/day; (b) 100 g single dose	Human plasma				Plasma antioxidant capacity. Hyty, Ty and vitamin E content in LDL		Bonanome et al., 2000
Olive mill waste water extracts with increasing concentrations of Hyty	Oral, single doses *(a)* 1 mg/kg of extract: 41.4 µg/kg of Hyty *(b)* 5 mg/kg of extract: 207 µg/kg of Hyty *(c)* 10 mg/kg of extract: 414 µg/kg of Hyty	Rat plasma and urine			Hyty gluc	Plasma antioxidant capacity		Visioli et al., 2001
Radiolabeled synthetic Hyty and Ty in different solutions	*(a)* Oral, single dose; 225 mg oil-based solution (23.5 mg Hyty or 14.7 mg Ty in 1300 mg EVOO) or water-based solution (25.5 mg Hyty or 14.4 mg Ty in 1300 mg water) *(b)* IV, 950 mg saline solution (6.5 mg Hyty or 9.8 mg Ty added to 5 ml of 9 g/l NaCl)	Rat urine and feces		*(a)* Oral in oil (%): Hyty 94.1, Ty 72.9 ; oral in water, Hyty 70.9, Ty 53.2; *(b)* IV (%): Hyty 94.9, Ty 74.4	Gluc and sulfate conjugates	Feces: Hyty: < 3% and Ty: 25-30% of administered amount (after 24 h)	HPLC-radiometric detection	Tuck et al., 2001
Radiolabeled synthetic Hyty	IV, 0.3 mg single dose: 1.5 mg Hyty/kg body weight	Rat blood, urine, feces, tissues and GI content	Hyty: 8% of administered radioactivity (after 5 min), 6% associated with plasma and 1.9% with cell fraction	Hyty: 90% of administrated radioactivity (after 5 h)	Sulfo-conjugated, HVAlc, HVA, DOPAC, DOPAL	Hyty (% of administered radioactivity): brain 0.89, heart 0.39, kidney 0.8, liver 3.19, lung 0.53, skeletal muscle 61, GI content 9 (after 5 min). Feces: 3.2% (after 5 h). Measurements of detected metabolites in urine and tissues as well	Radioactivity measures, HPLC-UV for metabolite identification	D'Angelo et al., 2001
Olive oil enriched with increasing concentrations of phenols	Oral, 50 ml single dose (mg): total OI Agl 12.6-39.5; free Hyty 1.9-7.1; total Hyty 7-23.2	Human urine		% of total metabolites: Hyty 16.8-23.7% ; HVA 53.9-61.8 %; HVAlc 22.0-22.4 %	HVAlc, HVA		GC/LC-MS	Caruso et al., 2001

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurements	Analysis Methods	Ref.
EVOO	Oral, 50 ml single dose: 1650 µg of Ty	Human urine		Ty 17-43%	Ty conjugates		GC-MS	Miró-Casas et al., 2001a
VOO	Ora; 50 ml single dose: 1055 µg of Hyty, and 655 µg of Ty	Human urine		Hyty 32-98.8%; Ty 12.1-52%; total free Hyty and Ty ~15%	Hyty and Ty conjugates		GC-MS	Miró-Casas et al., 2001b
Aqueous and oil solutions of radiolabelled synthetic Hyty	*(a)* Oral, oil solution *(b)* IV, aqueous solution (For a detailed description see Tuck et al., 2001)	Rat urine		*(a)* Free Hyty 4.10 %; Hyty sulfate 48.42%; Hyty gluc 9.53%; HVA 10.26%; other metabolites: 20.27%; *(b)* Free Hyty 2.35 %; Hyty sulfate 34.24%; Hyty gluc 3.58%; HVA 18.69%; other metabolites 30.87%	Hyty monosulfate, Hyty 3-*O*-gluc, HVA	Determination of the radical scavenging ability of authentic HVA and HVAlc and of each metabolite using DPPH radical scavenging test.	HPLC-radiometric detection; HPLC-MS/MS, ^{1}H NMR for metabolite identification	Tuck et al., 2002
Supplements containing nonpolar and polar phenols extracted from EVOO, and Ol glycoside (commercially available capsules)	Oral, single doses: 100 mg of phenols *(a)* Ileostomy subjects. Phenolic content (µmol): nonpolar 371; polar 498; Ol glycoside 190; *(b)* Subjects with a colon. Phenolic content (µmol): nonpolar 382; polar 526	Human urine and ileostomy effluent		*(a)* Nonpolar 12%; polar 6%; Ol glycoside 16% *(b)* Nonpolar 6%; polar 5%	Hyty and Ty in free form or gluc conjugated	Total excretion in ileostomy effluent over 24 h (µmol): nonpolar < 127; polar < 153; Ol glycoside < 51	HPLC-MS/MS, GC-MS, HPLC-DAD	Vissers et al., 2002
VOO from Arbequina cultivar	Oral *(a)* 50 ml single dose (µg): Ty 1720; Hyty 1370 *(b)* 25 ml /day sustained doses for 1 week (µg): Ty 860; Hyty 685	Human urine		*(a)* Ty 16.9%; Hyty 78.5% *(b)* Ty 19.4%; Hyty 121.5% (at the end of the sustained period)			GC-MS	Miró-Casas et al., 2003a
VOO	Oral, 25 ml single dose (mg/l): free Hyty 6.2; Hyty after acidic treatment 49.3	Human urine and plasma	Hyty conjugate 25.83 µg/l (after 32 min); HVAlc 3.94 µg/l (after 53 min)	Different results according to hydrolytic treatment (µg): acidic conditions: Hyty 714.7, HVAlc 188.0; enzymatic hydrolysis: Hyty 479.6, HVAlc 122.9 (after 12 h)	HVAlc, Hyty gluc		GC-MS	Miró-Casas et al., 2003b
EVOO and synthetic Hyty in ROO and low-fat yogurt	Oral, single dose *(a)* Rats: 50.3 µg total Hyty 0.5 ml in EVOO (201.2 µg/kg) *(b)* Humans: 3.2 mg total Hyty in 30 ml EVOO (45.7 µg/kg); 7 mg Hyty in 30 ml ROO; 20 mg synthetic Hyty in 125 g yogurt.	Rat and human urine		% of total Hyty administered: *(a)* Hyty + HVAlc 7.6 *(b)* EVOO: Hyty + HVAlc 44.2. ROO: Hyty + HVAlc 23.0; Yogurt: Hyty + HVAlc 6.7	HVAlc		GC/LC-MS	Visioli et al., 2003

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurements	Analysis Methods	Ref.
Ol in soya oil and distilled water	Oral, 350g single dose (Ol 100 mg/kg)	Rat plasma and urine	Ol 200 ng/ml (after 2 h)	Ol gluc 91%; Hyty gluc 97%	Ol and Hyty gluc		LC-MS/MS	Del Boccio et al., 2003
Ol in saline solution	IV, 100 µl single dose: 25 mg/kg of Ol in NaCl (0.9%, w/v)	Rat plasma	Approximate values: 3.5 µg/ml Ol; 20 ng/ml Hyty (after 10 min)				HPLC-fluorescence detection	Tan et al., 2003
EVOO and pure Ol (isolated from olive tree leaves)	Oral, sustained doses for 80 days (g/kg) *(a)* EVOO 50 *(b)* Ol 0.15	Rat urine		ng/ml: *(a)* free Ty 321; free Hyty 253.2; total Ty 1855.6; total Hyty 404.3 *(b)* free Ty 183.6; free Hyty 154.4; total Ty 814.5; total Hyty 1036.7	Gluc conjugates		GC-MS/MS (for urine samples) and HPLC-DAD (for EVOO extracts)	Bazoti et al.,2005
EVOO with increasing concentrations of polyphenols	Oral, sustained doses for 4 days. 25 ml EVOO: phenol content (mg/kg) *(a)* high 486 *(b)* moderate 133 *(c)* low 10 (%: Hyty 6.3, Ty 5.3, Ol Agl 40.0; Lig Agl 26.2; luteolin 11.7; apigenin 2.6)	Human urine		Approximate values (mmol): *(a)* Ty 1.2; Hyty 0.5; HVAlc 0.2 *(b)*Ty 2.8; Hyty 2.2; HVAlc 0.6; *(c)*Ty 3.5; Hyty 4.8; HVAlc 1.0			HPLC	Weinbrenner et al., 2004
VOO, COO or ROO from Picual cultivar	Oral, 25 ml sustained doses for 3 weeks. Total phenols in olive oil (mg/kg): VOO 366; COO 164; ROO 2.7	Human plasma and urine				Detection of etheno-DNA adducts in plasma. Measurement in 24 h urine of Ty and Hyty as biomarkers of the type of olive oil ingested	LC-MS/MS	Hillestrom et al., 2006
VOO from Picual cultivar	Oral, 50 ml VOO single dose	Human blood			Hyty mono-gluc, Hyty monosulfate, Ty gluc, Ty and HVA sulfate	Metabolites in LDL (ng/mg apo-B): Hyty mono-gluc 2.11; Hyty monosulfate 24.27; Ty gluc 1.16; Ty sulfate 14.87; HVA sulfate 27.16 (after 1 h)	HPLC-DAD-MS/MS	de la Tore-Carbot et al., 2006
EVOO from Picual cultivar	Oral, 50 ml single dose. Phenolic content (µg/ml): total 648; Hyty 70.6; Ty 27.01	Human blood			Hyty mono-gluc isomers, Hyty monosulfate, Ty and HVA sulfate, Ty gluc	Metabolites in LDL (ng/mg apo-B): total phenolics 105.43; Hyty mono-gluc I 2.45; Hyty mono-gluc II 2.55; Hyty monosulfate 34.22; Ty gluc 0.96; Ty sulfate: 17.23; HVA sulfate 48.02 (after 1 h)	HPLC-DAD-MS/MS	de la Tore-Carbot et al., 2007
VOO	Oral, 30 ml single dose: total phenols 400 mg/kg	Human plasma	11 free phenolicss and 9 metabolites (after 1 and 2 h)		Hyty and apigenin gluc; Hyty, Ty, HVA, vanillin, vanillic acid, dihydroferulic acid and coumaric acid sulfates		UPLC-ESI-MS/MS	Suárez et al., 2009

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurements	Analysis Methods	Ref.
EVOO enriched with Ol and pure Ol (isolated from olive tree leaves)	Oral, sustained doses for 80 days *(a)* EVOO: 1.1 g/kg *(b)* Ol supplement: 0.33 mg/kg	Rat plasma	After enzymatic treatment (ng/ml): *(a)* HVAlc 61.9 *(b)* HVAlc 53.5; (Hyty not detected)		HVAlc		LC-MS/MS	Bazoti et al., 2010
Spanish VOO (Picual cultivar) and ROO	Oral, 25 ml/day sutained doses for 3 weeks *(a)* VOO, phenol concentration (mg/l): total 629; Hyty 63.5; Ty 24.4; Ol derivatives 327.2; Lig derivatives 208; other 6 phenolics quantified *(b)* ROO	Human blood			Hyty, Ty and HVA sulfates	LDL composition before and after consumption of ROO and VOO	HPLC-DAD-MS/MS	de la Tore-Carbot et al., 2010
EVOO (50% v/v Arbequina and Picual cultivars)	Oral, 50 ml single dose: Phenolic content (mg/kg): Hyty 8.31; Ty 5.33; pinoresinol 3.25; luteolin 2.65; apigenin 0.64; EA 34.91; Lig Agl 40.58	Human urine		28 phase I and 32 and II metabolites	Phase I reactions: hydrogenation, hydroxylation, hydration, methylation Phase II reactions: gucuronidation, sulfoconjugation	Excretion kinetics for 6 h of main metabolites identified as biomarkers in human urine after EVOO intake	RRLC-ESI-TOF-MS	García-Villalba et al., 2010
Spanish VOO	Oral, 50 ml single dose. Phenolic content in µmol: Hyty 21.96; Ty 15.20; HVAlc 0.27	Human plasma and urine		% of ingested Hyty: Total Hyty 9.2; free Hyty 1.8; Hyty-4′-*O*-gluc 3.1; Hyty-3′-*O*-gluc 4.3; total Ty 12.9; free Ty 3.3; Ty-4′-*O*-gluc 9.6; Total HVAlc 4.5; free HVAlc: 1.4; HVAlc -4′-*O*-gluc: 3.1	HVAlc; Hyty, Ty and HVAlc gluc	Control of compliance with the dietary recommendations by analyzing the plasma and urine concentrations of Hyty, Ty and HVAlc	UPLC-ESI-MS and GC-MS	Khymenets et al., 2011

[a] Percentage of administered amount after 24 h, unless otherwise indicated

Abbreviations: apo-B: apolipoprotein-B; COO: common olive oil; DAD: diode array detector; DOPAC: 3,4-dihydroxyphenylacetic acid; DOPAL: 3,4-dihydroxyphenylacetaldehyde; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EA: elenolic acid; EVOO: extra virgin olive oil; gluc: glucuronide; GC: gas chromatography; GI: gastrointestinal; HPLC: high performance liquid chromatography; Hyty: hydroxytyrosol; HVA: homovanillic acid; HVAlc: homovanillic alcohol; IV: intravenous; LC: liquid chromatography; LDL: low-density lipoproteins; Lig Agl: ligstroside aglycone; MS: mass spectrometry; Ol: oleuropein; P_{app}: apparent permeability coefficient; ROO: refined olive oil; Ty: tyrosol; UV: ultraviolet; VOO: virgin olive oil

Table 2. Bioavailability of olive oil polyphenols in animals and humans.

A major limitation of the commented human studies is that they used phenolics extracts or olive oil samples artificially enriched with phenolics extracts, and therefore extrapolation of these results to typical olive oil consumption may not be realistic. Further studies have been performed administering VOO at doses close to that used in the Mediterranean countries (30-50 g/day) (Bonanome et al., 2000; de la Torre-Carbot et al., 2006, 2007; García-Villalba et al., 2010; Khymenets et al., 2011; Miró-Casas et al., 2001a, 2001b, 2003a, 2003b; Suárez et al, 2009). Results confirmed that Hyty and Ty are mainly excreted in their glucurono-conjugated form; in fact, the role of glucuronidation in metabolism of main olive oil phenols can be evaluated in about 65-75% of totally recovered in urine after dietary VOO consumption (Khymenets et al., 2011; Miró-Casas et al., 2003b), which suggests an extensive first-pass intestinal/hepatic metabolism of the compounds ingested. Suárez et al. (2009) considered for the first time the absorption and disposition of flavonoids and lignans after the ingestion of VOO. Besides the presence of those VOO polyphenols in their conjugated forms, an important variability in the concentrations was observed between the plasma samples obtained from different volunteers. This variability may be attributed to differences in the expression of metabolizing enzymes due to genetic variability within the population. The most comprehensive study regarding the identification of metabolites in human urine of practically all the olive oil polyphenols described was reported by García-Villalba et al. (2010). These authors were able to achieve the tentative identification of 60 metabolites; the most abundant were those containing a catechol group, such as Hyty and the secoiridoids Ol Agl and deacetoxy-Ol Agl. Phenolic compounds were subjected to various phase I and phase II reactions, mainly methylation and glucuronidation. The report suggests that most of the olive oil polyphenols are absorbed to a greater or lesser extent, although absorption and metabolism seems to differ greatly among the different compounds.

2.2 Conjugation and nature of metabolites

Low doses of polyphenols are delivered through human diet and, generally, do not escape first-pass metabolism. As a result, most olive oil polyphenols undergo structural modifications, i.e. conjugation process; in fact, conjugates are the predominant forms in plasma. Once absorbed, olive oil polyphenols are subjected to three main types of conjugation: methylation, glucuronidation and, to a lesser extent, sulfation, through the respective action of catechol-O-methyl transferases (COMT), uridine-5'-diphosphate glucuronosyltransferases (UDPGT) and sulfotransferases (SULT) (Manach et al., 2004).

Recently, García-Villalba et al. (2010) carried out a broad study of the metabolites of most olive oil phenolic compounds excreted in human urine, showing that most polyphenols were absorbed, metabolized and excreted to a lesser or greater extent. It was initially suggested in literature that Ol Agl and Lig Agl were hydrolyzed in the gastrointestinal tract (GI) tract and then, the resulting polar phenols, Hyty and Ty, were absorbed and metabolized (Vissers et al., 2002). Nevertheless, the results obtained in later experiments with Caco-2 cells (Pinto et al., 2011) and humans (García-Villalba et al., 2010), showed that, at least, part of the secoiridoids can be absorbed and metabolized; reduction (hydrogenation) is the most probable metabolic pathway of these compounds. Hydroxylation and hydration are also possible pathways for the secoiridoids. In fact, they can precede or follow the action of COMT on compounds such as Hyty, deacetoxy-Ol Agl, and Ol Agl. Some compounds can even suffer a double hydroxylation before or after the methylation (García-Villalba et al., 2010).

A notable metabolic pathway for Hyty is the methylation, giving rise to the formation of HVAlc (Caruso et al., 2001; Bazoti et al., 2010; Manna et al., 2000; Visioli et al, 2003). Oxidation and methylation-oxidation, rendering 3,4-dihydroxyphenilacetic acid (DOPAC) and HVA, respectively, have been also proposed (D'Angelo et al., 2001). It is noteworthy that many of the reported metabolites of Hyty are also the major molecular species deriving from dopamine metabolism (HVA, DOPAC, 3,4-dihydroxyphenyl acetaldehyde - DOPAL); in fact, Hyty can be also called DOPET, a well-known dopamine metabolite.

Besides, olive oil phenolic compounds and most of their corresponding phase I metabolites can be subsequently subjected to phase II reactions, preferentially glucuronoconjugation (García-Villalba et al., 2010). The presence of glucuronoconjugates of phenolic compounds belonging to most of chemical classes families described in olive oil has been widely detected in both urine and plasma, whereas the presence of sulfated metabolites has scarcely been reported in literature.

The metabolism of olive oil lignans has not been reported in detail so far and one of the few references appeared only recently (Soler et al., 2010). In this study, pinoresinol glucuronide and sulfate conjugates were identified after incubation of free pinoresinol using differentiated Caco-2/TC7 cell monolayers.

As far as flavonoids are concerned, products of methylation and glucuronidation have been observed (Soler et al., 2010; Suárez et al., 2009). Methyl-monoglucuronides of apigenin and luteolin have been identified as well (García-Villalba et al., 2010).

2.3 Binding of olive oil polyphenols to lipoproteins

Several reports converge on the *in vitro* ability of olive oil phenolic compounds to bind low density lipoproteins (LDL) and to protect them against oxidation (Covas et al., 2000; Visioli et al., 1995). Moreover, both animal and human *in vivo* studies (Coni et al., 2000; Marrugat et al., 2004) have provided evidence on the effects of olive oil ingestion on LDL composition and the incorporation of olive oil phenolics and their metabolites in LDL. In one of the first studies, Bonanome et al. (2000) determined the presence of Hyty and Ty in human lipoprotein fractions after olive oil ingestion. Both compounds were recovered in all of the fractions, except in the very low density lipoproteins one; concentrations peaked between 1 and 2 h. Covas et al. (2006) demonstrated that the postprandial oxidative stress can be modulated by the olive oil phenolic content and that the degree of LDL oxidation decreases in a dose-dependent manner with the phenol concentration of the olive oil ingested. They arrived to these conclusions administering a single dose of olive oil, but similar results were obtained in studies using sustained doses; olive oil consumption for 1 week led to an increase in the total phenolic content of LDL (Gimeno et al., 2002). In a later study, volunteers were requested to ingest virgin, common or refined olive oil daily for 3 weeks (Gimeno et al., 2007). The concentration of total phenolic compounds in LDL was directly correlated with the phenolic concentration of the oils and with the resistance of LDL to their *in vitro* oxidation.

De la Torre-Carbot et al. (2006, 2007) developed a rapid method for the determination in LDL of Ty, Hyty and several of their metabolites. The presence of these compounds in LDL strengthens claims that these compounds can act as *in vivo* antioxidants. The effect of the intake of virgin and refined olive oils after long-term ingestion of real-life doses on the

content of the metabolites in LDL was examined as well (de la Torre-Carbot et al., 2010). The phenols in VOO modulated the LDL content of 3 phenolic metabolites, Hyty, Ty, and HVA sulfates; the concentration of these compounds increased significantly after the ingestion of VOO, in contrast to the refined one. In parallel, the ingestion of VOO significantly reduced LDL and plasma oxidative markers, which suggests that the metabolic activities of phenols can be related to the capacity of these compounds to remain bound to LDL.

2.4 Plasma concentration and tissue uptake

In 1998, Bai et al. reported for the first time the absorption of Hyty into the bloodstream. Hyty was administered orally to rats and appeared in plasma 2 min after, reaching the highest level at 5-10 min. Its concentration was low compared to the administered amount. The experiment, however, did not take into account the presence of metabolites.

After this first approach, different methods for the simultaneous detection of Ol and Hyty in rat plasma have been optimized. Ruiz-Gutiérrez et al. (2000) determined Hyty in overnight-fasted rat plasma after its oral administration. Ol and Hyty plasma concentrations were measured after oral administration of a single dose of Ol to rats using soya oil and distilled water as administration vehicle (Del Boccio et al., 2003). Analysis of plasma showed the presence of unmodified Ol, reaching a peak value within 2 h, with a small amount of Hyty. In another study, Ol and Hyty plasma levels were monitored in rats after intravenous dosing of Ol (Tan et al., 2003). The dosing profile showed that at 10 min both Ol and Hyty were rapidly distributed.

Studies in which phenolic ingestion is closer to typical dietary patterns may be more appropriate for estimating bioavailability than the administration of pure compounds. Recently (Bazoti et al., 2010), the simultaneous quantification of Ol and its major metabolites in rat plasma was carried out after a control diet of 80 days supplemented with Ol or with EVOO. Basal levels of HVAlc were detected in the blood stream after the enzymatic treatment of the samples with β-glucuronidase. Before the enzymatic treatment, HVAlc was detected below the limits of quantification in plasma samples of rats supplement with Ol. Hyty was not detected, which indicates that it was metabolized to HVAlc. Ol was detected below the LOQ before and after the enzymatic treatment. These results are in accordance with the study made by Del Boccio et al. (2003), who demonstrated that Ol was rapidly metabolized and eliminated.

Miró-Casas et al. (2003b) quantified Hyty and HVAlc in human plasma and urine after real-life doses of VOO. Both compounds appeared rapidly in plasma mainly as glucuronides, with peak concentrations at 30 min for Hyty and 50 min. for HVAlc after the ingestion, supporting the premise that the small intestine is the major site of absorption for these compounds (Vissers et al., 2002).

Most of the studies described so far have centered their attention on Hyty, Ty and Ol derivatives. In a recent work, the absorption and disposition of other olive oil polyphenols (flavonoids and lignans) have been considered (Suárez et al., 2009). Samples were obtained from healthy humans 1 and 2 h after the ingestion of VOO. The major compounds identified and quantified in plasma corresponded to metabolites of Ty, although Ty sulfate was only detected in one subject, and especially Hyty, as glucuronide and sulfate conjugates. HVA sulfate could be the direct product of the Hyty methylation, and vanillin sulfate and vanillic

acid sulfate could be formed as products of alcohol dehydrogenase and aldehyde dehydrogenase activities. Suárez & co-workers also found hydroxybenzoic acid in all the plasma samples. The glucuronide metabolite of apigenin was tentatively quantified in all the samples analyzed, but showing a considerable inter-individual variation. Lignans (pinoresinol and acetoxypinoresinol) could not be detected in the plasma samples even in glucuronide or sulfate conjugated forms.

Once the polyphenols reach the bloodstream, they are able to penetrate tissues, particularly those in which they are metabolized. The nature of the tissular metabolites may be different from that of blood metabolites; data are still very scarce, even in animals, and their ability to accumulate within specific target tissues needs to be further investigated. An article written by D'Angelo et al. (2001) studied the fate of radiolabelled ^{14}C Hyty intravenously injected in rats in different biological fluids (plasma, urine and feces) and tissues (brain, heart, kidney, liver, lung, skeletal muscle and GI content). The pharmacokinetic analysis indicated a fast and extensive uptake of the molecule by the organs and tissues investigated, with a preferential renal uptake. Over 90% of the administered radioactivity was excreted in urine after 5 h and about 5% was detectable in feces and GI content. Less than 8% of the administered radioactivity was still present in the blood stream 5 min after injection. Regarding tissues, the time course analysis indicated that the highest level of radioactivity was detected 5 min after injection, followed by a rapid decrease. It is worth noting that Hyty is able to cross the blood-brain barrier, even though its brain uptake is lower compared with other organs. In all the investigated tissues, Hyty was enzimatically converted in four oxidized and/or methylated derivatives (HVAlc, HVA, DOPAC, DOPAL) and sulfoconjugated derivatives. Enzymatic methylation is presumably operative in the brain, HVAlc representing 41.9% of the detected, labeled species. This reflects the key role of COMT in the central nervous system. The occurrence in the analyzed organs of both labeled DOPAL and DOPAC implies a sequential oxidation of Hyty ethanol side chain catalyzed by alcohol, and aldehyde dehydrogenase, respectively. Labeled HVA, the product of both methylation and oxidation, was also identified. Sulfoconjugated metabolites were mainly found in plasma (43.3%) and urine (44.1%).

As data on plasma concentration of olive oil phenols are still scarce, an alternative is to look at olive oil phenols excreted in urine; these may provide information on the form in which phenols are present in plasma.

2.5 Elimination

The amount and form in which the olive oil phenols are excreted in urine may give an insight into their metabolism in the human body. The first experimental evidence of the absorption of Ty and Hyty from olive oil in humans was obtained by Visioli et al. (2000) from a single oral dose of 50 ml of phenolic-enriched olive oil. The proportions of Hyty and Ty recovered in glucuronidase-hydrolyzed urine, with respect to ingested dose, were in the ranges of 30–60% and 20–22%, respectively. This paper postulated that Hyty and Ty were dose-dependently absorbed in humans and excreted in urine as glucuronide conjugates.

Miró-Casas et al. (2001a) measured the urinary recovery of administered Ty during the 24 h after EVOO ingestion. Maximal Ty values were obtained in the 0-4 h urine samples and

decrease to reach basal values after 8-12 h. Ty was excreted in urine mainly in its glucuro-conjugated form, with only 6–11% excreted in the free form. In a later study (Miró-Casas et al., 2001b), the simultaneous determination of Hyty and Ty in human urine after the intake of VOO was reported. Like the previous study, Hyty and Ty levels in urine rose after VOO consumption, reaching a peak at 0–4 h and returning to basal values at 12–24 h. After hydrolytic treatment, the amount of total compounds recovered in 24 h urine was also determined for Hyty and Ty. Recoveries ranged between 32-98% for Hyty and 12.1-52% for Ty. Both compounds were mainly excreted in conjugated form since only 5.9% Hyty and 13.8% Ty of the total amount excreted were in free form. The hydrolysis procedure applied in this study was limited because it did not provide specific information about the type of conjugation involved. This paper also postulated that Ol is not the main source of Hyty after ingestion of olive oil. The absorption of Hyty and Ty was later confirmed in an experiment using single and sustained doses of VOO (Miró-Casas et al., 2003a). Urinary recoveries of Ty were similar for both cases; however, mean recovery values for Hyty after ingestion of 25 ml/day VOO for one week, were 1.5-fold of those obtained after a 50 ml single dose.

Vissers et al. (2002) studied the absorption of Hyty, Ty and, for the first time, Ol and Lig Agl, in ileostomy subjects and in volunteers with a colon. The results showed that 55-66% of the ingested olive oil phenols were absorbed in ileostomy subjects, which implies that most phenols are absorbed in the small intestine. Excreted phenolics, mainly in the form of Hyty and Ty, were determined to be 5–16% of the total ingested. Similar levels of Hyty and Ty were found in the urine of subjects with and without a colon, confirming that olive oil phenols are absorbed mainly in the small intestine. The obtained values, lower than those reported by others, could be underestimations because metabolites of olive oil phenols were not considered. In this work it is also suggested that an important step in the metabolism of the Ol glycoside and Ol and Lig-aglycones is their transformation into Hyty or Ty. This was supported by finding that 15% of an Ol glycoside supplement administered to healthy human subjects was excreted in urine as Hyty and Ty.

Tuck et al. (2001) investigated the *in vivo* fate of tritium labeled Hyty and Ty after intravenous (in saline, tail vein) and oral dosing (in oil- and water-based solutions) to rats. For both Hyty and Ty, the elimination of radioactivity in urine within 24 h for the intravenously and orally administered oil-based dosing was significantly greater (95 and 75%, respectively) than the oral aqueous dosing method (74 and 53%, respectively). The majority of the excreted dose was eliminated from the body within 2 h, when intravenously dosed, and within 4 h for both oral dosings. Later, urine samples collected after 24 h were re-examined (Tuck et al., 2002). After oral oil dosing Hyty represented 4.10% of compound eliminated, monosulfate conjugate 48.42%, glucuronide conjugate 9.53%, HVA 10.26% and other possible metabolites 20.27%. Other study with rats supplemented with Ol (Ol rats) or with EVOO (EVOO rats) was developed for the simultaneous determination of Hyty, Ty and EA in rat urine (Bazoti et al., 2005). The urinary levels of free Ty and Hyty were higher in EVOO rats than in Ol rats. When the urine sample were treated with β-glucuronidase, the total amount of metabolites measured for the EVOO rat was higher for Ty but lower for Hyty than in Ol rats. EA was not detected, probably because of its further metabolism to simpler molecules. Nevertheless, as already mentioned, caution should be taken interpreting the results achieved from rats (Visioli et al., 2003).

The urinary excretion of HVAlc and HVA in humans was reported for the first time by Caruso et al. (2001) after the intake of different VOOs. HVAlc contributes to 22% of the total excretion of Hyty and its metabolites, and HVA 56%. The excretion of both metabolites correlated with the administered dose of Hyty. Even at low doses, HVAlc and HVA were excreted. In a later study, Miró-Casas et al. (2003b) observed how urinary amounts of Hyty and HVAlc increased in response to VOO ingestion, reaching the maximum peak at 0-2 h. Urinary recovery 12 h after olive oil ingestion was rather different depending on the hydrolytic treatment applied. Under acidic conditions recoveries were higher for both Hyty and HVAlc than with enzymatic hydrolysis. It was apparent that 65% of Hyty was in its glucuronoconjugated form and 35% in other conjugated forms.

To understand the impact of glucuronidation on the metabolic pathway of olive oil phenols, the simultaneous determination of Ty, Hyty, HVAlc and their corresponding *O*-glucuronides in human urine was carried out by Khymenets et al. (2011). It was the first time that the glucuronides of these compounds have been directly identified and quantified in urine samples of volunteers supplemented with EVOO, because previous methods measured either free or total phenols after hydrolysis. The maximum excretion of Hyty and Ty occurred during the first 6 h after administration, which is in agreement with earlier reported data. The free Ty and Hyty, as well as HVAlc, were detected at significant concentrations in all urine samples collected 6 h after EVOO acute intake. Concentrations of *O*-glucuronide metabolites (4-*O*-gluc-Ty, 4-*O*-gluc-Hyty, 3-*O*-gluc-Hyty, and 4-*O*-gluc-HVAlc) were substantially higher in 6 h postprandial samples when compared to their parent compounds. About 13% of the consumed olive oil polyphenols were recovered in 24 h urine, 75% of which were in the form of glucuronides and 25% as free compounds.

In another recent work, specific information about the type of conjugates in human urine was provided (García-Villalba et al., 2010). The authors were able to indentify more than 60 metabolites. This was the first report in which metabolites of simple phenols, flavonoids, lignans and secoiridoids have been found in human urine samples. Phenolic compounds were subjected to different phase I (hydrogenation, hydroxylation, methylation) and phase II (mainly glucuronidation) reactions. Ten metabolites were identified as possible biomarkers of olive oil intake and their levels in urine after the olive oil ingestion were monitored, finding the highest level of most of them 2 h after administration.

In summary, data on urinary excretion indicate that at least 5% of ingested olive oil phenols is recovered in urine mainly as glucuronidated Ty and Hyty. The remaining phenols are metabolized into other compounds, such as *O*-methylated Hyty. Monosulfate conjugates might be other metabolites, as shown in two rat studies; however, if olive oil phenols are also metabolized into these conjugates in humans remains to be elucidated.

3. Need and difficulties of carrying out bioavailability studies of polyphenols

In this section, we will discuss some common mistakes that can be made when bioavailability studies are carried out, the difficulties that the analyst can find and the limitations of some of the studies made so far. **Figure 1** gives a general idea of the most important topics commented in the section.

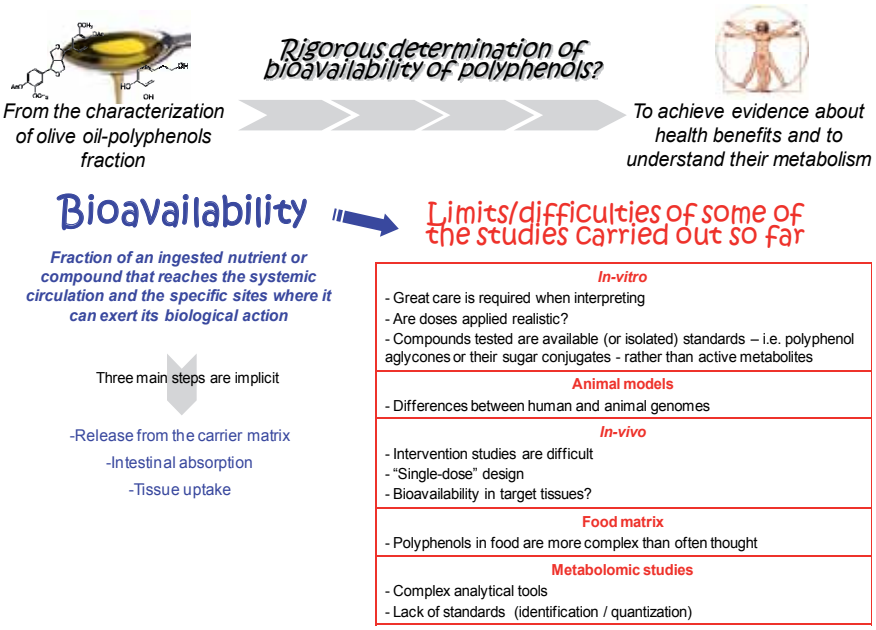

Fig. 1. Definition of bioavailability and the limits affecting bioavailability studies of polyphenols

Since intervention studies are very difficult to carry out, in many cases the researchers have to turn to *in vitro* or animal studies. *In vitro* studies are a pillar of pharmacological research and build the bases for future *in vivo* assays; however, the interpretation and extrapolation of the achieved data have to be made very carefully (Kroon et al., 2004). When the biological activity of polyphenols is assessed by using culture cells as tissue models, in most of the cases, cells are treated with aglycones or polyphenols-rich extract derived from plants or, in this case, from olive oil, and data are reported at concentrations which elicited a response. It is absolutely necessary to bear in mind that plasma and tissues are not exposed *in vivo* to polyphenols in these forms: the molecular forms reaching the peripheral circulation and tissues are different from those present in the olive oil (Day et al., 2001). Moreover, the polyphenols concentration tested should be of the same order as the maximum plasma concentration attained after a polyphenol-rich meal (0.1-10 μmol/l).

Matters of practicality determine the use of rats rather than humans as the model of choice for *in vivo* studies, although interspecies variability renders comparisons between the model species (animals, humans) complex and sometimes questionable (Visioli et al., 2003), since the rats and rodents in general are not the best model for the study of dietary problem of human metabolism.

When *in vivo* studies are carried out, we can say that most of researches have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol, provided as pure compound, plant extract, or whole food/beverage. Using this "single-dose" design, the increase in the blood concentration is transitional and reflects mainly the ability of the organism to take up the polyphenol from the food matrix. Consequently, most

of the data from humans presented in the literature on the bioavailability refer only to the release of the polyphenols from the food matrix and their consequent absorption (D'Archivio et al., 2010; Vissers et al., 2004).

To address the bioavailability of olive oil phenols, we should exclude studies without a control diet and studies in which the amount of ingested phenols is not reported or could not be estimated (Miró-Casas et al., 2001a; Visioli et al., 2000a; Vissers et al., 2004). In other words, it is essential to characterize in depth the polyphenolic extract of the olive oil before starting bioavailability studies to assure their usefulness; since this fraction is quite complex and heterogeneous, it represents another requirement which difficults the whole process.

Advances in the understanding of olive oil polyphenols metabolism have been made possible by improvements in the analytical methodologies used, particularly high-resolution chromatographic systems with mass spectrometry as detector (Bai et al., 1998; Del Boccio et al., 2003; García-Villalba et al., 2010; Khymenets et al., 2011; Miró-Casas et al., 2003b). Performing metabolomic studies is challenging and requires measurements of a very high quality using powerful platforms. Even if the analyst uses proper tools, the fully structural assignment of the metabolites under study is sometimes very difficult due to the lack of the metabolite standards; fact which makes difficult the correct quantification too (D'Archivio et al., 2010; García-Villalba et al., 2010). The amount of information about the sample under study achieved in metabolomic studies is considerable, that is why for meaningful interpretation the appropriate statistical tools must be employed to manipulate the large raw data sets in order to provide understandable and workable information (Manach et al., 2009).

A very interesting review written by D'Archivio et al. (2010) gives a critical overview about the difficulties and the controversies surrounding the studies aimed at determining the bioavailability of polyphenols. Summarizing, there are some essential steps to be followed to establish conclusive evidences for the effectiveness of polyphenols in disease prevention and in human health improvement: 1) determination of the distribution of these compounds in our diet, estimating their content in each food; 2) identification of which of the existing polyphenols are likely to provide the greatest effects in the context of preventive nutrition, and 3) assesment of the bioavailability of polyphenols and their metabolites, to evaluate their biological activity in target issues.

Even though the bioavailability studies are properly designed, we have to be aware of how many different endogenous and exogenous variables are involved and the difficulties that have to be faced. The main factors recognized as affecting olive oil polyphenols bioavailability can be grouped in the following categories: factors related to the polyphenol characteristics, food/food processing related factors, external factors and factors related to the host, as it can be observed in **Figure 1.** An in-depth discussion of every factor influencing the bioavailability of olive oil polyphenols has been made by Manach et al. (2004) and Cicerale et al. (2009).

4. Conclusions

To explore and understand the mechanism of action of olive oil polyphenols and their role in disease prevention and human health improvement, extensive studies of absorption, metabolism, excretion, toxicity, and efficacy are needed. Although *in vitro* studies can be

very useful and provide valuable information, they have to be completed with extensive *in vivo* research. The first requirement for a beneficiary dietary compound is that it enters into the blood circulation; therefore to demonstrate *in vivo* effects of olive oil phenolics it is necessary to assess first their *bioavailability*.

Analysis of plasma and urine provide valuable information on the identity and pharmacokinetics of circulating metabolites after ingestion. Since the metabolites sequestered in body tissues are not usually taken into account, results from urine samples could be an underestimation. There have been several studies which have determined the metabolites of the various olive oil polyphenols (mainly Hyty, Ty, and Ol) in human plasma and urine after oral intake, although the information is still scarce. The conjugation mechanisms that occur in the small intestine and later in the liver are highly efficient. The resulting metabolites are mainly glucuronate and sulfate conjugates with or without methylation across the catechol group (many are multiply conjugated).

Bioavailability studies are gaining increasing interest as food industries are continually involved in developing new products, defined as ''functional'' by virtue of the presence of specific antioxidants or phytochemicals. The difference between functional foods and medicines calls for moderation when the "medicinal" properties of individual food items, be it olive oil, are indicated. The correct message should be to select foods whose components have proven, albeit limited in magnitude, biological activities and build a balanced diet round them, to reduce several chronic diseases.

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19

Oleocanthal: A Naturally Occurring Anti-Inflammatory Agent in Virgin Olive Oil

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1. Introduction

Research on the non-steroidal anti-inflammatory olive oil phenolic, (-)- decarboxymethyl ligstroside aglycone (more commonly known as oleocanthal) has supported speculation that this compound may confer some of the health benefits associated with the traditional Mediterranean diet. Oleocanthal elicits a peppery, stinging sensation at the back of the throat similar to that of the non-steroidal anti-inflammatory drug (NSAID), ibuprofen (Beauchamp et al., 2005) and this localized irritation is due to stimulation of the transient receptor potential cation channel A1 (TRPA1) (Peyrot des Gachons et al., 2011). The perceptual similarity between oleocanthal and ibuprofen spurred the hypothesis that these two compounds may possess similar pharmacological properties. Further investigation demonstrated that oleocanthal inhibits inflammation in the same way as ibuprofen, and moreover, is substantially more potent on a equimolar basis (Beauchamp et al., 2005). Subsequent studies have shown that oleocanthal exhibits various modes of action in reducing inflammatory-related disease, including neuro-degenerative disease (Pitt et al., 2009, Li et al., 2009), joint-degenerative disease (Iacono et al., 2010) and specific cancers (Elnagar et al., 2011). Therefore, long term consumption of extra virgin olive oil (EVOO) containing oleocanthal may contribute to the health benefits associated with the Mediterranean dietary pattern. This chapter summarizes the current knowledge on oleocanthal, in terms of its sensory and physiological properties, its extraction from the oil matrix and subsequent identification and quantification, and finally the factors that may influence the concentration of oleocanthal in EVOO.

2. Olive oil, a hallmark of the Mediterranean diet

The health benefits of following a Mediterranean eating pattern were first acknowledged in the Seven Countries Study (Keys, 1970). Thereafter over a period of 30 years, a number of investigators have reported that the Mediterranean diet is associated with low rates of degenerative diseases such as cardiovascular disease (CVD) (Estruch et al., 2006, Pitsavos et al., 2005), coronary heart disease (CHD) (Fung et al., 2009), stroke (Fung et al., 2009), certain types of cancers (La Vecchia, 2004, Dixon et al., 2007), diabetes (Martinez-Gonzalez et al., 2008), Alzheimer's disease (Scarmeas et al., 2009) and non-alcoholic fatty liver disease (Fraser et al., 2008). Research has also demonstrated that Mediterranean populations have

increased life expectancy (Hu, 2003, Visioli et al., 2005, Trichopoulou et al., 2005), reduced risk of developing disorders such as metabolic syndrome (Tortosa et al., 2007, Babio et al., 2008) and have decreased levels of systematic inflammation (Dai et al., 2009, Fragopoulou et al., 2010, Panagiotakos et al., 2009).

The traditional Mediterranean diet is defined as the pattern of eating observed in the olive growing areas of the Mediterranean region, namely Greece and Southern Italy in the 1960s. An integral component of this dietary pattern is the consumption of EVOO (Stark and Madar, 2002, Kok and Kromhout, 2004). EVOO, the pillar of Mediterranean recipes, is commonly incorporated into cooked dishes as well as in salads. Typically, the intake of EVOO ranges from 25 to 50 ml per day in the Mediterranean diet (Corona et al., 2009). Therefore, the apparent health benefits have been partially attributed to the dietary consumption of EVOO by Mediterranean populations. Figure 1 displays the Mediterranean diet pyramid featuring EVOO as a core component of this dietary pattern.

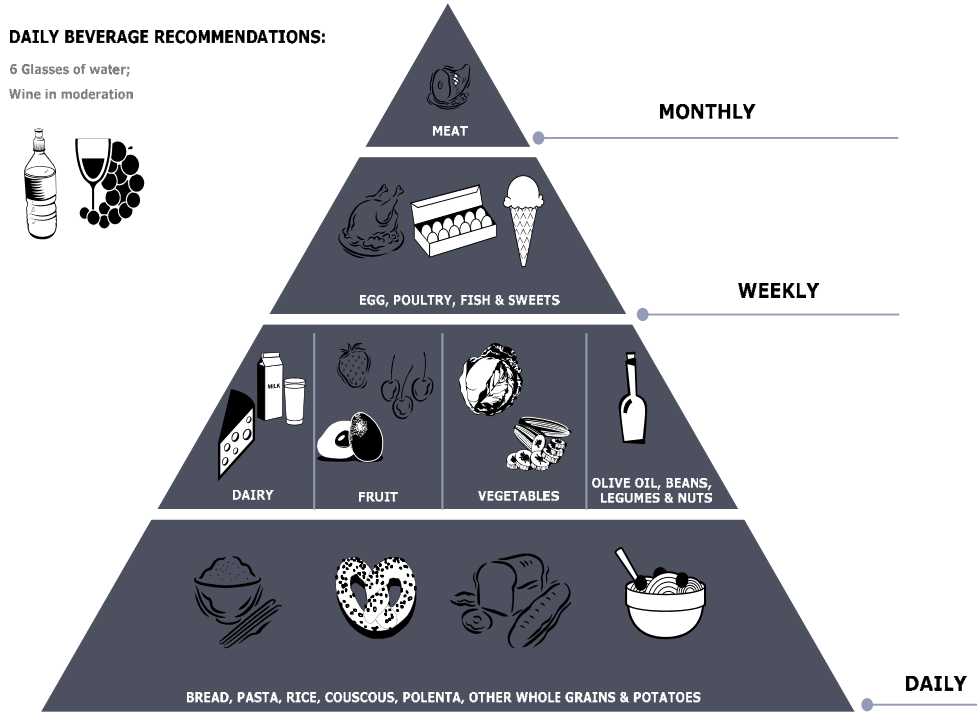

Fig. 1. Food pyramid reflecting the traditional Mediterranean diet. As depicted, EVOO is an integral food component of this diet residing in the consume 'daily' food group.

3. Olive oil phenolic compounds

Historically, the health promoting properties of EVOO were attributed to the high concentration of monounsaturated fatty acids (MUFAs), in particular oleic acid, contained in EVOO. However other seed oils (i.e. sunflower, soybean, and rapeseed), which also contain high concentrations of oleic acid, do not exhibit the same health benefits as EVOO (López-

Miranda et al., 2010, Harper et al., 2006, Aguilera et al., 2004). In addition to oleic acid, EVOO contains a minor, yet significant phenolic fraction that other seed oils lack and this fraction has generated much interest regarding its health promoting properties.

Currently, 36 phenolic compounds have been identified in EVOO and studies (human, animal, *in vivo* and *in vitro*) have demonstrated that olive oil phenolics have positive effects on certain physiological parameters such as plasma lipoproteins, oxidative damage, inflammatory markers, platelet and cellular function, antimicrobial activity, and bone health (for review see (Cicerale et al., 2010)), possibly reducing the risk of chronic disease development.

4. Discovery of oleocanthal

The phenolic compound (-)- decarboxymethyl ligstroside aglycone was first reported in EVOO by Montedoro *et al.* (Montedoro and Servili, 1993) in 1993 (Figure 2). A decade after its discovery, Andrewes and colleagues (Andrewes et al., 2003) reported that decarboxymethyl ligstroside aglycone was responsible for the throat irritation and pungency elicited by some EVOOs. In 2005, Beauchamp *et al.* (Beauchamp et al., 2005) confirmed that the phenolic compound, decarboxymethyl ligstroside aglycone was indeed responsible for the throat irritation elicited by EVOOs post-ingestion. This confirmation was carried out by isolating the compound from various EVOOs and measuring the throat irritation elicited. However, at that stage, there was a possibility that co-elution of a minor component or a mixture of components along with decarboxymethyl ligstroside aglycone may collectively cause the localized throat irritation. Therefore, the authors chemically synthesized decarboxymethyl ligstroside aglycone and dissolved it in non-irritating corn oil. Throat irritation elicited by the synthesized decarboxymethyl ligstroside aglycone was found to be dose-dependent on the concentration of this phenolic in corn oil and mimicked that of EVOO containing this compound naturally. Decarboxymethyl ligstroside aglycone was thus deemed the sole throat irritant in EVOO and was named oleocanthal (*oleo* for olive, *canth* for sting, and *al* for aldehyde) (Beauchamp et al., 2005).

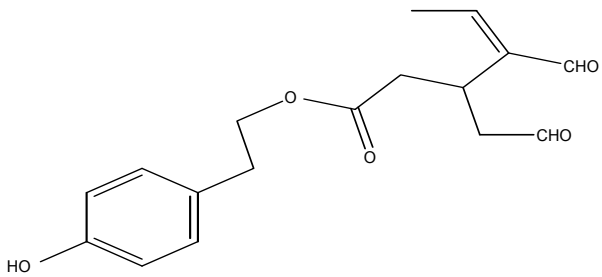

Fig. 2. Oleocanthal structure

5. Sensory properties of oleocanthal

The intake of EVOO is often associated with a peppery sting that is localized to the oropharyngeal region in the oral cavity (Figure 3). There is wide inter-individual variation in sensitivity to oleocanthal which can range from a slight irritation in the throat, to an irritation that is strong enough to produce a cough in those highly sensitive. Of particular

interest is the spatial location of irritation produced by oleocanthal. Irritating, pungent compounds often aggravate all regions in the oral cavity rather than acting on one localized area (Peyrot des Gachons et al., 2011), which implies a sensory receptor specific to oleocanthal exists in the oropharyngeal region of the oral cavity. Recent investigations have indeed verified that the TRPA1 is the receptor linked to oleocanthal and the anatomical location of this receptor has been found in the oropharyngeal region of the oral cavity (Peyrot des Gachons et al., 2011).

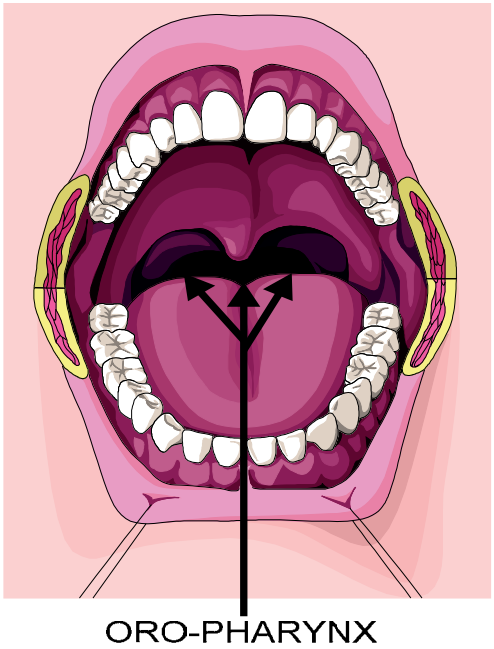

Fig. 3. Oleocanthal irritation occurs solely in the oropharyngeal region (area shaded in black) of the oral cavity.

A large variability among subjects in the perceived irritation from oleocanthal has been noted (Cicerale et al., 2009a). Such individual variation in perception of oleocanthal may be related directly to the quantity of the TRPA1 receptor, as has been reported for other oral stimuli such as the bitter compounds: 6-n-propylthiouracil (PROP) and phenylthiocarbamide (PTC) (Hansen et al., 2006), which activate the TAS2R38 bitter receptor (Hayes et al., 2011). The important link between the perceptual aspects of oleocanthal and health benefits is the notion that variation in sensitivity to oleocanthal irritation may relate to potential differences in sensitivity to the anti-inflammatory action of this compound. However, further research is required to investigate this.

6. Physiological properties of oleocanthal and its putative health benefits

Research conducted by Beauchamp and colleagues (Beauchamp et al., 2005) demonstrated that oleocanthal inhibits cyclooxygenase (COX) enzymes in a dose-dependent manner, mimicking the anti-inflammatory action exerted by ibuprofen. Cyclooxygenase 1 and 2 (COX 1 and COX 2) enzymes are responsible for the conversion of arachidonic acid to

prostaglandins and thromboxane which are produced in response to inflammatory or toxic stimuli. COX 1 and COX 2 can be harmful to the body. In particular, COX 2 has been implicated in the pathogenesis of several cancers in both human and animal studies (Harris et al., 2003, Boland et al., 2004, Subbaramaiah et al., 2002, Ristimäki et al., 2002), and may also play a role in atherosclerosis (Chenevard et al., 2003). The novel findings presented by Beauchamp and colleagues (Beauchamp et al., 2005) demonstrate that oleocanthal not only mimics the mode of ibuprofen action, it exhibits increased potency (compared with ibuprofen) in inhibiting COX 1 and COX 2 enzymes at equimolar concentrations. For instance, oleocanthal (25 μM) inhibited 41-57% of COX activity in comparison to ibuprofen (25 μM) which inhibited only 13-18% of COX activity.

Moreover, Beauchamp and colleagues (Beauchamp et al., 2005) put forth the suggestion that chronic ingestion of small quantities of oleocanthal via EVOO consumption, may be responsible, in part, for the lowered prevalence of disease associated with the Mediterranean diet. Thus, if an olive oil consumer ingests around 50 g of EVOO a day containing approximately 200 μg/kg of oleocanthal, the person would ingest approximately 10 mg/day of oleocanthal. This would equate to a relatively low (10%) equivalent dose of ibuprofen (recommended for adult pain relief). Chronic low doses of ibuprofen and other COX inhibitors such as aspirin are known to have important health benefits in the prevention of cancer development (e.g. colon and breast) (Garcia-Rodriguez and Huerta-Alvarez, 2001, Harris et al., 2006) and CVD (Hennekens, 2002). Therefore, long term ingestion of oleocanthal via EVOO consumption may contribute to a reduction in chronic disease development and certainly emerging evidence supports this notion.

Finally, it is important to note oleocanthal's bioavailability within the body. Only one study has investigated this to date. A study by Garcia-Villalba et al. (Garcia-Villalba et al., 2010) noted that oleocanthal was readily metabolized however further studies are required to gain a more thorough understanding of the metabolism and bioavailability of this compound.

6.1 Oleocanthal and neuro-degenerative disease

Ibuprofen is known to exert beneficial effects on markers of neuro-degenerative disease (Van Dam et al., 2008) and based on the similar oral irritant properties and shared anti-inflammatory mode of action via COX inhibition, oleocanthal was investigated for potential neuro-protective properties. Li and collegues (Li et al., 2009) presented significant findings demonstrating that oleocanthal inhibits tau fibrillization *in vitro* by forming an adduct with PHF6 peptide, which is a VQIXXK motif that resides in the microtubule binding region, and is crucial in the formation of tau fibrils (Li and Virginia, 2006) Hyperphosphorylated tangles of tau are lesions that are observed in neuro-degenerative disease (i.e. Alzheimer's disease) and PHF6 enables the phosphorylation of tau, thus the convalent modification of PHF6 peptide disrupts tau-tau interaction and subsequent fibril formation (Li et al., 2009) (Figure 4). B-amyloid peptides (Aβ) are another type of lesion that are characteristic of Alzheimer's disease (Guela et al., 1998), as Aβ derived diffusible ligands (ADDLs) are neurotoxic factors proposed to instigate the onset of Alzheimer's disease (Pitt et al., 2009). Pitt and colleagues (2009) demonstrated that *in vitro*, oleocanthal alters the structure of ADDLs and augments antibody clearance of ADDLs, therefore protecting hippocampal neurons from ADDL toxicity. This data supports research showing a 40% decrease in Alzheimer's disease in populations consuming a Mediterranean style diet (Scarmeas et al., 2009).

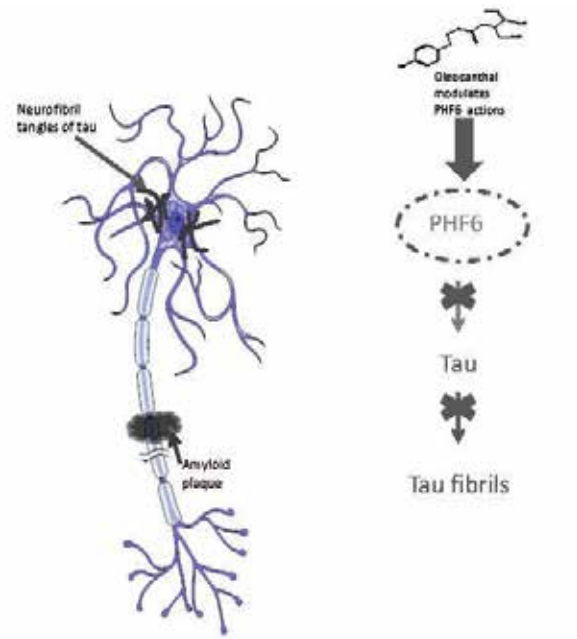

Fig. 4. Oleocanthal inhibits tau fibrilisation by covalently modifying the PHF-6 peptide which is crucial for the formation of tau fibrils. The fibrilisation of tau leads to neurofibrillary tangles which are inherently associated with neurodegenerative diseases such as Alzheimer's disease.

6.2 Oleocanthal and joint-degenerative disease

In vitro research draws attention to oleocanthal, as a potential therapeutic compound that may be of interest in the quest to find suitable natural NSAIDs for the treatment of joint degenerative disease. Pro-inflammatory cytokines up-regulate the synthesis of cartilage to degrading enzymes, and stimulate nitric oxide (NO) production (Scher et al., 2007), as well as increase prostaglandin PGE_2 production, which have all been implicated in the development of arthritic pain and thus joint-degenerative disease. COX enzymes are a catalyst for the formation of prostaglandins and have also been reported to be highly expressed in arthritic spine in an animal model (Procházková et al., 2009). Therefore, oleocanthal may influence arthritic pain through inhibition of PGE_2 synthesis accompanying COX inhibition.

NO plays an integral role in joint-degenerative disease and the stable end product of NO, nitrite (NO_2), is significantly expressed in arthritic synovial fluid (Iacono et al., 2010). In osteoarthritis arthritis (OA) pathogenesis, diseased cartilage synthesizes NO spontaneously from diseased chrondocytes (Tung et al., 2002). NO is biosynthesized by nitric oxide synthase (NOS). Another form of NOS is inducible NOS (iNOS) which is largely responsible for the inflammatory actions of NOS (Espey et al., 2000) (Figure 5). Iacano and collegues (2010) have shown that oleocanthal and synthesized derivatives, decrease production of iNOS protein expression in LPS challenged murine chondrocytes, dose dependently, further highlighting the anti-inflammatory actions of oleocanthal and the pharmacological potential. Also, as oleocanthal mediates prostaglandin synthesis via inhibitory actions on COX enzymes, it is possible that oleocanthal may exert pharmacological actions in the treatment of both rheumatoid arthritis and osteoarthritis through COX inhibition.

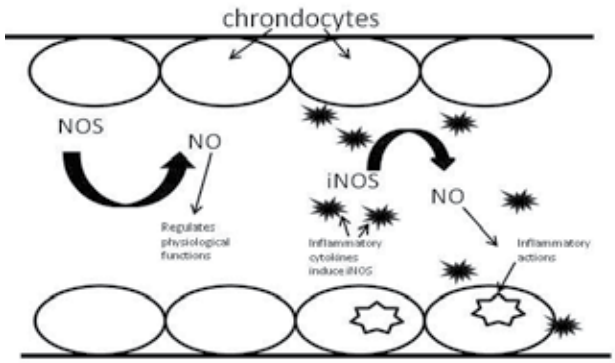

Fig. 5. Nitric oxide (NO) derived from nitric oxide synthase (NOS), functions as a neurotransmitter and vasodilator, and is important in normal physiological responses. Inducible nitric oxide synthase (iNOS) is a third form of NOS and is not present in resting cells, but rather is induced by inflammatory cytokines. NO produced from iNOS promotes inflammation in chrondocytes and is associated with cartilage degenerative diseases such as osteoarthritis.

6.3 Oleocanthal and cancer

Recent evidence demonstrates that oleocanthal may exert therapeutic properties against the pathogenesis of c-Met kinase induced malignancies. Elnagar and colleagues (Elnagar et al., 2011) have reported that oleocanthal possesses anti-proliferative effects in human breast and prostate cancer lines (Elnagar et al., 2011). Also Khanal and collegues (Khanal et al., 2011) recently showed that oleocanthal has an anti-proliferative effect and prevents tumour induced cell transformation in mouse epidermal JB6 Cl41cells. The mechanism of action in which oleocanthal achieved this was via the inhibition of extracellaullar signal-regulated kinases 1/2 and p90RSK phosphorylation. Furthermore, oleocanthal has also been shown to promote cell apoptosis by activating caspase-3 and poly-adenosine diphosphate-ribose polymerase, the phosphorylation of p53 (Ser15), and also induced fragmentation of DNA in HT-29 cells derived from human colon adenocarcinoma (Khanal et al., 2011). These findings suggest that oleocanthal may have potential as a therapeutic agent in the inhibition of carcinoma progression and supports substantial evidence that populations residing in the Mediterranean region have a reduced incidence of prostate, breast, lung and gastrointestinal cancer (Trichopoulou et al., 2000, La Vecchia, 2004, Fortes et al., 2003, Dixon et al., 2007). It is important to note that while there is strong evidence that oleocanthal is an effective anti-inflammatory agent and demonstrates pharmacological characteristics *in vitro,* future *in vivo* studies are required to fully elucidate the efficacy of this natural NSAID. Caution is required when extrapolating results from a single compound out of the matrix in which it normally exists. Oleocanthal is one of many phenolic compounds contained in EVOO, and it is probable that the synergistic and interactive actions of these phenolics combined are responsible for the low incidence of chronic inflammation associated with EVOO intake. Furthermore, the bioavailability of oleocanthal needs to be firmly established in future research to consolidate the pharmacological potential of this compound.

7. Extraction, identification and quantification of oleocanthal

The method used for the extraction, identification and quantification of oleocanthal described herein, is selective for oleocanthal and was developed by Beauchamp and co-workers (Beauchamp et al., 2005). More recently, this method was adapted and involves the quantification of oleocanthal using an internal standard (ISTD), 3,5 dimethoxyphenol (Beauchamp et al., 2005, Cicerale et al., 2009b).

The extraction of oleocanthal from the oil matrix involves liquid-liquid partitioning using both hexane and acetonitrile, whereby the phenolic fraction partitions into the acetonitrile phase. Acetonitrile is then removed and the dried down extract is dissolved in methanol:water and analyzed by HPLC. Separation of the oleocanthal phenolic compound is carried out using a HPLC system with a diode array detector set to 278 nm. A reverse phase-C18 column (250 mm × 4.6 mm ID, 5 µm) is used for the separation at a constant temperature of 25°C using the gradients listed in Table 1. A flow rate of 1 ml/min is used, and the injection volume is 20 µl. See Figure 6 for HPLC chromatogram.

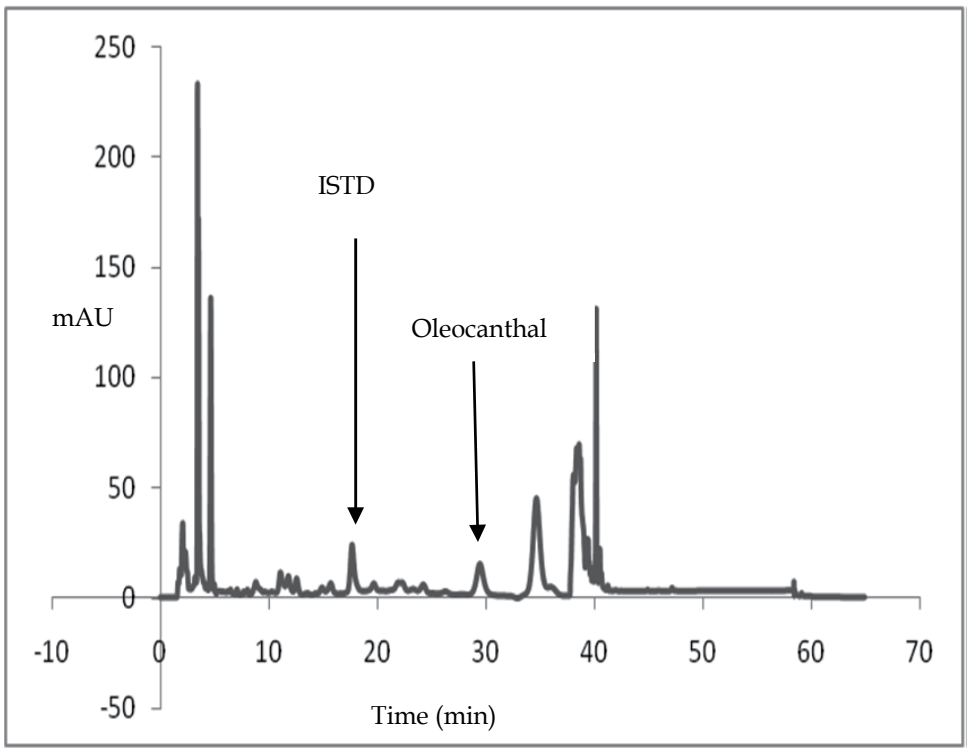

Fig. 6. HPLC chromatogram of olive oil phenolic extract containing oleocanthal and 3,5 dimethoxyphenol (ISTD) (Cicerale et al., 2009b).

Via mass spectrometry (6210 MSDTOF), oleocanthal is further identified under the following conditions: drying gas, nitrogen (N_2) (7 mL^{-1}, 350°C); nebulizer gas, N_2 (15 psi); capillary voltage 4.0 kV; vaporizer temperature, 350°C; and cone voltage, 60 V. Figure 7 displays the negative ion mass spectrum of oleocanthal with the characteristic $[M-H]^-$ ion at *m/z* 303.12 highlighted.

Time	Gradient
0-35 min	75% water, 25% acetonitrile
36-55 min	90% acetonitrile, 10% methanol
56-65 min	75% water, 25% acetonitrile

Table 1. Mobile phase gradient for oleocanthal separation.

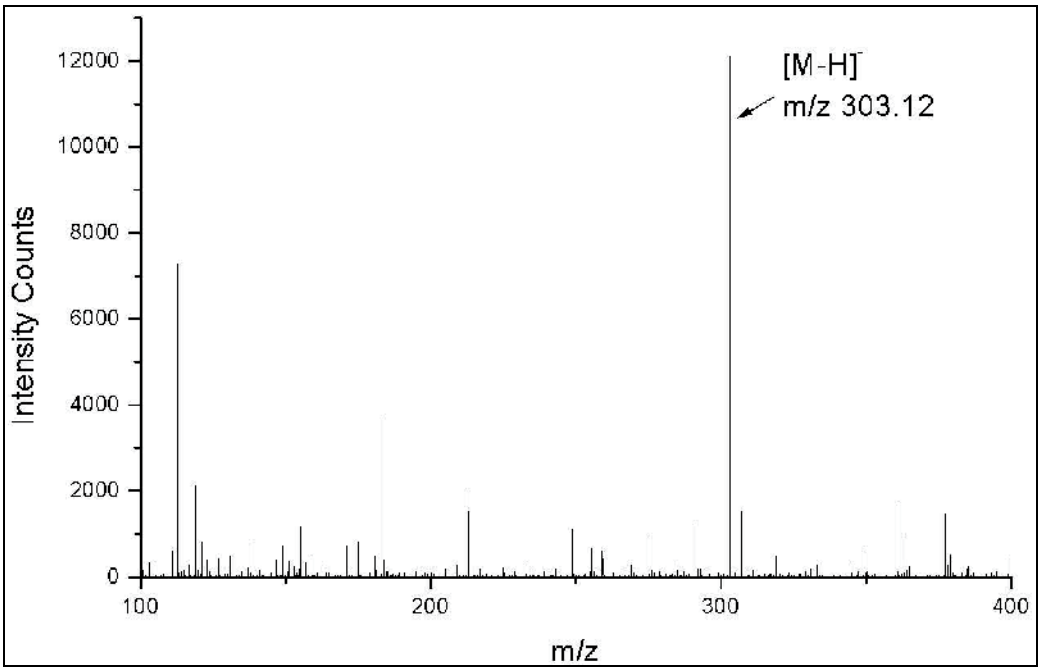

Fig. 7. Negative ion mass spectrum of oleocanthal with the characteristic $[M-H]^-$ ion at *m/z* 303.12 (Cicerale et al., 2009b).

8. Oleocanthal concentration in olive oil and factors which may affect its concentration

Oleocanthal concentration in EVOO is highly variable, ranging from as little as 0.2 mg/kg to as high as 498 mg/kg (Gomez-Rico et al., 2006). Such variation in oleocanthal concentration amongst differing EVOOs may be due to multiple factors that have the capacity to modify the concentration of this compound (Carrasco-Pancorbo et al., 2005). These factors include: method of phenolic extraction and quantification, geographic region of olive growth, olive tree cultivar, agricultural techniques applied to cultivate olives, olive maturity, processing of the olives to oil, storage of oil, and domestic heat application to oil.

8.1 Extraction and quantification

The analytical method used to quantify phenolics present in EVOO has an influence on the reported concentration and therefore is an important consideration when interpreting and comparing the data of such investigations (Carrasco-Pancorbo et al., 2005). Regarding the

analysis of oleocanthal, Beauchamp and colleagues (Beauchamp et al., 2005) in collaboration with Impellizzeri et al. (Impellizzeri and Lin, 2006), developed an extraction and quantification method specific for this compound. Utilizing this method, Impellizeri et al. (Impellizzeri and Lin, 2006) and Franconi et al. (Franconi et al., 2006) found the concentration of oleocanthal ranged between 8.3 ± 4.0 and 189.9 ± 2.7 mg/kg. An adaption of the Beauchamp and co-workers' (Beauchamp et al., 2005) methodology, was also utilized in studies by Cicerale and colleagues (Cicerale et al., 2009a, Cicerale et al., 2009b, Cicerale et al., 2011b, Cicerale et al., 2011a). The resultant oleocanthal concentrations in these investigations were similar to those found by Franconi et al. (Franconi et al., 2006) and Impellizzeri et al. (Impellizzeri and Lin, 2006) (53.9 ± 7.7 to 152.2 ± 10.5 mg/kg). However, a number of methods not specific for oleocanthal have also been used to quantify this compound and may account partially for the large variation in oleocanthal concentration observed (5.0 ± 0.3 – 498.0 ± 47.0 mg/kg) (Vierhuis et al., 2001, Servili et al., 2007b, Servili et al., 2007a, Romero et al., 2002, Morello et al., 2004, Tovar et al., 2001, De Stefano et al., 1999, Montedoro et al., 1992, Gomez-Alonso et al., 2003, Gomez-Rico et al., 2006, Allouche et al., 2007)

8.2 Geographic region

Geographic region in which olives are grown has been shown to be an important factor in regards to phenolic composition and concentration in general (Vinha et al., 2005, Cerretani et al., 2005). Beauchamp et al. (Beauchamp et al., 2005) demonstrated that EVOOs produced in differing countries had variable oleocanthal concentrations. For instance, EVOO produced in the U.S.A., contained a low concentration of oleocanthal (22.6 ± 0.6 mg/kg), however EVOOs produced in Italy contained some of the highest quantities of this compound (up to 191.8 ± 2.7 mg/kg).

8.3 Cultivar

Several studies have demonstrated differences between olive cultivar and oleocanthal concentration in the oil produced. In one study, the Coratina cultivar EVOO contained 78.2 ± 0.5 mg/kg oleocanthal, whereas the Oliarola cultivar EVOO contained 21.0 ± 0.8 mg/kg oleocanthal, a 3-fold difference (De Stefano et al., 1999). In another study, EVOO produced from the Frantoio cultivar had an oleocanthal concentration of 43.8 ± 3.1 mg/kg, whilst EVOO obtained from the Coratina cultivar, contained a 2-fold higher oleocanthal content at 92.8 ± 7.8 mg/kg (Servili et al., 2007b). A study by Franconi and colleagues (Franconi et al., 2006) also showed significant differences in oleocanthal concentration amongst differing olive cultivars. For instance, an oleocanthal concentration of 8.3 ± 4.0 mg/kg and 53.0 ± 12.0 mg/kg in EVOOs produced from the Taggiasca and Seggianese cultivars respectively, was noted.

8.4 Agricultural methods

The concentration of phenolic compounds in EVOO is greatly affected by agricultural techniques used in the cultivation of olive fruit (Gomez-Rico et al., 2006, Ayton et al., 2007). Tovar and co-workers (Tovar et al., 2001) demonstrated that with increased irrigation applied to the olive tree, oleocanthal concentration decreased. For instance, in the EVOO obtained from the least irrigated olive trees (46 mm water per year) oleocanthal concentration was determined to be 50.9 ± 6.5 mg/kg. For the EVOO produced from highly irrigated olive trees (259 mm water per year), oleocanthal concentration was 23.1 ± 1.3 mg/kg. Gomez-Rico et al.

(Gomez-Rico et al., 2006) also demonstrated the negative effect of irrigation on oleocanthal concentration. Rain-fed olive trees produced EVOO containing higher concentrations of oleocanthal (229.0 ± 48.0 to 498 ± 47.0 mg/kg) compared to those that underwent the highest amount of irrigation (206 mm water per year), (119.0 ± 36.0 to 336.0 ± 81.0 mg/kg). Two additional studies (Romero et al., 2002, Servili et al., 2007a) are also in agreement with this data in that, they both observed a 37-38% decrease in oleocanthal concentration amongst the EVOOs from the highly irrigated olive trees compared to those least irrigated.

8.5 Olive maturation

Maturation of the olive fruit at harvest is an important predictor of the phenolic composition and concentration in EVOO. In regards to oleocanthal, one study found that with extended picking date and increased olive fruit ripeness, the concentration of oleocanthal in EVOO decreased by 43% (148.0 mg/kg to 84.0 mg/kg) over a short two month period (Morello et al., 2004). The researchers, Gomez-Rico et al. (Gomez-Rico et al., 2006) also observed a similar decrease of 20% and 54% in oleocanthal with increasing maturity index using two olive cohorts.

8.6 Processing

In general, the processing of olive fruit to oil has a substantial effect on the concentrations of phenolic compounds in EVOO (Kalua et al., 2006b, Cerretani et al., 2005, Vierhuis et al., 2001, Romero et al., 2004, Gimeno et al., 2002). EVOO produced by the traditional processing method (whereby the entire olive fruit is crushed, including the stone), was found to contain lower quantities of oleocanthal (43.8 ± 3.1 mg/kg) compared to EVOO produced by the stoning method in which the olive stone is removed before crushing (54.8 ± 3.1 mg/kg). The researchers from this study hypothesized that the differences may be due to the increased peroxidase (POD) activity that tends to accompany the crushed olive stone, which has an oxidizing effect on oleocanthal concentration (Servili et al., 2007b).

EVOO produced under nitrogen (N_2) flushing and with use of enzymatic treatment (which aids cell wall degradation and thus improves phenolic extraction) (Vierhuis et al., 2001), was found to contain oleocanthal concentrations of 31.4 ± 1.0 mg/kg. EVOO produced with no added enzymes and without nitrogen (N_2) flushing (therefore allowing oxygen (O_2) to be present) was found to contain a lower amount of oleocanthal (24.8 ± 1.9 mg/kg). EVOOs produced with use of N_2 flushing alone and enzymatic treatment alone, contained 28.4 ± 1.4 mg/kg and 29.4 ± 0.8 mg/kg oleocanthal respectively (Vierhuis et al., 2001).

EVOOs produced using two-phase centrifugation which uses no added water in the processing method, was found to contain a higher phenolic concentration compared to EVOOs obtained from three-phase centrifugation which utilizes a considerable amount of water (approximately 400 L/h) (De Stefano et al., 1999). De Stefano and et al. (De Stefano et al., 1999) found oleocanthal concentration in EVOO obtained from the two-phase centrifuge to be higher (78.2 ± 0.5 mg/kg) than that produced from the three-phase method (67.3 ± 2.6 mg/kg). The addition of water in the three-phase centrifugation method, may have a reducing effect on the more water-soluble phenolics from the oil phase during processing, thus reducing the concentration of oleocanthal in the resultant EVOO (Cicerale et al., 2009c).

8.7 Storage of EVOO

Immediately following oil extraction from the olive fruit, there is potential for the phenolic quality of the oil to decline, via oxidation catalysed by oxygen (O_2) and light (Kalua et al., 2006a, Morello, 2004). One study to date, has investigated the effect O_2, light, and storage time have on oleocanthal concentration. In this study, oleocanthal concentration decreased somewhat (15 - 37%) over a 10-month storage period, depending on the storage conditions. The largest decrease was seen in EVOO stored under exposure to O_2 and light (37%) and the smallest loss was found in the EVOO stored under O_2 and light limiting conditions (15%). Oils stored under sole exposure to O_2 or light were found to have a similar rate of oleocanthal degradation over the 10-month period (28% and 25% respectively) (Cicerale et al., 2011b).

8.8 Domestic heat application

In general, research has shown that olive oil phenolic compounds are subject to degradation upon the application of heat during cooking (Brenes et al., 2002, Gomez-Alonso et al., 2003). However, oleocanthal has demonstrated to be relatively stable upon heating when the EVOO contains a considerable quantity of this compound initially.

One study found a 20% decrease in oleocanthal (96.7 ± 8.5 to 77.5 ± 2.4 mg/kg) upon 12 frying operations (each frying operation 10 min in length, at a temperature of 180°C) (Gomez-Alonso et al., 2003). Similarly, another study observed a 24% decrease (41.5 ± 0.3 to 31.4 ± 0.1 mg/kg) in oleocanthal after heating at 180°C for 36 hr (Allouche et al., 2007). However, for EVOO which naturally contained a lower quantity of oleocanthal to begin with (7.9 ± 0.5 mg/kg), oleocanthal degradation was substantially higher at 71%. It appears that oleocanthal possesses an antioxidative effect, in that oleocanthal is able to withstand heating and therefore protect itself to a greater degree when there is a higher concentration of it in EVOO.

Cicerale and co-workers (Cicerale et al., 2009b) also demonstrated oleocanthal to be stable upon heating at high temperatures (240°C) for extended periods of time (up to 90 min). The authors postulated that the minimal degradation of oleocanthal may be partially due to the chemical structure of this compound and subsequent antioxidant activity. The antioxidant capacity of phenolic compounds is dependent upon the number of hydroxyl groups bonded to the aromatic ring (Sroka and Cisowski, 2003). When free radicals are produced through oxidation, phenolic compounds with a higher number of hydroxyls and therefore increased antioxidant capacity, quickly diminish because they react rapidly with lipid radicals and are therefore consumed (Gomez-Alonso et al., 2003). Oleocanthal possesses one hydroxyl group. Moreover, the site of bonding and mutual position of hydroxyls in the aromatic ring was also postulated to play a role in the antioxidant potential of phenolic compounds (Sroka and Cisowski, 2003, Rice-Evans et al., 1996). Rice-Evans and co-workers (Rice-Evans et al., 1996) reported that a hydroxyl group in the *ortho* position in the aromatic ring results in increased antioxidant capacity compared to compounds with hydroxyl groups in the *meta* and *para* positions. Oleocanthal contains its one hydroxyl group in the *para* position. These structural features may help in explaining why a minimal degradation of oleocanthal was observed (Cicerale et al., 2009c).

9. Perspectives and future directions

In summary, EVOO a key component of the Mediterranean diet contains a number of phenolics, one being oleocanthal. The unique sensory qualities and anti-inflammatory actions

of oleocanthal, have prompted research to further verify its therapeutic potential. Oleocanthal has been shown, *in vitro*, to exert beneficial physiological effects in terms of neuro-degenerative disease, joint-degenerative disease and cancer. Therefore, it has been postulated that the long term ingestion of this compound via EVOO consumption may have significant health promoting action over time, thereby reducing the development of chronic disease.

However, the studies conducted on the health promoting potential of oleocanthal have involved *in vitro* investigations and it is difficult to extrapolate data from *in vitro* studies to actual physiological benefits. *In vivo* research is therefore required to substantiate the *in vitro* findings. Furthermore, the bioavailability of oleocanthal has not been adequately investigated. To date, only one study has reported on the post-ingestive fate of oleocanthal, noting that it was readily metabolized however the mechanism was not investigated. The degree to which oleocanthal is metabolized is an important consideration when reviewing the health benefits associated with ingestion, and further research on this is warranted. The link between the perceptual aspects of oleocanthal and health benefits is the notion that variation in sensitivity to oleocanthal oro-pharyngeal irritation may relate to potential differences in sensitivity to the anti-inflammatory action of this compound and this is also worthy of future investigations.

Finally, it was not the purpose of this overview to attribute the health benefits of the Mediterranean diet solely to one component and we did not aim to credit oleocanthal as being the lone therapeutic agent contained in EVOO. Other constituents of the Mediterranean diet and EVOO will contribute, either with independent actions, or in a synergistic and complementary manner to impart beneficial health effects (Lucas et al., 2011, Fogliano and Sacchi, 2006). However, the studies conducted to date investigating the pharmacological actions of oleocanthal are encouraging and show that this compound possesses substantial health benefiting properties. Further research will no doubt provide new insight into the pharmacological potential of oleocanthal and assess the role that oleocanthal has in the clinical treatment of chronic disease.

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Biological Properties of Hydroxytyrosol and Its Derivatives

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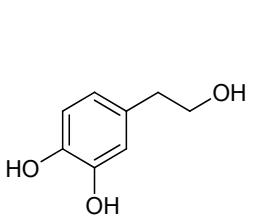
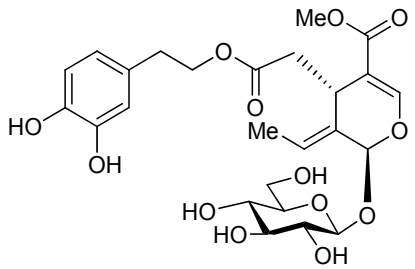
1. Introduction

Polyphenols are a wide family of compounds found in fruits and vegetables, wine, tea, cocoa, and extra-virgin olive oil, which exhibit strong antioxidant activity by scavenging different families of Reactive Oxygen Species (ROS). One of the most effective members of the polyphenol family in terms of free radical scavenging is hydroxytyrosol, 2-(3,4-dihydroxyphenyl)ethanol (Fernández-Bolaños et al., 2008), a simple phenol found predominantly in olive tree (*Olea europaea*).

Hydroxytyrosol (HT) can be found in leaves and fruits of olive, extra virgin olive oil and it is specially abundant in olive oil mill wastewaters from where it can be recovered (Fernández-Bolaños et al., 2008; Sabatini, 2010). Hydroxytyrosol is a metabolite of oleuropein (Fig. 1), another major phenolic component of olive products; they both give to extra-virgin olive oil its bitter and pungent taste (Omar, 2010a). Hydroxytyrosol shows a broad spectrum of biological properties due to its strong antioxidant and radical-scavenging properties. More active than antioxidant vitamins and synthetic antioxidants, hydroxytyrosol exerts its antioxidant activity by transforming itself into a catechol quinone (Rietjens, 2007).



Hydroxytyrosol



Oleuropein

Fig. 1. Structures of hydroxytyrosol and oleuropein

2. Biological activity of hydroxytyrosol

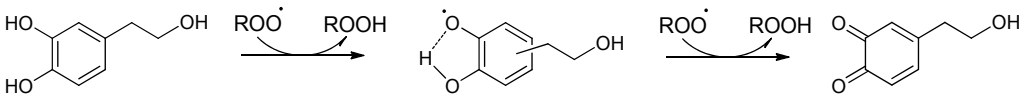
Historically, olive tree leaves were used for traditional therapy by ancient civilizations. Extracts from olive tree leaves were found to have a positive effect on hypertension by the

middle of last century (Scheller, 1955; Perrinjaquet-Moccetti et al. 2008; Susalit et al., 2011), and, since then, the benefits of minor olive components have been extensively investigated (Tripoli et al., 2005).

2.1 Antioxidant activity

The antioxidant activity is the most studied property of olive phenolic compounds. The interest of hydroxytyrosol is based on its remarkable pharmacological and antioxidant activities. Reactive oxygen species, which are continuously being formed as a result of metabolic processes in the organism, may cause oxidation and damage of cellular macromolecules, and therefore, may contribute to the development of degenerative diseases, such as atherosclerosis, cancer, diabetes, rheumatoid arthritis and other inflammatory diseases (Balsano & Alisi, 2009).

The high antioxidant efficiency of HT, attributed to the presence of the *o*-dihydroxyphenyl moiety, is due to its high capacity for free radical scavenging during the oxidation process and to its reducing power on Fe^{3+} (Torres de Pinedo et al., 2007). The antioxidant properties of the *o*-diphenols are associated with their ability to form intramolecular hydrogen bonds between the hydroxyl group and the phenoxy radical (Visioli et al., 1998); therefore, the catechol avoids the chain propagation by donating a hydrogen radical to alkylperoxyl radicals ($ROO^{\cdot}$) formed in the initiation step of lipid oxidation (Scheme 1).



Scheme 1. Mechanism of free radical scavenging by hydroxytyrosol

Oxidation of low-density lipoproteins (LDLs) is a lipid peroxidation chain reaction, which is initiated by free radicals. It has been shown that hydroxytyrosol can inhibit LDL oxidation efficiently due to its capacity to scavenge peroxyl radicals (Arouma et al., 1998; Turner et al., 2005). Hydroxytyrosol reduces oxidation of the low-density lipoproteins carrying cholesterol (LDL-C), which is a critical step in the development of atherosclerosis and other cardiovascular diseases (Gonzalez-Santiago et al., 2010; Vázquez-Velasco et al., 2011); hydroxytyrosol has also a potential protective effect against oxidative stress induced by *tert*-butyl hydroperoxide (Goya et al., 2007).

It has been reported that hydroxytyrosol enhances the lipid profile and antioxidant status preventing the development of atherosclerosis. This compound may also reduce the expression of vascular cell adhesion molecules (Carluccio et al., 2007) and inhibit platelet aggregation in rats (González-Correa et al., 2008a) and hypercholesterolaemia in humans (Ruano et al., 2007).

2.2 Anticancer activity

Numerous studies about the relationship between olive oil consumption and cancer prevention have been carried out (Pérez-Jiménez et al., 2005). Antioxidant compounds supplied in the diet can reduce the risk of cancer due to the fact that they can minimize DNA damage, lipid peroxidation and the amount of ROS generated (Omar, 2010a; Hillestrom, 2006; Manna, 2005).

It has been reported that HT may exert a pro-apoptotic effect by modulating the expression of genes involved in tumor cell proliferation of promyelocytes (HL60 cells) (Fabiani et al., 2006, 2008, 2009, 2011). Moreover, it has been shown that HT inhibits proliferation of human MCF-7 breast cancer cells (Siriani et al., 2010; Bulotta et al. 2011; Bouallagui et al., 2011a), human HT29 colon carcinoma cells (Guichard et al., 2006), human M14 melanoma cells (D'Angelo et al., 2005) and human PC3 prostate cells (Quiles et al., 2002).

Pre-treatment of HepG2 cells with hydroxytyrosol prevented cell damage, what could be due to the fact that hydroxytyrosol may prepare the antioxidant defense system of the cell to face oxidative stress conditions (Goya et al., 2007, 2010).

2.3 Osteoporosis

Hydroxytyrosol may have critical effects on the formation and maintenance of bone, and could be used as an effective remedy in the treatment of osteoporosis symptoms, as it can stimulate the deposition of calcium and inhibit the formation of multinucleated osteoclasts in a dose-dependent manner. HT also suppressed the bone loss of spongy bone in femurs of ovariectomized mice (Hagiwara et al., 2011).

2.4 Antimicrobial activity

Antimicrobial activity of oleuropein, tyrosol and hydroxytyrosol has been studied *in vitro* against bacteria, viruses and protozoa (Bisignano et al., 1999).

The *in vitro* antimycoplasmal activity of HT has been investigated, concluding that this compound might be considered as an antimicrobial agent for treating human infections caused by bacterial strains or casual agents of intestinal or respiratory tract (Furneri et al., 2004).

It has been shown that polyphenols from olive oil are powerful anti-*Helicobacter Pylori* compounds *in vitro* (Romero et al., 2007), a bacteria linked to a majority of peptic ulcers and to some types of gastric cancer.

2.5 Antiinflammatory activity

Inflammation and its consequences play a crucial role in the development of atherosclerosis and cardiovascular diseases. Polyphenols have been shown to decrease the production of inflammatory markers, such as leukotriene B4, in several systems (Biesalski, 2007).

The effect of hydroxytyrosol on platelet function has been tested. Hydroxytyrosol was proven to inhibit the chemically induced aggregation, the accumulation of the pro-aggregant agent thromboxane in human serum, the production of the pro-inflammatory molecules leukotrienes and the activity of arachidonate lipoxygenase (Visioli et al., 2002).

Recently, it has been described that HT-20, an olive oil extract containing about 20% of hydroxytyrosol, inhibits inflammatory swelling and hyperalgesia, and suppresses proinflammatory cytokine in a rat inflammation model (Gong et al., 2009).

2.6 Antiviral activity

Hydroxytyrosol and oleuropein have been identified as a unique class of HIV-1 inhibitors that prevent HIV from entering into the host cell and binding the catalytic site of the HIV-1

integrase. Thus, these agents provide an advantage over other antiviral therapies in which both, viral entry and integration, are inhibited (Lee-Huang et al., 2007a, 2007b, 2009).

HT and its derivatives are also useful, when applied topically, as microbicide for preventing HIV-infection, as well as other sexually transmitted diseases caused by fungi, bacteria or viruses (Gómez-Acebo et al., 2011). Furthermore, it has been reported that hydroxytyrosol inactivated influenza A viruses, suggesting that the mechanism of the antiviral effect of HT might require the presence of a viral envelope (Yamada et al., 2009).

2.7 Hydroxytyrosol as an antinitrosating agent

The antinitrosating properties of hydroxytyrosol and other plant polyphenols of dietary relevance have been investigated (De Lucia et al., 2008). It has been shown that HT reacts with sodium nitrite at pH 3 to give 2-nitrohydroxytyrosol, supporting a protective role of HT as an efficient scavenger of nitrosating species (Fig. 2).

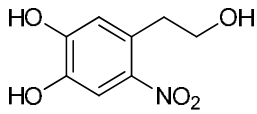


Fig. 2. 2-Nitrohydroxytyrosol formed by nitrosation of HT

3. Hydroxytyrosol derivatives

3.1 Lipophilic hydroxytyrosol esters

Many different hydroxytyrosol lipophilic analogues occur naturally in olive fruit and in virgin olive oil. The amount of these compounds is related to olive variety and ripeness, climate, location, type of crushing machine and oil extraction procedures. As an example, the concentration of hydroxytyrosyl acetate is similar to that of HT in some olive oil varieties such as Arbequina, twice as high in the Picual variety, and between one third and one fourth in the Manzanilla and Hojiblanca oils (Romero et al., 2007).

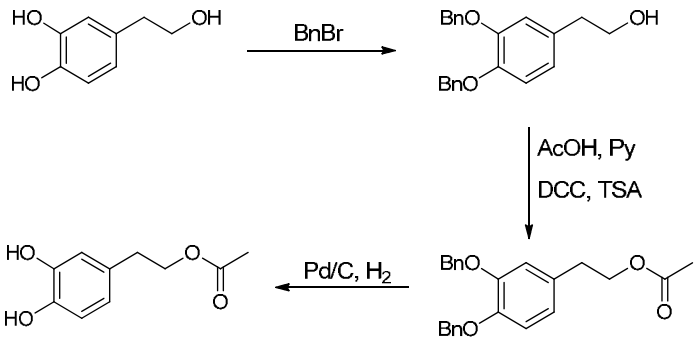
Due to the limited solubility of HT in lipid media, the search for new lipophilic hydroxytyrosol esters with enhanced properties is of great interest, both in food industry and in medicine. Studies on olive polyphenols have shown the importance of the lipophilicity of the antioxidants on the cell uptake and membrane crossing, and on the substrate to be protected (membrane constituents or LDL), (Grasso et al., 2007). These facts explain the efforts made in the development of new synthetic analogues with increased lipophilicity.

3.1.1 Synthetic approaches

Phenolic acids, such as caffeic acid, have been esterified with good chemoselectivity in the presence of strong protic acids (Fischer esterification), but the severe reaction conditions together with the large excess of alcohol required make this strategy of limited applicability (Burke et al., 1995). Under basic catalysis, phenols can be easily deprotonated, so the esterification of phenolic alcohols and phenolic acids via acyl nucleophilic substitution requires previous protection of the phenolic hydroxyl groups, due to the competition between aliphatic and phenolic hydroxyl groups (Appendino et al., 2002; Gambacorta et al., 2007).

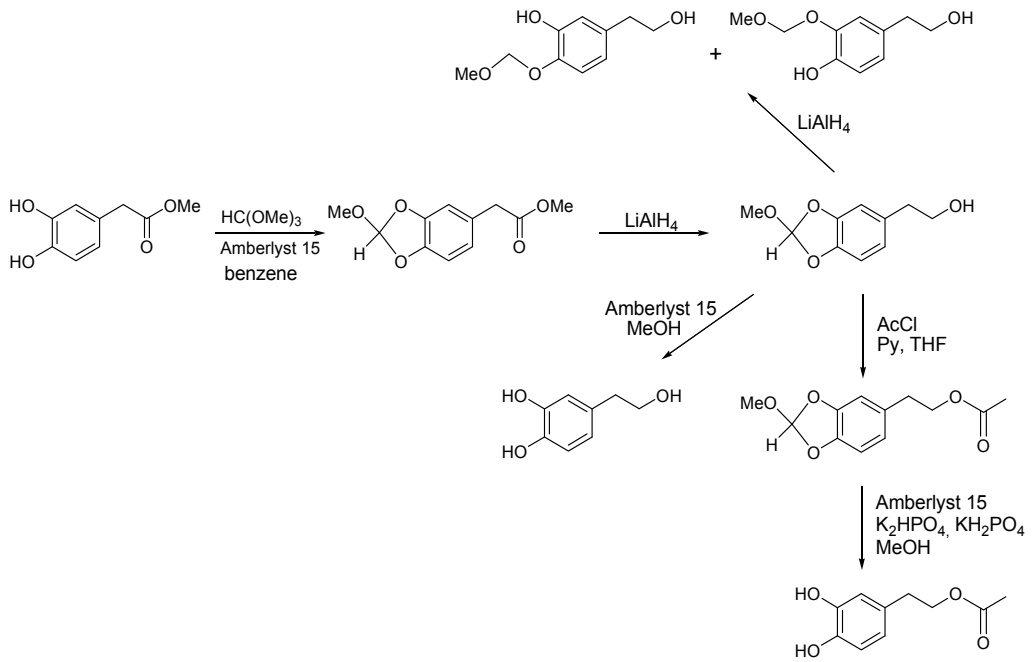
3.1.1.1 Protection of phenolic hydroxyl groups

As an example, benzyl groups have been used to carry out the HT esterification under basic conditions, followed by catalytic hydrogenation to remove the protective groups (Gordon et al., 2001), as depicted in Scheme 2.



Scheme 2. Synthesis of hydroxytyrosyl acetate via benzylation of phenolic hydroxyls

A two-step procedure involving the reaction of methyl orthoformate-protected hydroxytyrosol with acetyl chloride, and hydrolytic deprotection in phosphate buffer under very mild conditions (pH=7.2) to get hydroxytyrosyl acetate (87% overall yield) (Scheme 3) was also described as a successful procedure for the preparation of HT-derived esters (Gambacorta et al., 2007). The key synthetic orthoester intermediate was also used for the synthesis of HT upon reduction with $LiAlH_4$ and acidic deprotection.

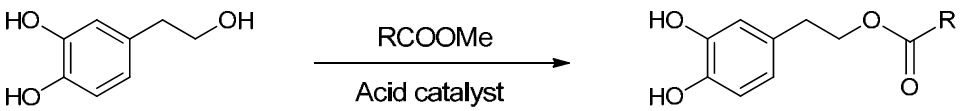


Scheme 3. Synthesis of hydroxytyrosyl acetate via methyl orthoformate-protected hydroxytyrosol.

In order to overcome the problems associated to the protection and deprotection steps of the phenolic hydroxyl groups, different methods for the preparation of hydroxytyrosyl esters by reaction of hydroxytyrosol with various acylating agents have been described, such as esterification with free acids (Appendino et al., 2002), transesterification with methyl or ethyl esters (Alcudia et al., 2004; Trujillo et al., 2006), acyl chlorides (Torregiani et al., 2005) and the use of enzymatic methodologies (Grasso et al., 2007; Mateos et al., 2008; Torres de Pinedo et al., 2005; Buisman, 1998).

3.1.1.2 Acid catalyzed transesterification

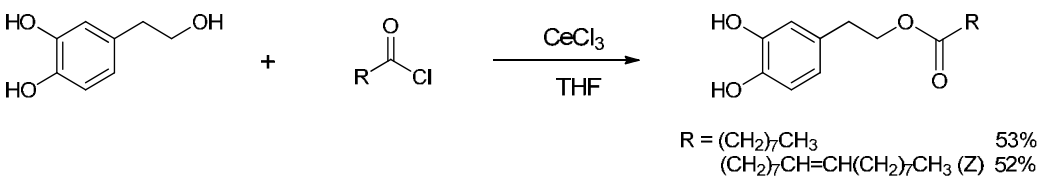
HT transesterification using methyl or ethyl esters and *p*-toluenesulfonic acid as catalyst has been described as a method without the need of protection of the aromatic hydroxyl groups due to its total chemoselectivity (Alcudia et al., 2004; Trujillo et al., 2006). This method involves heating a solution solution of hydroxytyrosol in the corresponding ethyl or methyl ester, containing a catalytic amount of *p*-toluenesulfonic acid (Scheme 4). This protocol has been optimized for HT acetate (86%), and also for longer aliphatic chains like hydroxytyrosyl butyrate, laureate, palmitate, stearate, oleate and linoleate, obtained in acceptable to good yields (62-76%) (Mateos et al., 2008).



Scheme 4. General procedure of acid-catalyzed transesterification

3.1.1.3 Acylation of polyphenolic alcohols with the couple $CeCl_3$–RCOCl

Cerium (III) chloride has been reported to be an efficient promoter for the chemoselective esterification of unprotected polyphenolic alcohols with acyl halides as acyl donors, thereby making it possible to avoid the protection of phenolic hydroxyl groups and providing polyphenolic esters of interest (Torregiani, 2005). This reaction is one example of the so-called Lewis acid catalysis by lanthanide salts (Ishihara et al., 1995). The reaction presumably involves the formation of an electrophilic Lewis adduct between acyl chlorides and cerium (III) chloride, which is quenched by the more nucleophilic aliphatic hydroxyl group of the substrate, with formation of the ester, and regeneration of the lanthanide promoter. The yields obtained are acceptable for HT using nonanoyl and oleoyl chlorides (53 and 52%), respectively (Scheme 5).

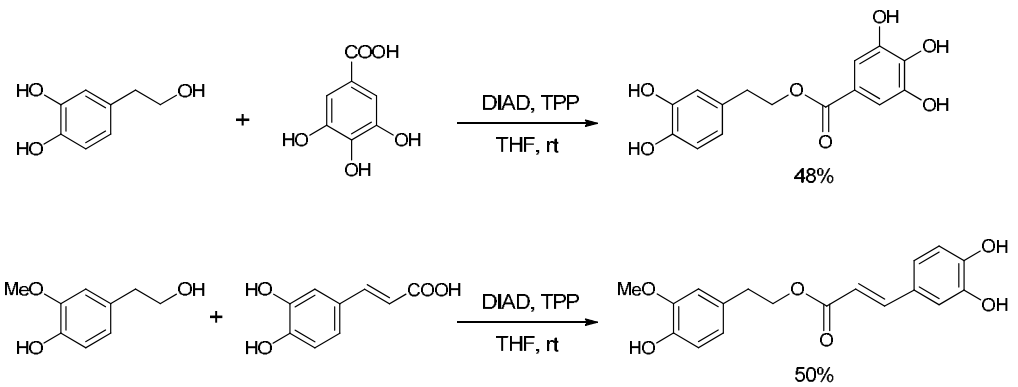


Scheme 5. Acylation of hydroxytyrosol with acyl chlorides and Ce(III)

3.1.1.4 Esterification with free acids: Mitsunobu esterification

The Mitsunobu reaction has been also applied to the chemoselective esterification of phenolic acids with phenolic alcohols (Appendino et al., 2002) as demonstrated by the condensation of hydroxytyrosol with gallic acid, and of vanillyl alcohol with caffeic acid in a one step

procedure with 48% and 50% yields, respectively. The esterification is carried out using DIAD (diisopropyl azodicarboxylate) and TPP (triphenylphosphine) in THF (Scheme 6). The removal of byproducts arising during the Mitsunobu reaction, a major problem of this type of reactions, could be solved by gel-permeation chromatography on Sephadex LH-20.

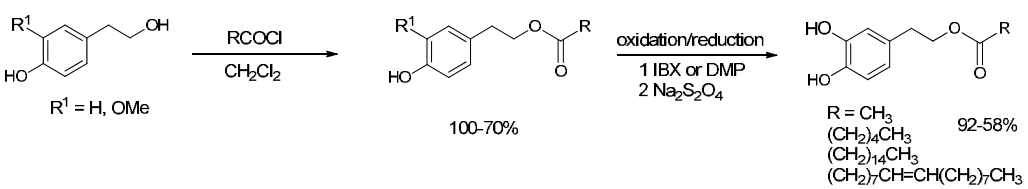


Scheme 6. Mitsunobu esterification of hydroxytyrosol and vanillyl alcohol

3.1.1.5 Syntheses of hydroxytyrosol esters from tyrosol and homovanillyl alcohol

The syntheses previously described in the previous sections had all in common hydroxytyrosol as a precursor of its esters, but some efforts have also been done to get hydroxytyrosyl esters starting from different and cheaper reagents. In this context, the syntheses of hydroxytyrosol esters from tyrosol and homovanillyl alcohol have been proposed (Bernini et al., 2008b). This procedure involves the selective esterification of tyrosol and homovanillyl alcohol with acyl chlorides in dicholoromethane as solvent, to give tyrosyl and homovanillyl acetates in 90% and quantitative yields, respectively, by using only a little excess of acetyl chloride in dichloromethane without any catalysts. The authors suggested acid catalysed acylation due to traces of hydrochloric acid derived from the hydrolysis of the acetyl chloride under the experimental conditions. A similar selectivity was observed by using several saturated or unsaturated acyl chlorides with longer chains such as hexanoyl, palmitoyl, oleoyl and linoleoyl chlorides.

The subsequent oxidation with 2-iodoxybenzoic acid (IBX) or Dess-Martin periodinane reagent (DMP) and *in situ* reduction with sodium dithionite ($Na_2S_2O_4$) of tyrosyl and homovanillyl esters led to the corresponding hydroxytyrosol derivatives. In general, the oxidation of tyrosol derivatives proceeded with higher yields (92-77%) compared to those of homovanillyl derivatives (88-58%). The use of DMP gave similar results to those obtained with IBX. The procedure of oxidation/reduction with IBX/$Na_2S_2O_4$ to obtain the different esters is under protection of two patents (Bernini et al. 2007, 2008c).



Scheme 7. Synthesis of hydroxytyrosol esters from tyrosol and homovanillyl alcohol

3.1.1.6 Lipase-catalyzed transesterification

The use of enzymes, like lipases, as catalysts in non-aqueous solvents to prepare lipophilic derivatives directly from HT has been widely described in the last few years (Grasso et al., 2007; Torres de Pinedo et al., 2005; Mateos et al., 2008; Buisman et al., 1998). This procedure avoids the use of toxic reagents and allows mild reaction conditions.

The esterification of phenols with carboxylic fatty acids and lipases as biocatalysts was firstly investigated by Buisman et al., (1998), using hydroxytyrosol, octanoic acid in hexane, and immobilized lipases from *Candida antartica* (CAL-B). Furthermore, a strong dependence of the yield on the solvent used was observed; so, in diethyl ether a conversion of 85% was obtained within 15 hours (35 ºC), while conversions of roughly 20% were found in the case of solvents like chloroform, dichloromethane or THF. Yields of 70–80% were observed using *n*-pentane and *n*-hexane, in spite of the poor solubility of HT in such solvents.

Different enzymes have been tested on hydroxytyrosol (Grasso et al., 2007) including lipases from *A. niger, C. cylindracea, M. javanicus, P. cepacia, M. miehei, C. viscosum, P. fluorescens, R. arrhizus, R. niveus, C. antarctica*, porcine pancreas and wheat germ, using vinyl acetate as reagent and *tert*-butyl methyl ether as solvent. The best results were obtained with *C. antarctica* in terms of short reaction time, chemioselective conversion and good yield. *C. antarctica* lipase (CAL) was selected for acylation of hydroxytyrosol and homovanillic alcohol with vinyl esters of different acyl chains on a preparative scale, as shown in Table 1. The use of *C. antarctica* with increasing alkyl chain length required longer reaction times. The homovanillyl alcohol and its esters were found to exhibit scarce effectiveness both as radical scavengers and antioxidant agents.

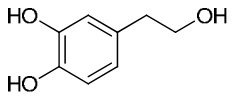
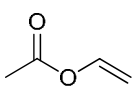
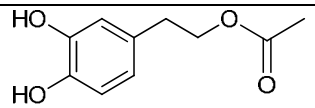
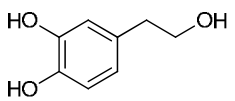
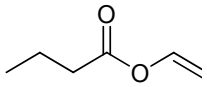
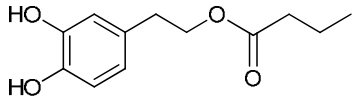
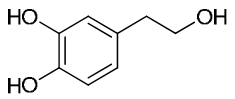
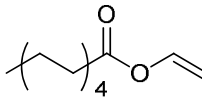
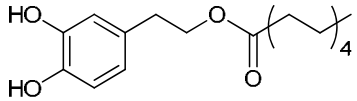
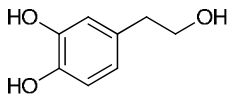
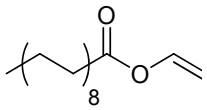
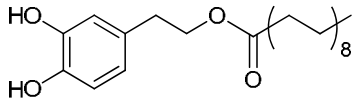
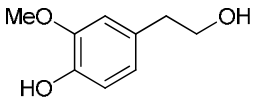
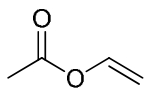
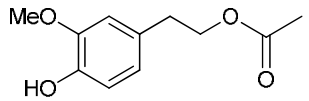
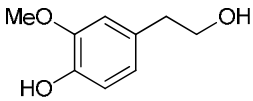
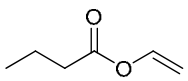
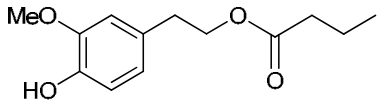
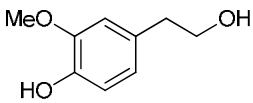
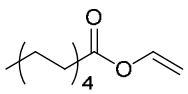
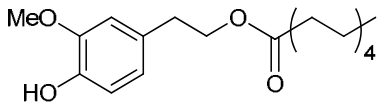
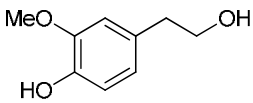
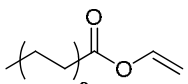
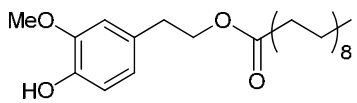
Transesterification of HT with ethyl saturated, mono- and poly-unsaturated fatty acid esters, catalized by Novozym® 435 (immobilized *C. antarctica* lipase B), in vacuum under solventless conditions, has been successfully developed (Torres de Pinedo et al. 2005). This procedure gave hydroxytyrosyl esters in 59-98% yield for the saturated fatty acid esters, and 32-97% yield for the mono- and poly-unsaturated fatty acid esters.

3.1.2 Biological activity

3.1.2.1 Antioxidant activity

The antioxidant activity of hydroxytyrosyl esters has been measured with different methods, including DPPH (1,1-diphenyl-2-picrylhydrazyl radical), ABTS (2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonic acid), FRAP (ferric reducing antioxidant power) and Rancimat (Mateos et al., 2008; Gordon et al., 2001; Bouallagui et al., 2011b). The Rancimat test is a method commonly used to evaluate the antioxidant power in lipophilic food matrices, such as oils and fats, while the ABTS and FRAP assays are used for the evaluation of antioxidant activity in hydrophilic medium; the ABTS assay evaluating the radical-scavenging capacity, and the FRAP method determining the reducing activity.

The Rancimat test revealed a lower activity for ester derivatives compared to HT, in agreement with the so-called polar paradox, according to which hydrophilic antioxidants are more effective in less polar media, such as bulk oils, whereas lipophilic antioxidants are more effective in relatively more polar media, such as in oil-in-water emulsions or liposomes (Frankel et al., 1994; Shahidi & Zhong, 2011).

Phenol	Acylating agent	Product	Time (min)	Yield (%)
			35	95.0
			35	96.5
			75	93.3
			180	92.3
			60	96.8
			90	90.9
			90	97.5
			240	98.0

Phenol: acylating agent 1:20, *C. antarctica* lipase, *t*-BuOMe, 40 ºC

Table 1. Enzymatic esterification of HT and homovanillyl alcohol (Grasso et al., 2007)

The order of the scavenging activities toward the ABTS radical was hydroxytyrosyl esters ≥ α-tocopherol > hydroxytyrosol > tyrosyl >tyrosyl esters ≅ BHT. In a similar trend, comparison of FRAP values obtained for the free hydroxytyrosol and tyrosol with the corresponding esters revealed that while hydroxytyrosyl esters showed a significantly higher reducing activity than their precursor, all the tyrosyl esters showed a lower antioxidant activity than that of tyrosol. The same conclusion was obtained from DPPH assay of the radical scavenging activity (Grasso et al., 2007).

In connection with the size of the acyl chain, the reported literature seems to conclude that the antioxidant capacity of hydroxytyrosyl esters is better for medium-sized (C4–C9) alkyl chains in comparison with HT, whereas further elongation of the acyl chain does not improve the antioxidant activity. This confirms that antioxidant capacity does not depend

only on lipophilicity. A possible explanation could be related to the fact that the conformational freedom of the ester chain increases with the acyl chain length, and this could result in folded structures in which catechol hydroxyls are shielded (Tofani et al., 2010; Pereira-Caro et al., 2009; Medina et al., 2009).

This antioxidant activity has also been proved in biological assays, in order to check the ability of hydroxytyrosyl esters to protect proteins and lipids against oxidation caused by peroxyl radicals, using a brain homogenate as an *ex vivo* model (Trujillo et al., 2006) and cumene hydroperoxide to induce oxidation. The results obtained showed a protective effect in these systems, which was more effective in preventing the generation of carbonyl groups in proteins than the generation of malondialdehyde in lipid; hydroxytyrosyl linoleate showed the greatest activity. This fact proves that the introduction of a lipophilic chain in the hydroxytyrosol molecule increases both protein and lipid protection.

Dichlorodihydrofluorescein (DCF) fluorometric assay on whole cells, carried out to check the antioxidant activity of a large serie of hydroxytyrosyl esters (Tofani et al., 2010) on rat muscle cells, showed that hydroxytyrosol esters had a better antioxidant activity compared to HT due to the better penetration into the cells of the lipophilic derivatives.

Hydroxytyrosol fatty acid esters have shown a nonlinear tendency in antioxidant capacity in fish oil-in-water emulsions (Lucas et al., 2010), where a maximum of antioxidant efficiency appeared for hydroxytyrosol octanoate in a study of hydrosytyrosyl esters with alkyl chains varying from C2 to C18. These results seem to be in disagreement with the antioxidant polar paradox.

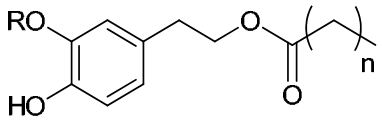
3.1.2.2 Cardiovasvular diseases

Platelet aggregation is considered one of the main events in arterial thrombosis; therefore aggregation prevention is a major goal of cardiovascular research. It has been proved that hydroxytyrosol acetate inhibits platelet aggregation induced by ADP, collagen or arachidonic acid and stimulates nitric oxide production, more efficiently than hydroxytyrosol, and as effectively as acetylsalicylic acid; the latter is the most widely used drug in the world to prevent ischaemic cardiovascular diseases because of its antiplatelet aggregating action. This conclusion has been achieved *in vivo* in a study of oral administration of this ester to rats (González-Correa et al., 2008b), and *in vitro* in both human whole blood and platelet-rich plasma (González-Correa et al., 2009).

3.1.2.3 DNA damage oxidative protection

The atypical Comet test on whole blood cells has been applied to several hydroxytyrosyl esters to check their capacity to counteract the oxidative stress caused by H_2O_2 and the basal DNA damage.

The results obtained show that antidamaging properties on DNA of HT acetate and propanoate are comparable to those of HT, whereas the protective effect progressively decreases in the order butanoate < decanoate ≅ estearate (Fig. 3). This behavior was not observed for the lipophilic analogues of homovanillyl alcohol which appear to be scarcely protective, indicating that *o*-diphenols are more effective antioxidants than simple phenols (Grasso et al., 2007).



R = H, Me n = 0,1,2,8,16

Fig. 3. Hydroxytyrosol lipophilic analogues

3.1.2.4 Prevention of oxidative stress

The ability of hydroxytyrosol and its esters to prevent iron-induced oxidative stress has been studied on human cervical cells (HeLa cells) by the TBARS protocol (Bouallagui et al., 2011b). Pre-incubation of HeLa cells in the presence of 100 µM phenolic compounds led to a significant improvement of the oxidative status. In fact, thiobarbituric acid-reactive substance (TBARS) production was decreased by 30%, 36% and 38% with hydroxytyrosol, hydroxytyrosyl acetate and hydroxytyrosyl oleate, respectively.

3.1.2.5 Transport, absorption and metabolism

The study of the metabolism of hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate has been carried out using human hepatoma cells (HepG2) as a model system of the human liver (Mateos et al., 2005). The results showed extensive uptake and metabolism of hydroxytyrosol and scarce metabolism of tyrosol, while hydroxytyrosyl acetate showed an interesting behavior, with formation of deacetylated hydroxytyrosol after only 2 h. Because hydroxytyrosyl acetate was stable in the culture medium, the hydroxytyrosol detected in the extracellular medium should be attributed to the action of the hepatic cells.

3.1.2.6 Neuroprotective effect of hydroxytyrosyl and hydroxytyrosol acetate

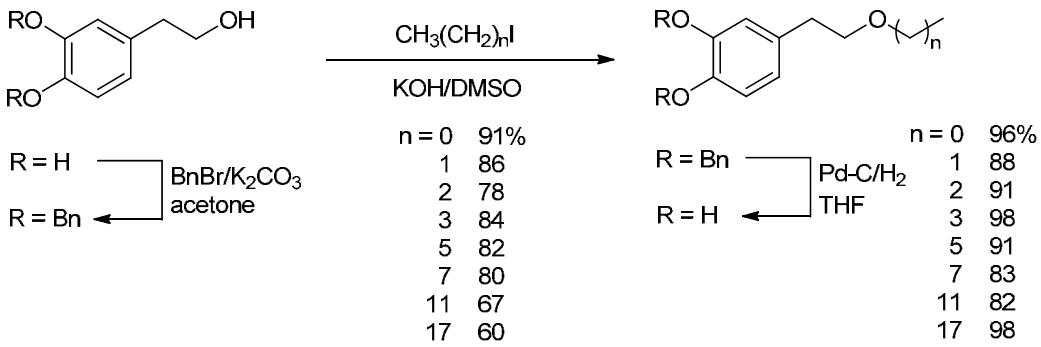
Neuroprotection exerted by HT derivatives has been investigated in rat brain slices subjected to hypoxia-reoxygenation, both *in vitro* and after oral administration (González-Correa et al., 2008). This study was carried out to confirm to the previously demonstrated neuroprotective effects of virgin olive oil in rats (González-Correa et al., 2007). Although the studies gave positive results in the neuroprotective activity of both HT and hydroxytyrosyl acetate, mechanisms that underlie this effect are still unknown.

3.2 Lipophilic hydroxytyrosyl alkyl ethers

3.2.1 Synthetic approaches

Hydroxytyrosyl alkyl ethers have been obtained (Madrona et al., 2009) in a three-step procedure starting from hydroxytyrosol isolated from olive oil waste waters (Scheme 8). This procedure requires first the selective protection of the aromatic hydroxyl groups *via* benzylation with benzyl bromine in the presence of K_2CO_3, and then the addition of an alkyl iodide under basic conditions, and the subsequent deprotection by catalytic hydrogenation (Pd/C) to obtain the corresponding ethers.

The yield for the alkylation step varies depending on the length of the alkyl chain; as depicted in Scheme 8, the yields decrease as the length of the alkyl chain increases, due to the reduced solubility of the corresponding long chain alkyl iodides in the solvent (DMSO).



Scheme 8. Synthesis of hydroxytyrosyl alkyl ethers by alkylation with alkyl iodides

The oxidative stability of lipid matrix in the presence of these compounds, measured by the Rancimat method, has shown that these derivatives retain the high protective capacity of free hydroxytyrosol and similar induction times, having higher induction times than butylhydroxytoluene (BHT) and α-tocopherol (Madrona et al., 2009). These results are in agreement with those obtained in the case of hydroxytyrosyl esters, covered in the previous section (Mateos et al., 2008). The antioxidant activity has been checked by the DPPH, FRAP and ABTS assays in a hydrophilic medium (Pereira-Caro et al., 2009). The antioxidant activity of the lipophilic hydroxytyrosyl ethers was slightly lower in bulk oils and higher in hydrophilic media in comparison with their reference HT, supporting the polar paradox. The length of the alkyl chain did have a positive influence in hydrophilic medium for ethers with a short alkyl chain (methyl, ethyl, propyl), while ethers with longer alkyl chains (from butyl to octadecyl) maintained or decreased their antioxidant activity, probably due to the steric effect of the hydrocarbon chains.

3.2.2 Biological activity

In order to evaluate the safety and potential biological activity of these ethers, studies of their transport, absorption and metabolism in cellular and animal models have been developed (Pereira-Caro et al., 2010a, 2010b) using a human hepatoma cell line (HepG2) as a model system of the human liver and human enterocyte-like Caco-2/TC7 cells, which are commonly used to characterize the intestinal absorption of a range of drugs, nutrients, and other xenobiotics.

The results showed a direct relationship between the lipophilic nature of each compound and the level of metabolization; as an example, hydroxytyrosyl butyl ether biotransformation was complete after 18 h, whereas small amounts of the others remained after the same time. Furthermore, an intestinal absorption increase was observed from methyl to *n*-butyl ethers.

Protective effects against oxidative stress have also been studied (Pereira-Caro et al., 2011) using HepG2 cells, the ones previously employed to assess the metabolism of the synthesized HT ethers. The results obtained show the potential to prevent cell damage induced by *tert*-butyl hydroperoxide (*t*-BuOOH) and the ability to maintain unaltered cellular redox status, partially after 2 hours of pretreatment and almost completely after 20 hours. These results are in accordance with those obtained with hydroxytyrosol (Martín et al., 2010), but they also show the relevance of the role of the lipophilic character of the

phenolic compounds on their antioxidant potential against cell damage: HT methyl and ethyl ethers are less effective than HT propyl and butyl ethers.

3.3 Hydroxytyrosol-derived isochromans

Isochroman fragment is a ubiquitous scaffold that can be found in natural products, drugs and agrochemicals (Larghi & Kaufman, 2006). Access to dihydroxyisochromans derived from HT can be achieved by using the oxa-Pictet-Spengler reaction, by reaction of arylethanols with aldehydes, ketones or masked-carbonyl derivatives (Guiso et al. 2001). The reaction is highly regioselective, as intramolecular cyclization takes place mainly in the less hindered position, as it can be deduced from its reaction mechanism, shown in Scheme 9. Two of the synthetized isochromans (Fig. 4) have been detected in olive oil (Bianco et al., 2001).

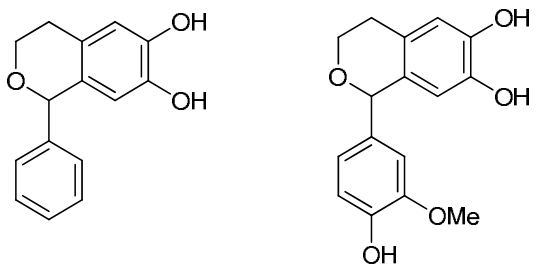
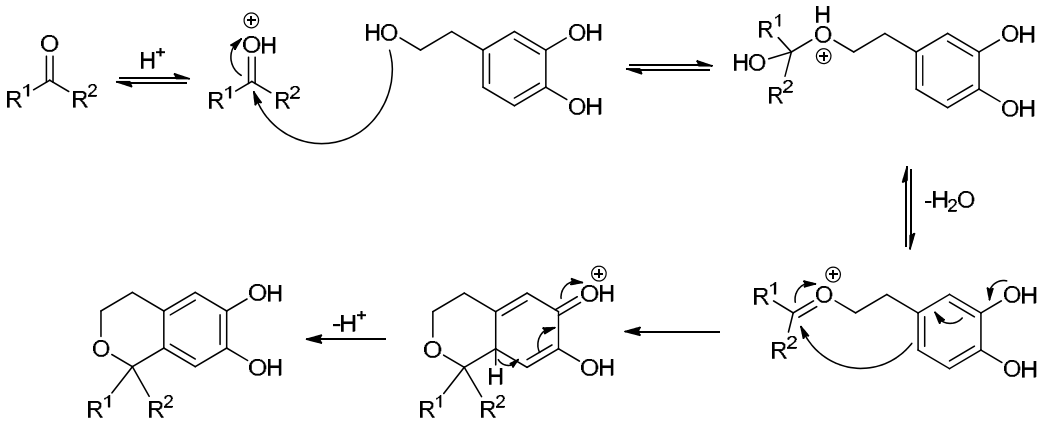


Fig. 4. Isochromans naturally present in olive oil



Scheme 9. Synthesis of hydroxytyrosol isochroman derivatives

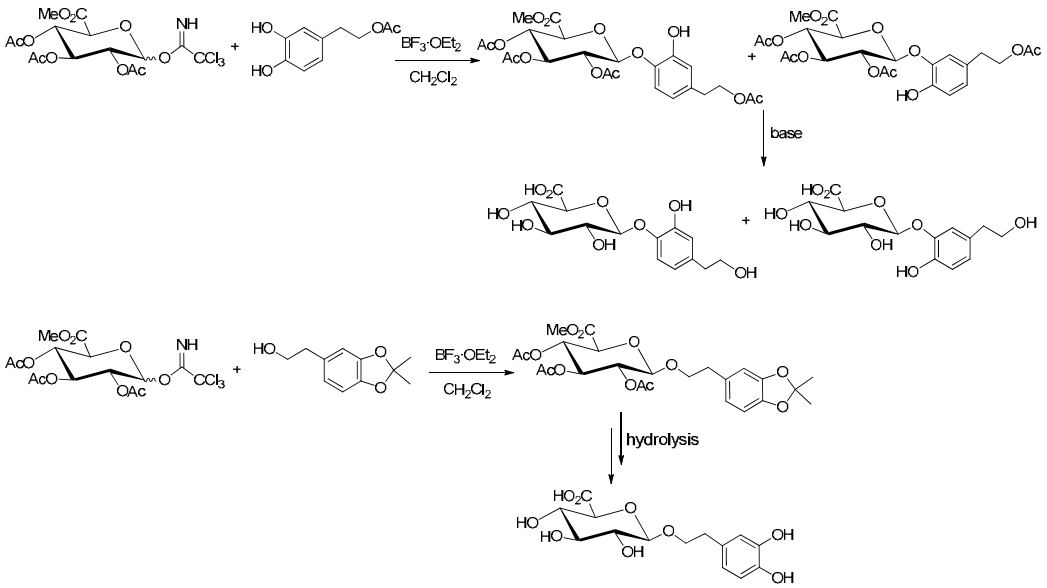
Hydroxytyrosol isochroman derivatives shown in Fig. 4 were effective free radical scavengers able to inhibit platelet aggregation and thromboxane release (Togna, 2003).

3.4 Hydroxytyrosol glucoronide derivatives

One of the major metabolic pathways found *in vivo* for dietary phenolic compounds such as hydroxytyrosol is *O*-conjugation via glucuronidation and sulfation. Therefore, it is of interest to study these metabolites and their biological activities.

Biocatalyzed syntheses of hydroxytyrosol and other phenolic glucuronides have been developed using porcine liver microsomes (Khymenets et al., 2006, 2010). This type of glucuronides has also been synthesised stereoselectively (Lucas et al., 2009) in the phenolic or aliphatic hydroxyl groups using efficient chemical method from *O*-partially protected hydroxytyrosol and glucuronosyl trichloroacetimidate donors (Scheme 10).

The antioxidant activities of hydroxytyrosol conjugates have been evaluated, concluding that none of these glucuronides displayed significant antioxidant activities at the concentration tested (Khymenets et al., 2010).



Scheme 10. Synthesis of hydroxytyrosol glucuronides

3.5 Hydroxytyrosol glucosides

The three isomers of hydroxytyrosol β-D-glucopyranosides (Fig.5) have been reported to be present in olives (Bianco et al., 1998). The 4-glucoside (Romero et al., 2004) and the 1-glucosides (Medina et al., 2007) have been found in table olive brines, and have been analysed as antimicrobial compounds against *Lactobacillus pentosus* with negative results. It has been recently shown that hydroxytyrosol 4-glucoside was the main phenolic compound in the aqueous phase of fresh alpeorujo, followed by hydroxytyrosol, and hydroxytyrosol 1-glucoside.

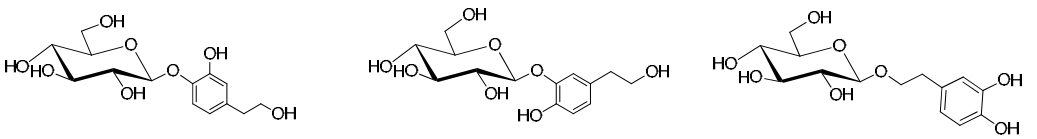
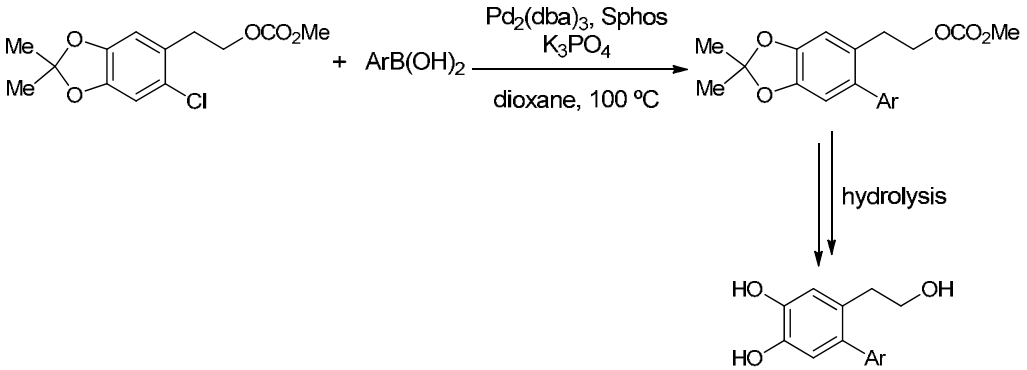


Fig. 5. The three isomers of hydroxytyrosol • –Δ–γλυχοπψρανοσιδεσ

3.6 Arylhydroxytyrosol derivatives

The synthesis of 2-arylhydroxytyrosols from 2-halohydroxytyrosol derivatives has been described (Bernini et al., 2008a). The reaction of the corresponding 2-chloro precursors via Suzuki-Miyaura cross-coupling reaction with arylboronic acids containing electron-donating, electron-withdrawing, as well as *ortho* substituents, yielded this family of compounds in high to excellent yields (Scheme 11).



Scheme 11. Synthesis of 2-arylhydroxytyrosol derivatives

3.7 Complexation of hydroxytyrosol with β-cyclodextrins

The complexation of hydroxytyrosol with commercially-available β-cyclodextrin (β-CD) (López-García et al., 2010; Rescifina et al. 2010) and hydroxypropyl-β-cyclodextrin (HP-β-CD) (López-García et al., 2010) in aqueous solutions has been studied. The stoichiometries, the association constants and the geometry of the complexes have been determined by NMR techniques. The stoichiometries of both complexes are 1:1 and the association constants are 93±7 M^{-1} for HT/β-CD complex and 43±1 M^{-1} for HT/HP-β-CD complex (López-García et al., 2010). In both cases, the insertion of the catechol moiety took place by directing the hydroxyalkyl chain to the primary rim. The postulated geometry of the 1:1 HT/β-CD inclusion complex is depicted in Fig. 6.

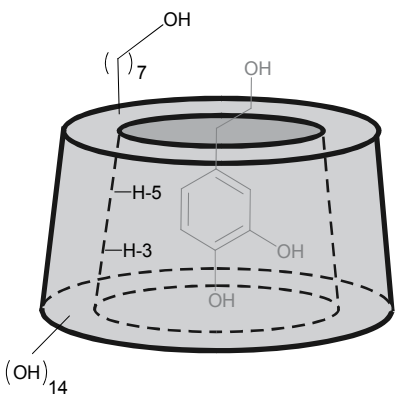


Fig. 6. Postulated geometry of the 1:1 HT/β-CD inclusion complex

Moreover, the antioxidant activity of encapsulated HT, together with the photoprotection effect of β-CD on HT, has been evaluated by scavenging of the stable DPPH radical. It has been proven that β-Cyclodextrin acts as a secondary antioxidant and provides a moderate improvement of the radical scavenging activity of HT measured by the DPPH assay.

β-Cyclodextrin exerts a strong photoprotection of HT upon UV irradiation, which could be deduced from the EC_{50} values (Table 2). For equimolecular mixtures of HT and β-CD at 1.2 mM, the observed degradation after 24 h and 48 h is similar to the degradation found for HT at the same concentration and time (entries 4 and 5) showing no protection at 24 h and only a slight protection after 48 h. However, using 1:4 mixtures of HT (1.2 mM) and β-CD (4.8 mM), a remarkable reduction of the degradation rate was observed when compared with pure HT. In this way, the complexation of HT with cyclodextrins might enhance stability, improve its performance as antioxidant and extend its storage life (López-García et al., 2010).

Entry	Antioxidant	[HT] (mM)	Irradiation time (h)	EC_{50} (g HT / kg DPPH)
1	HT	1.2	12	119.0 ± 1.4
2	HT	1.2	24	353.6 ± 23.4
3	HT	1.2	48	1436.1 ± 73.2
4	HT-βCD (1:1)	1.2	24	357.2 ± 30.7
5	HT-βCD (1:1)	1.2	48	1011.6 ± 171.9
6	HT-βCD (1:4)	1.2	12	112.4 ± 6.4
7	HT-βCD (1:4)	1.2	24	198.0 ± 4.6
8	HT-βCD (1:4)	1.2	48	387.2 ± 13.3

Table 2. Effect of the encapsulation of HT on its photostability

4. Conclusions

Hydroxytyrosol is a phenolic compound that can be isolated from olive oil mill wastewaters. The remarkable biological properties of this compound, mainly due to its strong antioxidant activity, has stimulated the synthesis of a series of derivatives, some of them are also naturally-occurring in the olive tree. Among these derivatives hydroxytyrosyl esters and ethers are of great interest, as some of them show strong antioxidant activity and improved bioavailability.

5. Acknowledgement

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21

Differential Effect of Fatty Acids in Nervous Control of Energy Balance

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1. Introduction

Energy homeostasis is kept through a complex interplay of nutritional, neuronal and hormonal inputs that are integrated at the level of the central nervous system (CNS). A disruption of this regulation gives rise to life-threatening conditions that include obesity and type-2 diabetes, pathologies that are strongly linked epidemiologically and experimentally. The hypothalamus is a key integrator of nutrient-induced signals of hunger and satiety, crucial for processing information regarding energy stores and food availability. Much effort has been focused on the identification of hypothalamic pathways that control food intake but, until now, little attention has been given to a potential role for the hypothalamus in direct control of glucose homeostasis and nergy balance. Recent studies have cast a new light on the role of the CNS in regulating peripheral glucose via a hypothalamic fatty acid (FA)-sensing device that detects nutrient availability and relays, through the autonomic nervous system, a negative feedback signal on food intake, insulin sensitivity and insulin secretion. Indeed, accumulating evidences suggest that FA are used in specific areas of CNS not as nutrients, but as cellular messengers which inform "FA sensitive neurons" about the energy status of the whole body (Blouet & Schwartz, 2010; Migrenne et al., 2006; Migrenne et al., 2011). Thus it has been described that up to 70% of hypothalamic arcuate nucleus (ARC) and ventromedian nucleus (VMN) neurons are either excited or inhibited by long chain fatty acids such as oleic acid (Jo et al., 2009; Le Foll et al., 2009; Migrenne et al., 2011). Within the VMN, 90% of the glucosensing neurons also have their activity altered by FA. In a large percentage of these neurons, glucose and FA have opposing effects on neuronal activity, much as they do on intracellular metabolism in many other cells (Randle et al., 1994). Neuronal FA sensing mechanisms include activation of the K_{ATP} channel by long chain fatty acid acyl CoA (Gribble et al., 1998) or inactivation by generation of ATP or reactive oxygen species during mitochondrial β-oxidation (Jo et al., 2009; Le Foll et al., 2009; Migrenne et al., 2011; Wang et al., 2006). Many fatty acid sensing neurons are activated by interaction of long chain fatty acids with the fatty acid transporter/receptor, FAT/CD36, presumably by activation of store-operated calcium channels by a mechanism that is independent of fatty acid metabolism (Jo et al., 2009). Importantly, most neurons utilize FA primarily for membrane production rather than as a metabolic substrate (Rapoport et al., 2001; Smith & Nagura, 2001) and only nanomolar concentrations of fatty acid are required to

alter the activity of fatty acid sensing neurons in the absence of astrocytes (Jo et al., 2009). While cerebral lipids are both produced in the brain and transported into it from the periphery (Rapoport et al., 2001; Smith & Nagura, 2001), the mechanism of this transport and the actual levels of various FA in the extracellular space in the brain remains largely unknown. As mentioned above, hypothalamic FA sensing may be involved in the control of feeding behaviour, hepatic glucose production and insulin secretion. It seems also that intracellular FA metabolism is important to relay their effects (β oxidation has been showed to be involved in oleate effect in hypothalamus) (Cruciani-Guglielmacci et al., 2004; Obici et al., 2003). In addition differential effect of FA in regard to feeding behaviour or glucose production may be related to their chain length and degree of saturation. For exemple, it has been showed in rodents that oleate both inhibits food intake and hepatic glucose production whereas octanoate has no effect on these parameters (Obici et al., 2002). In another study we showed that intracerebroventricular infusion of palmitate induced an hepatic insulin resistance and an impaired insulin signaling in hypothalamus (Benoit et al., 2009). In contrast oleate has no deleterious effect in this parameter (Benoit et al., 2009). Poly-unsaturated fatty acids (PUFA) such as n-3 or n-6 may have also different effects in neuronal activity and cognitive function such as memorization. The present work was aimed at studying differential effect of FA or triglycerides emulsion infused in rats in glucose homeostasis. In addition, in order to identify molecular mechanisms involved in specific effects of FA, mRNA expression of key genes involved in FA metabolism as well as ceramides and diacylglycerol (DAG) content have been measured in hypothalamus. Regarding physiopathology aspects it must be pointed out that dysfunction of central FA sensing could be a contributing factor to the early development of type 2 diabetes mellitus and/or obesity which leads to further dysfunction in predisposed subjects. A better understanding of these mechanisms, as well as further characterization of FA sensitive neurons and their role in physiological and pathological processes, might lead to identification of novel pharmacological targets for the prevention and treatment of diabetes and obesity.

2. FA sensing in hypothalamus

There is now growing amount of evidence suggesting, at least in rodents models, that some neurons located in hypothalamus (and brainstem) are sensitive to FA, ie their electrical activity is either increased or decreased in presence of variations of FA concentration. This has been evidenced both in vivo and in vitro. A key point is the transport of FA across the blood brain barrier (BBB). It cannot be excluded that FA may be produce directly in neurons from hydrolysis of intracellular troglycerides (TG).

2.1 Transport of FA uptake into the brain and neurons

Cerebral lipids are an essential component of both membranes and intracellular signalling pathways. They represent 50% of brain dry weight; the highest organ lipid content after adipose tissue (Edmond, 2001; Watkins et al., 2001). However, the mechanism by which FA are transported into the brain remains poorly understood. A growing body of evidence suggests that cerebral lipids are derived both from local synthesis and uptake from the blood (Rapoport et al., 2001). Several studies show that some poly-unsaturated FA (PUFA) have the ability to cross the BBB (Rapoport et al., 2001; Smith & Nagura, 2001). The question

of whether brain FA uptake occurs by passive diffusion or involves a protein which facilitates the transport is still matter of debate. However, once across the BBB, it is likely that neurons can take up FA since some neurons do appear to have FA transporters. For example, dissociated neurons from the VMN of rats express mRNA's for FA transport proteins (FATP)-1 and 4 and the FA transporter/receptor FAT/CD36 (Le Foll et al., 2009). Also, while it is unlikely that neurons derive much of their energy supply from FA, these same neurons do express mRNA's for the intracellular metabolism of FA such as long chain acyl-CoA synthetase, carnitine palmitoyltransferase-1a and 1c and uncoupling protein-2 (Le Foll et al., 2009). They also express enzymes for de novo FA synthesis such as FA synthetase (Le Foll et al., 2009). But, it seems likely that much of the reported oxidation of FA such as palmitate in the brain probably occurs in astrocytes (Escartin et al., 2007) whereas other FA such as arachidonate are largely incorporated into phospholipids (Rapoport et al., 2001).

2.2 Some hypothalamic neurons are lipid responsive

The presence of neurons sensitive to variations in extracellular glucose levels is clearly demonstrated in the brain (Gilbert et al., 2003) and in particular in the hypothalamus (review in (Luquet & Magnan, 2009; Migrenne et al., 2011; Penicaud et al., 2002). Thirty-five years ago Oomura and colleagues first showed that FA activated lateral hypothalamic neurons which suggested a role for FA as neuronal signaling molecules (Oomura et al., 1975). As shown in Figure 1, FA also modify neuronal firing rate in hypothalamic arcuate nucleus (ARC) (Wang et al., 2006). Both FA "excited" (around 20% of arcuate neurons) and "inhibited" neurons (about 12%) are detected in arcuate nucleus of rat using this patch clamp technique (Wang et al., 2006). These FA sensitive neurons are also detected *in vivo* using multi-unit recording approaches (Wang et al., 2006). Therefore we demonstrated that single injection of oleic acid (OA) through carotid artery induced either increased or decreased neuronal activity depending on location of microelectrode in hypothalamus. It seems that some areas are mainly composed with FA "excited" neurons whereas others are mainly composed with FA "inhibited" neurons. Such data also suggest that physiological variations of plasma FA concentrations (reflecting the metabolic state and energy availability) can be detected and integrated by FA sensing neurons in critical brain areas involved in the regulation of feeding behaviour, glucose and lipid metabolism (Clement et al., 2002; Obici et al., 2002). Indeed increased plasma FA concentration during fasted or starvation may be detected by FA excited neurons which in turn may have an impact on nervous control of energy balance. On the contrary decreased plasma FA concentration during a meal could be also detected by these sensitive neurons which may act a satiety signal like insulin and glucose do during a meal when acting on hypothalamus sensitive neurons (Gilbert et al., 2003).

The physiological relevance of brain FA sensing is supported by various studies showing that local increases in brain and hypothalamic FA levels are associated with changes in insulin secretion and hepatic glucose output with variable effects on food intake (Clement et al., 2002; Obici et al., 2002; Ross et al., 2010; Schwinkendorf et al., 2010). For example, a 6 hour intracerebroventricular (icv) infusion of the monounsaturated FA, oleic acid (OA), reduced food intake as well as hepatic glucose production (HGP) (Obici et al., 2002). Reducing hypothalamic FA oxidation by inhibition of carnitine palmitoyl transferase -1 (CPT1), the enzyme that promotes β-oxidation by facilitating transport of medium- and

long-chain FA into mitochondria, mimicked these effects on food intake and HGP induced by icv infusion of OA (Obici et al., 2003). In another study a direct bilateral infusion of OA into the mediobasal hypothalamus decreased hepatic glucose production (Ross et al., 2010). In addition, it seems that the hypothalamus differentially senses FA. For example, icv infusions of OA or docosahexanoic acid, but not palmitic acid, reduce food intake and body weight (Schwinkendorf et al., 2010). However, icv and direct infusions of FA into the brain are not physiological. Thus, they might produce non-specific effects by evoking an inflammatory response by irritating ependymocytes and tanycytes lining the ventricles or by exciting microglia and astrocytes in the brain parenchyma.

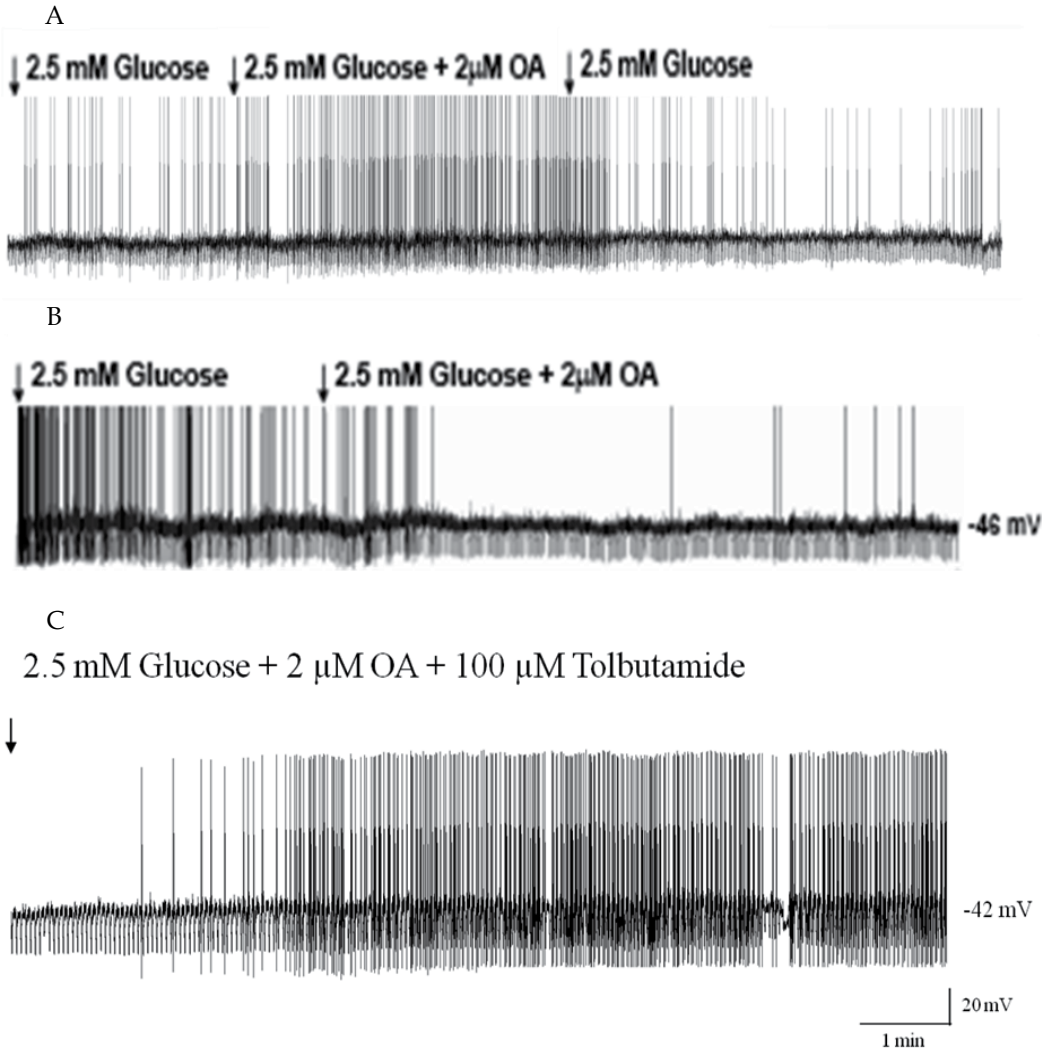


Fig. 1. Fragments of whole cell current clamp recordings of oleic acid (OA) excited (A) and inhibited (B) neuron in arcuate nucleus of rat (Adapted from Wang et al, 2006 et Migrenne et al, 2006).The inhibitory effect of OA on neuronal activity is inhibited by tolbutamide (C), suggesting involvement of K_{ATP} channels in OA effect.

More physiological routes include elevating systemic levels of FA or infusing them directly into the carotid arteries, the major route by which FA reach the forebrain. For example, a two-fold increase in plasma triglycerides produced by a two day systemic infusion of triglycerides was associated with decreased sympathetic activity. This reduced sympathetic tone, which is also produced by central FA infusions (Magnan et al., 1999), might contribute to the associated FA-induced exaggeration of glucose-induced insulin secretion (GIIS), a condition which is similar to what occurs in the prediabetic state (Magnan et al., 1999). Also, this exaggerated GIIS and a reduction in HGP were mimicked by infusing triglycerides into the carotid artery (Cruciani-Guglielmacci et al., 2004). These exaggerated responses were reduced by central inhibition CPT1 (Magnan et al., 1999). Similarly, central CPT1 inhibition was associated with an increase in the acyl CoA intracellular pool which was postulated to be the "final" satiety signal rather than FA themselves (review in (Lam et al., 2005; Luquet & Magnan, 2009).

However, there are at least two potential problems involved in the interpretation of such in vivo data. First, the idea that increases in brain FA levels act as a satiety signal to inhibit feeding (Obici et al., 2003) is counterintuitive given the fact that plasma FA levels do not rise substantially after food ingestion, but do rise significantly during fasting (Ruge et al., 2009). Second, the vast majority of FA oxidation in the brain occurs in astrocytes rather than neurons (Escartin et al., 2007). While a select group of neurons in the hypothalamus clearly responds directly to changes in ambient FA levels by altering their activity (Le Foll et al., 2009; Oomura et al., 1975), only a relatively small percentage of these responses depend upon neuronal FA metabolism (Le Foll et al., 2009). Furthermore, although β-oxidation and formation of malonyl-CoA and FA metabolites such as acyl-CoA may be mediators of the in vivo effects produced by FA infusions (Dowell et al., 2005; Migrenne et al., 2011) it is likely that most of these occur at the level of the astrocyte. If so, then there must be a mechanism by which alterations in astrocyte FA metabolism can provide a signal to those neurons which regulate HGP and food intake. We suggest that this communication between astrocyte FA metabolism and neuronal FA sensing involves the production and export of ketone bodies from astrocytes (Escartin et al., 2007) and subsequent uptake by neurons. Finally another important issue is the nature of the FA and its effect on sensitive neurons. As previously mentioned OA and octanoate have differential effect regarding food intake or hepatic glucose production, suggesting that medium or long chain fatty acids may have different effects (Obici et al., 2002). Thus, the aim of the present study was to test whether different FA may have different effect on glucose homeostasis when infused in rats brains through carotid artery. Triglyceride emulsion either enriched in ω3, ω6 FA or saturated FA (lard oil) have been also tested.

3. Methods

All animal care and experimental procedures were approved by the animal ethics committee of the university Paris-Diderot. Four weeks-old male wistar rats were purchased from Charles Rivers (Lyon, France) and housed at 21°C with normal light/dark cycle and free access to water and food.

3.1 First serie of experiments

Rats received an intracerebroventricular (icv) infusion of FA during 3 days. Briefly, rats anesthetized with isoflurane were stereotactically implanted with a chronic stainless steel

cannula in the right lateral cerebral ventricle. The cannula was connected via a polyethylene catheter to a subcutaneously osmotic minipump filled up with FA (oleate, octanoate or linolenate) or saline. Infusions started 6h after surgery. The rate of infusion was 0.5 µl/h. Blood was daily removed (~ 80 µl) from caudal vessels for measurement of plasma substrate (FA and glucose) and insulin. Food intake was daily measured. At day 3 of infusion glucose-induced insulin secretion (GIIS) was measured in response to a single intraperitoneal injection of glucose (0.5g/kg bw). was made in overnight fasted rats. The glycemia was determined by a glucometer (AccuChek, Rabalot, France) from 2 µl collected from the tip of the tail vein at time 0, 5, 10, 15, 20, 30 and 60 min. In addition 20 µl of blood was sampled at the same time for insulin measurement (RIA, Diasorin, France). In another serie of experiments, etomoxir (CPT1 inhibitor) was concomitantly infused with FA. At the end of experiment brain were removed and five hypothalamus nuclei (arcuate, lateral, ventromedian, paraventricular and dorsomedian) were micropunched in order to measure gene expression (acetylCoA carboxylase, ACC, carnitine palmitoyl transferase, CPT1, FA synthase, FAS, G protein related peptide GPR41). Briefly, total RNA was isolated from the hypothalamus using RNeasy Lipid kit (Qiagen). To remove residual DNA contamination, the RNA samples were treated with DNAse RNAse-free (Qiagen). 4 µg of total RNA from each sample was reverse transcribed with 40 U of M-MLV Reverse Transcriptase (Invitrogen, life technologies) using random hexamer primers.

3.2 Second serie of experiments

In a second serie of experiments our goal was to test the effect of different triglyceride emulsion on glucose tolerance and both diacylglycerol and ceramides content in hypothalamus. To that end rats received an intracarotid infusion during 24 h of lard oil, mainly composed of saturated FA (SFA), ω3-enriched (Omegaven, Santec, France) or ω6-enriched (Ivelip, Rabalot, France) polyunsaturated FA (PUFA) triglyceride emulsion. The long-term unrestrained infusion technique was used, as previously described (Gilbert et al., 2003). Briefly, 5 days before the beginning of the infusion, rats were anaesthetized with isoflurane for catheterization of right carotid artery, towards the brain. Catheter was then exteriorized at the vertex of the head, and animals were allowed to recover for 5 days. For infusion, catheter is connected to a swiveling infusion device, allowing the animal free access to water and food and infused with a triglyceride emulsion. Food intake was measured after the 24h infusion period. In another set of experiments, oral glucose tolerance test (3g/kg, OGTT) was also performed.

3.3 Extraction and analysis of ceramids and DAG content in the hypothalamus

Diacylglycerol and ceramide levels in tissues extracts were measured by the diacylglycerol kinase enzymatic method as previously described (Escalante-Alcalde et al., 2003; Le Stunff et al., 2002). Briefly, aliquots of the chloroform phases from cellular lipid extracts were re-suspended in 7.5% (w/v) octyl-β-D-glucopyranoside/5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6.6). The enzymatic reaction was started by the addition of 20 mM DTT, 0.88 U/ml E. coli diacylglycerol kinase, 5 µCi/10 mM [γ-^{32}P]ATP and the reaction buffer (100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM MgCl2, and 2 mM EGTA). After incubation for 1 h at room temperature, lipids were extracted with

chloroform/methanol/HCl (100:100:1, v/v) and 1 M KCl. [γ-32P]-phosphatidic acid was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a Molecular Dynamics Storm PhosphorImager. Known amounts of diacylgycerol and ceramide standards were included with each assay. Ceramide and diacylglycerol levels were expressed as pmol by nmol of phospholipid (PL) levels. Total phospholipids present in cellular lipid extracts used for ceramide analysis were quantified as described previously (Escalante-Alcalde et al., 2003; Le Stunff et al., 2002) with minor modifications. Briefly, a mixture of 10N H_2SO_4/70% perchloric acid (3:1, v/v) was added to lipid extracts which were incubated for 30 min at 210°C. After cooling, water and 4.2% ammonium molybdate in 4 N HCl/0.045% malachite green (1:3 v/v) was added. Samples were incubated at 37°C for 30 min, and absorbance was measured at 660 nm.

4. Results

In the first serie of experiment, whatever the FA ie oleate or linolenate), there was no change in food intake during experiment (data not shown). Basal plasma glucose, FA, and insulin concentrations were also similar in all groups. As displayed in figure 2, in response to glucose load in linoleate group time course of glycemia was similar to control but was associated with an increased glucose induced insulin secretion (GIIS), suggesting an insulin resistance state which was compensated by this increased GIIS. In oleate infused group there was no change in plasma glucose or GIIS compared to controls.

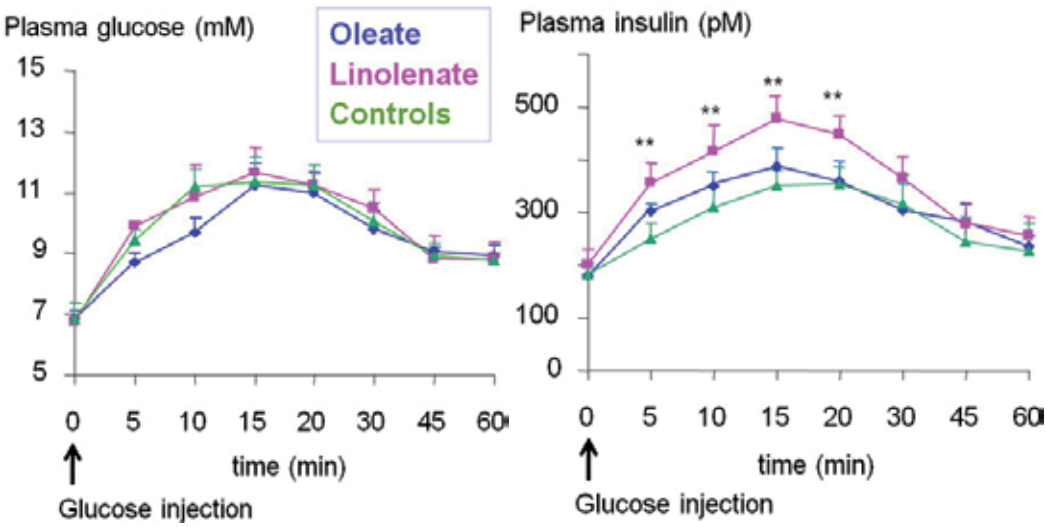


Fig. 2. Time course of plasma glucose and insulin concentration in response to glucose injection in oleate, linolenate, and NaCl (controls) icv 24h infused rats. **, $p < 0.01$ vs oleate and controls.

In order to test whether β oxidation was required to relay FA effect, GIIS was measured in presence or not of etomoxir a specifix inhibitor of CPT1 activity, a rate-limiting enzyme of β oxidation (figure 3). Results are expressed as insulinogenic index (ie ratio of areas under the curve of insulin to glucose during GIIS). Effect of linolenate on GIIS was reversed by etomoxir.

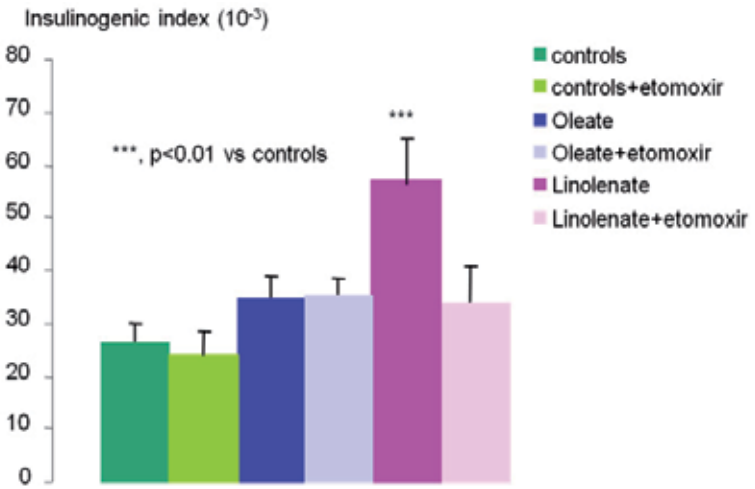


Fig. 3. Insulinogenic index in control rats, oleate and linolenate infused rats with or without etomoxir, a specific inhibitor of CPT1.*** p<0.01 vs controls

Hypothalamic gene expression have been measured in five areas (figure 4) involved in nervous control of energy balance: arcuate nucleus (ARC), ventromedian (VMH), lateral (LH), dorsomedian hypothalamus (DMH) and paraventricular nucleus (PVN). Studied genes were CPT1 (carnitine palmitoyl transferase 1), FAS (fatty acid synthase), GPR41 (G protein related receptor 41, GPR40 and 43 have been also tested but were not detected in our models), ACCβ (Acetyl carboxylase β) and AMPKα2. Results are displayed in figure 5.

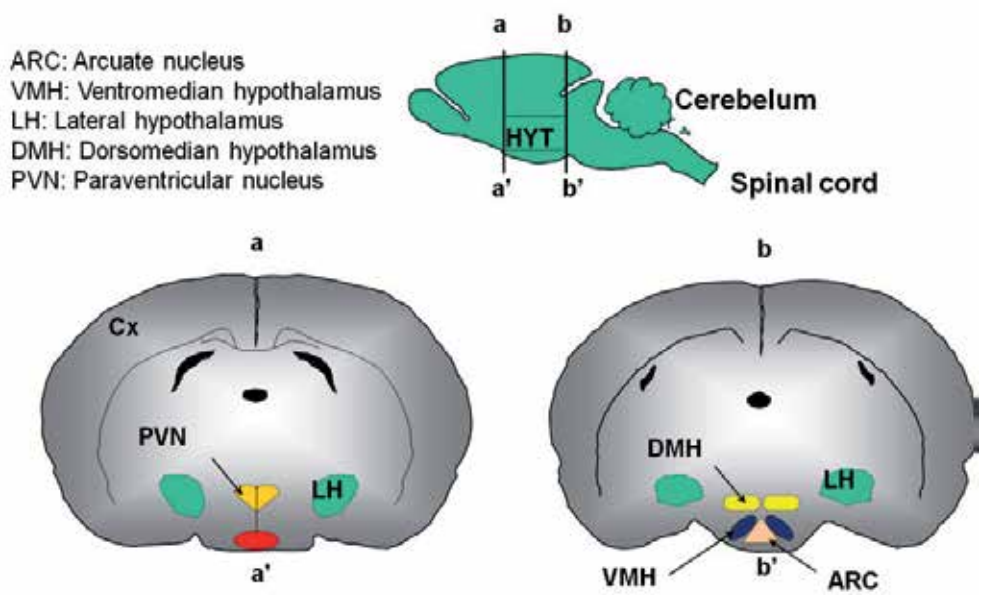


Fig. 4. Map of hypothalamic areas involved in nervous control of energy homeostasis. Arcuate nucleus (ARC), ventromedian (VMH), lateral (LH), dorsomedian hypothalamus (DMH) and paraventricular nucleus (PVN).

Hypothalamic gene expression were also modified in some areas depending on FA (figure 5). For example, FAS expression was inhibited in all nuclei except in VMH of linolenate infused rats. GPR41 was up-regulated in ARC of linolenate infused rats.

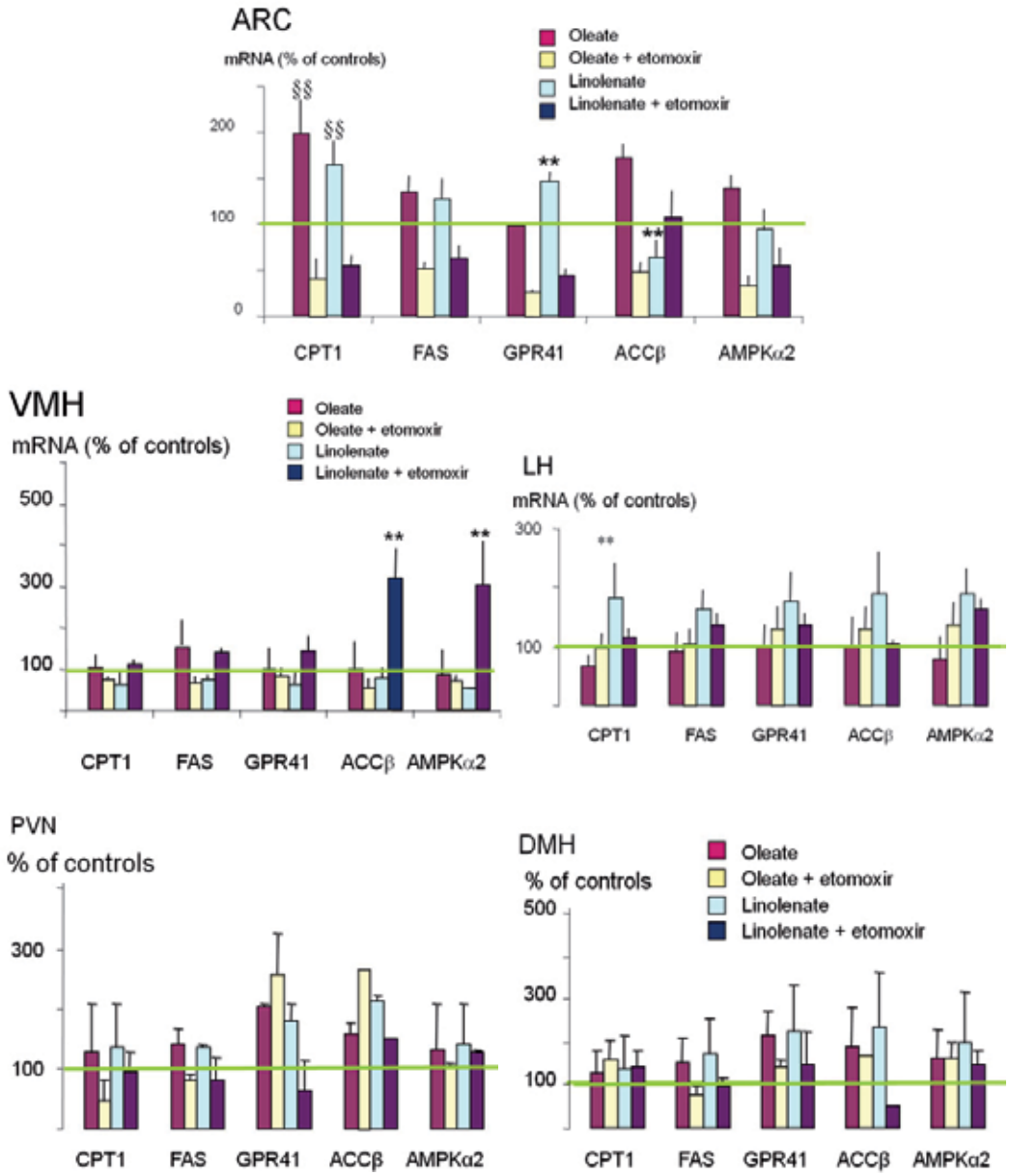


Fig. 5. mRNA expressionof target gene in different hypothalamic areas in oleate or linolenate +/- etomoxir. The green line represents gene expression in control rats (infused with NaCl). **, p <0.01 vs controls. §§, p <0.01 vs controls. ARC: arcuate nucleus; VMH: ventromedian hypothalamus; LH: lateral hypothalamus; DMH: dorsomedian hypothalamus; PVN: paraventricular nucleus.

In the second serie of experiments, we first measured food intake (figure 6). As depicted, there was a decreased in food intake with omegaven and ivelip infusion but not with lard oil.

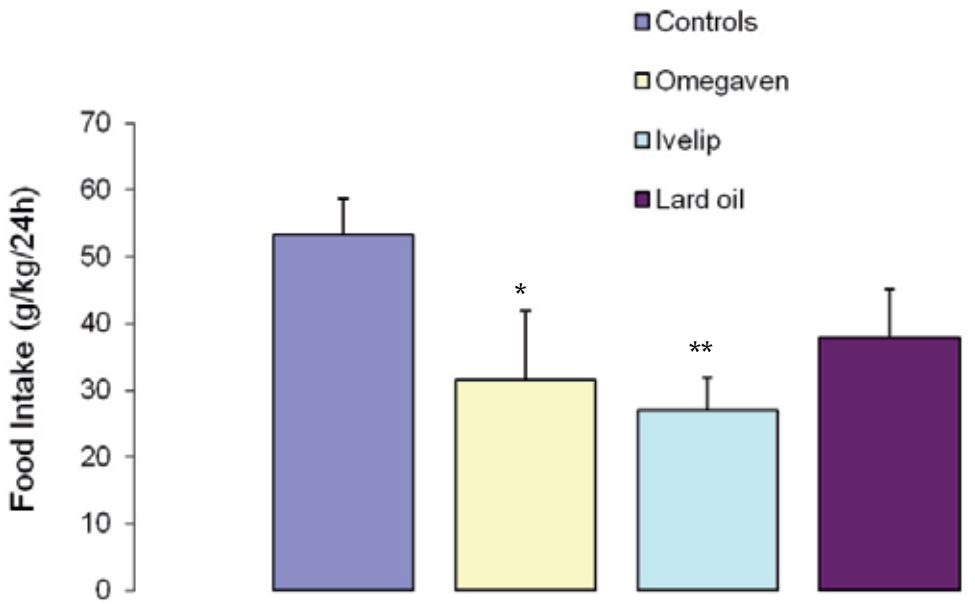
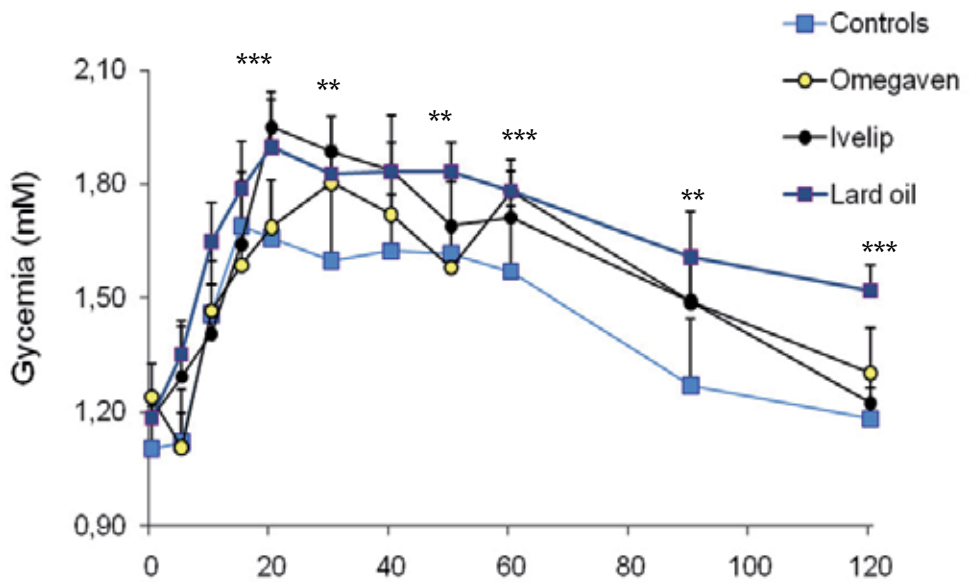


Fig. 6. Measurement of food intake. *, p<0.05 vs controls, **p<0.01 vs controls.

Figure 7 depicted time course and area under the curse of glycemia in response to oral glucose tolerance test. Lard oil induced glucose intolerance compared to controls.



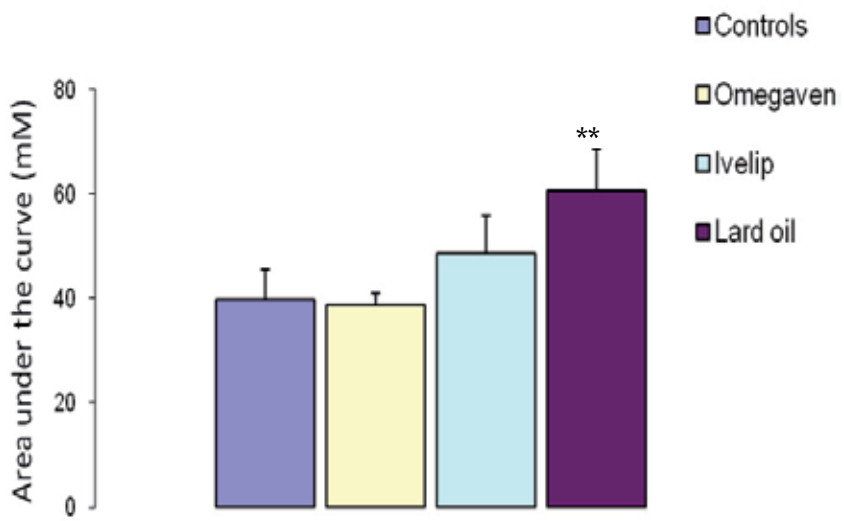


Fig. 7. Time course of glycemia (left) and area under the curve of glycemia during oral glucose tolerance test (right). **p<0.01, *** p<0.001 vs controls.

Interestingly, the effect of lard oil was associated with an accumulation of ceramide in the hypothalamus (figure 8). Therefore, our data suggest that ceramide accumulation in the hypothalamus following icv infusion of saturated fatty acid could contribute to the installation of an insulin resistant state by altering nervous output and consequently nervous control of insulin secretion and action.

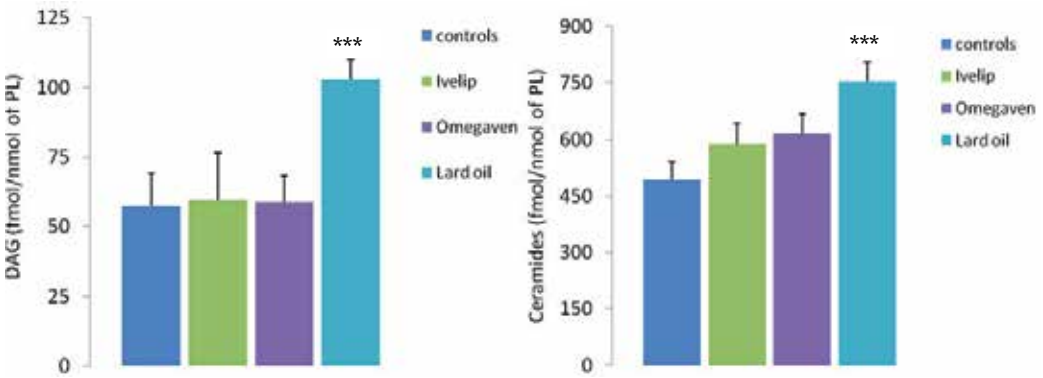


Fig. 8. Hypothalamic content of diacylglycerol (DAG) and ceramides in rats infused through carotid artery with NaCl (controls), Ivelip, Omegaven or Lard oil during 24h. ***p<0.001 vs controls.

4. Discussion

It is now clearly evidenced that hypothalamic FA sensing is an important regulator of nervous control of energy balance. In the present study we highlighted the differential

effects of FA regarding their chain length and degree of saturation. We firstly evidenced here that oleate and linolenate have differential effects in regard to glucose homeostasis and GIIS. Especially linolenate induced increased GIIS compared to both oleate and control group whereas time course of glycemia remained similar. Thus there is a difference between effect of monounsaturated and polyunsaturated fatty acids when infused toward the brain. This suggest activation of different pathways. It must be pointed out that we previously demonstrated that short term infusion of oleate (6h and 24h) induced an increased in insulin secretion induced by glucose compared to control rats (Migrenne et al., 2006; Wang et al., 2006). In the present study infusion was made during 3 days which can explain different effect in short vs long term infusion peridod. Indeed we cannot exclude an adaptation to oleate effect when infusion stay longer. In the same way of ideas, inhibitory effect of central infusion of oleate on food intake was also lost after 3 days of infusion as previously evidenced by obici et al (Obici et al., 2002). In contrast, in the present study effect of linolenate was still present after 3 days of infusion. Linolenate effect may induce an insulin-resistance state and increased GIIS could be an adaptation to this insulin resistance state. By acting on FA sensitive neurons, linolenate may affect nervous output from CNS, especially autonomic nervous system. This change in autonomic nervous system balance will in turn modify nervous control of insulin secretion and action. We previously demonstrated that lipid infusion induced changes in both sympathetic and parasympathetic nervous activity in both rodents (Magnan et al., 1999) and humans (Magnan et al., 2001). In both studies decreased sympathetic nervous activity induced an increased insulin secretion in response to glucose and insulin resistance. In addition, in the present study we showed that linolenate effect involved its metabolism since it had no more effect in presence of etomoxir an inhibitor of β oxidation. The involvement of β oxidation to relay FA effect on sensitive neurons have been also described in different models by us (Cruciani-Guglielmacci et al., 2004) and others (Obici et al., 2003). Finally that specific effects of linolenate compared to oleate could be, at least in part, related to differential gene transcription involved in FA metabolism such as CPT1, FAS or ACC in key areas of hypothalamus. More precisely in ARC and LH CPT1 expression was increased in linolenate infused rats and etomoxir induced a decreased in this gene and its return to basal value. However, it is difficult to further analyze these results since in other nuclei, there is no change of CPT1 expression. In addition, in some area others genes are differently expressed such as AMPKα2 or ACCβ, both key enzymes of glucose and FA metabolism. Altogether these data suggest that oleate or linolenate may act on different subpopulations of neurons (or astrocytes) thus highlighting the fact that FAs may have different effect in regard of the area in which they act. It is also interesting that expression of gene such as GPR41 can be also modified by linolenate infusion. Indeed it has been recently evidenced that short-chain fatty acids and ketones directly regulate sympathetic nervous system via GPR41 at the level of sympathetic ganglion (Kimura et al., 2011). Thus changes in hypothalamic GRP41 gene expression may have an impact during starvation, a situation in which ketone bodies production is increased. thereby control body energy expenditure in maintaining metabolic homeostasis.

In the second part of our work we demonstrated a differential role of PUFA vs saturated FA (SFA) regarding induction of insulin resistance and ceramides production in hypothalamus by using triglyceride emulsion infusion, in order to mimic a more "physiological approach".

Indeed 24h of lard oil infusion in carotid which had no effect on plasma TG or FA concentrations (data not shown) induced a glucose intolerance suggesting a deregulation of insulin sensitivity and or secretion. This deleterious effect of lard oil in nervous control of glucose homeostasis was associated with an increased in DAG and ceramides content in hypothalamus. An important role for ceramides has emerged from research on the pathogenesis of metabolic diseases associated with obesity, such as diabetes (Holland & Summers, 2008). Indeed, ceramides appear to be particularly deleterious components of the lipid milieu that accrues in obesity, and levels of ceramides are often elevated in skeletal muscle, liver, and/or serum of obese humans and rodents (Adams et al., 2004; Clement et al., 2002). DAG and ceramides are known to activate kinase such as PKC, which phosphorylate insulin receptor substrate and Akt leading to an inhibition of the insulin signaling (Mullen et al., 2009; Newton et al., 2009). A recent study also evidenced that sphingolipids such as ceramide might be key components of the signaling networks that link lipid-induced inflammatory pathways to the antagonism of insulin action that contributes to diabetes (Holland et al., 2011). We also recently demonstrated that the atypical protein kinase C, PKCΘ, is expressed in discrete neuronal populations of the ARC and the dorsal medial hypothalamic nucleus (Benoit et al., 2009). CNS exposure to saturated palmitic acid via direct infusion or by oral gavage increased the localization of PKCΘ to hypothalamic cell membranes in association impaired hypothalamic insulin and leptin signaling (Benoit et al., 2009). This finding was specific for palmitic acid, as the monounsaturated FA, OA, neither increased membrane localization of PKCΘ nor reduced insulin signaling. Finally, ARC-specific knockdown of PKCΘ attenuated diet-induced obesity and improved hypothalamic insulin signaling (Benoit et al., 2009). These results suggest that many of the deleterious effects of high fat diets, specifically those enriched with palmitic acid, are CNS mediated via PKCΘ activation, resulting in reduced insulin activity. Therefore, our data suggest that ceramide accumulation in the hypothalamus following icv infusion of saturated fatty acid could contribute to the installation of an insulin resistant state by altering nervous output and consequently nervous control of insulin secretion and action.

Further studies are needed to clearly identify molecular mechanism relaying ceramides production. However there is now several experiments highlighting some of these mechanisms in FA sensitive neurons as described below.

4.1 Molecular mechanisms involved in neuronal FA sensing

In FA sensitive neurons, exposure to long chain FA can alter the activity of a wide variety of ion channels including Cl^-, $GABA_A$ (Tewari et al., 2000), potassium, K^+-Ca^{2+} (Honen et al., 2003) or calcium channels (Oishi et al., 1990). Additionally, FA inhibit the Na^+-K^+ ATPase pump (Oishi et al., 1990). For example, OA activates ARC POMC neurons by inhibiting ATP-sensitive K^+ (K_{ATP}) channel activity (Jo et al., 2009) and the effect of OA on HGP is abolished by icv administration of a K_{ATP} channel inhibitor (Jo et al., 2009). However, K_{ATP} channels are ubiquitously expressed on neurons throughout the brain, not only in FA sensing neurons, making the mechanism and site of such in vivo manipulations difficult to discern (Dunn-Meynell et al., 1998). Using in vivo and in vitro electrophysiological approaches, OA sensitive-neurons have been characterized using whole cell patch clamp

records in ARC slices from 14 to 21 day old rats (Wang et al., 2006). Of these 13 % were excited by OA and 30% were inhibited by OA (Oomura et al., 1975). The excitatory effects of OA appeared to be due to closure of chloride channels leading to membrane depolarization and increased action potential frequency (Migrenne et al., 2006). On the other hand, inhibitory effect of OA may involve the K_{ATP} channels since this inhibition was reversed by the K_{ATP} channel blocker tolbutamide (Migrenne et al., 2006). Using fura-2 Ca^{2+} imaging in dissociated neurons from the ventromedial hypothalamic nucleus (VMN) neurons, we found that OA excited up to 43% and inhibited up to 29% of all VMN neurons independently of glucose concentrations (Le Foll et al., 2009). However, in these neurons, inhibition of the K_{ATP} channel mediated FA sensing in only a small percentage of FA sensing neurons. Importantly, although a relatively large percentage of hypothalamic neurons are FA sensors, a select population that also sense glucose are highly dependent upon ambient glucose concentrations for the resultant effect of FA on the activity of these neurons (Le Foll et al., 2009). Such data suggest that the responses of hypothalamic FA sensitive neurons are dependent upon the metabolic state of the animal and thus might be expected to respond differently during fasting (when FA levels rise and glucose levels fall) vs. the overfed state when glucose levels rise while free FA levels remain relatively unchanged (Le Foll et al., 2009). However, it must be pointed out that FA are naturally complexed to serum albumin in the blood and the concentration of circulating free FA is less than 1% of total FA levels. All the studies investigating FA sensing in the hypothalamus either use non-complexed FA or cyclodextrin-complexed FA *in vitro* or *in vivo*. The concentration of free FA in cyclodextrin-complexed FA preparation is unknown. Whether or not the FA concentration used mimics FA levels in physiological states needs to be determined.

4.2 Metabolic-dependent FA sensing effects

The effects of FA on activity of some neurons are dependent upon intracellular metabolism of FA. Enzymes involved in FA metabolism such as FA synthase (FAS), CPT1 and acetyl-CoA carboxylase (ACC) are expressed in some hypothalamic neurons as well as in glial cells (reviewed in (Blouet & Schwartz; Le Foll et al., 2009). Malonyl-CoA may be an important sensor of energy levels in the hypothalamus. It is derived from either glucose or FA metabolism via the glycolysis or β-oxidation, respectively. The steady-state level of malonyl-CoA is determined by its rate of synthesis catalysed by ACC relative to its rate of turnover catalysed by FAS. The synthesis of malonyl-CoA is the first committed step of FA synthesis and ACC is the major site of regulation in that process. Thus, when the supply of glucose is increased, malonyl CoA levels increase in keeping with a decreased need for FA oxidation. This increase in both malonyl CoA and acyl CoA levels is associated with reduced food intake. Central administration of C75, an inhibitor of FAS, also increases malonyl-CoA concentration in the hypothalamus, suppresses food intake and leads to profound weight loss (Proulx & Seeley, 2005). It has been proposed that centrally, C75 and cerulenin (another inhibitor of FAS) alter the expression profiles of feeding-related neuropeptides, often inhibiting the expression of orexigenic peptides such as neuropeptide Y (Proulx et al., 2008). Whether through centrally mediated or peripheral mechanisms, C75 also increases energy expenditure, which contributes to weight loss (Clegg et al., 2002; Tu et al., 2005). In vitro and in vivo studies demonstrate that at least part of C75's effects are mediated by the

modulation of AMP-activated kinase, a known energy-sensing kinase (Ronnett et al., 2005). Indeed, icv administration of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a 5'-AMP kinase activator, rapidly lowers hypothalamic malonyl-CoA concentration and increases food intake (Tu et al., 2005). These effects correlate closely with the phosphorylation-induced inactivation of ACC, an established target of AMP kinase. Collectively, these data suggest a role for FA metabolism in the perception and regulation of energy balance. However, it must be also pointed out that C75 and AICAR may also have non-specific or even opposite effects. For example, a major effect of C75 is to activate CPT-1 rather than lead to its inhibition *in vitro* (Aja et al., 2008). Finally the route of administration and the type of FA used are also critical. For example, bolus intracerebroventricular injections of OA, but not palmitic acid, reduce food intake and body weight, possibly mediated through POMC/MC4R signaling (Schwinkendorf et al., 2010). Again, such bolus icv injections could cause non-specific effects related to inflammation of ependymocytes and tanycytes. Also because so much of FA metabolism takes place in astrocytes, such manipulations done in vivo and in slice preparations are likely to alter FA metabolism that takes place in astrocytes which could then indirectly alter neuronal FA sensing (Escartin et al., 2007).

4.3 Non metabolic-dependent neuronal FA sensing

While intracellular FA metabolism may be responsible for altering neuronal activity in some FA sensitive neurons such as ARC POMC neurons (Jo et al., 2009) it accounts for a relatively small percent of the effects of OA on dissociated VMN neurons (Le Foll et al., 2009). In those neurons, inhibition of CPT1, reactive oxygen species formation, long-chain acyl CoA synthetase and K_{ATP} channel activity or activation of uncoupling protein 2 (UCP2) accounts for no more than 20% of the excitatory or approximately 40% of the inhibitory effects of OA (Le Foll et al., 2009). On the other hand, pharmacological inhibition of FAT/CD36, a FA transporter/receptor that can alter cell function independently of intracellular FA metabolism reduced the excitatory and inhibitory effects of OA by up to 45% (Le Foll et al., 2009). Thus, in almost half of VMN FA sensing neurons, CD36 may act primarily as receptor, rather than a transporter, for long chain FA as it does on taste cells on the tongue where it activates store-operated calcium channels to alter membrane potential and release of serotonin (Gaillard et al., 2008). These effects all occur in the presence of nanomolar concentrations of OA, whereas micromolar concentrations are generally required to effect similar changes in neuronal activity in brain slice preparations (Jo et al., 2009; Migrenne et al., 2011; Wang et al., 2006). Thus, in the absence of astrocytes, OA can directly affect VMN neuronal activity through both metabolic and non-metabolic pathways. Alternatively, FA might act as signaling molecules by covalent attachment to proteins (N-terminal acylation) to alter the function of membrane and intracellular signaling molecules. For example, palmitoylation facilitates the targeting and plasma membrane binding of proteins which otherwise would remain in the cytosolic compartment (Resh, 1999). Some membrane proteins (TGFα, synaptosomal associated protein of 25KDa (required for exocytosis) and plasma membrane receptors (seven transmembrane receptors such as α_{2a}- and β_2-adrenoceptors) are typically palmitoylated on one or several cysteine residues located adjacent to or just within the transmembrane domain (Resh, 1999) Such mechanisms might also modulate neuronal FA sensing.

4.4 Which neurotransmitters or neuropeptides?

The ultimate consequence of the activation or inactivation of a neuron is the release of neurotransmitters and neuropeptides. Since FA decrease food intake, they might be expected to alter activity neurons specifically involved in the regulation of feeding. In fact, OA activates catabolic POMC neurons directly, apparently via ß-oxidation and inactivation of the K_{ATP} channel in hypothalamic slice preparations (Jo et al., 2009). In vivo, Obici et al. (Obici et al., 2003) reported that icv administration of OA markedly inhibits glucose production and food intake, accompanied by a decrease in the hypothalamic expression of the anabolic peptide, neuropeptide Y. This decrease in the expression of such a critical anabolic peptide might contribute to the reduced food intake associated with direct central administration of OA. On the other hand, an n-3 FA enriched diet increases food intake in anorexic tumor-bearing rats, in association with reduced tumor appearance, tumor growth and onset of anorexia (Ramos et al., 2005). In these treated rats, neuropeptide Y immunoreactivity increased 38% in ARC and 50% in paraventricular nucleus, whereas α-melanocyte stimulating hormone (a catabolic peptide cleavage product of POMC) decreased 64% in the ARC and 29% in the paraventricular nucleus (Ramos et al., 2005). Finally, in the hippocampus, docosahexaenoic acid (22:6(n-3) increased the spontaneous release of acetylcholine (Aid et al., 2005).

4.5 Pathological implications of excess FA

Besides physiological regulation of energy balance by hypothalamic neuronal FA sensing, impaired regulation of such sensing might contribute to the development of metabolic diseases such as obesity and type 2 diabetes in predisposed subjects exposed to a chronic lipid overload (Luquet & Magnan, 2009; Migrenne et al., 2011). Excessive brain lipid levels may indeed alter control of glucose and lipid homeostasis through changes of autonomic nervous system activity. Increasing brain FA levels reduces sympathetic activity and increases GIIS in rats (Clement et al., 2002; Obici et al., 2003) a condition which would exacerbate the development of type 2 diabetes mellitus. Also, a lipid overload due to high-fat diet intake alters both hypothalamic monoamine turnover (Levin et al., 1983) and peripheral sympathetic activity in rats (Young & Walgren, 1994). In humans, overweight is often associated with an altered sympathetic tone (Peterson et al., 1988) suggesting a relationship between lipids and autonomic control centers in brain.

5. Conclusion

In conclusion, there is now increasing evidence that specialized neurons within hypothalamus and other areas such as the brainstem or hippocampus can detect changes in plasma FA levels by having FA directly or indirectly alter the of FA sensitive neurons involved in the regulation of energy and glucose homeostasis. Central FA effects on insulin secretion and action are related to their chain length or degree of saturation. Such effects are also mediated through differential changes in gene expression.

The neuronal networks of these FA sensitive neurons that sense and respond to FA are likely very complex given the fact that FA can either inhibit or excite specific neurons. In addition, many of these neurons also utilize glucose as a signaling molecule and there is often an inverse responsiveness of such "metabolic sensing" neurons to FA vs. glucose.

Thus, these neurons are ideally suited to respond differentially under a variety of metabolic conditions such as fasting, feeding, hypo- or hyperglycemia. However, while it is clear that specific neurons can respond to changes in ambient FA levels, many questions remain. We still do not know for certain how FA are transported into the brain, astrocytes or neurons and whether those FA that are transported are derived from circulating free FA or triglycerides. Since most studies suggest that rising FA levels reduce food intake, then we must explain why plasma FA levels are most elevated during fasting when the drive to seek and ingest food should be at its strongest. Another major issue relates to the interaction between astrocytes and neurons with regard to the metabolism and signaling of FA. Also, we still know little about the basic mechanisms utilized by neurons to sense FA, where such FA sensitive neurons reside throughout the brain and what neurotransmitters and peptides they release when responding to FA.

Finally, it has been postulated that diabetes may be a disorder of the brain (Elmquist & Marcus, 2003). If so, dysfunction of these FA sensitive neurons could be, at least in part, one of the early mechanisms underlying impairment of neural control of energy and glucose homeostasis and the development of obesity and type 2 diabetes in predisposed subjects. A better understanding of this central nutrient sensing, including both FA and glucose, could provide clues for the identification of new therapeutic targets for the prevention and treatment of both diabetes and obesity.

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Part 4

Innovative Techniques for the Production of Olive Oil Based Products

22

Meat Products Manufactured with Olive Oil

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1. Introduction

Consumer perception of processed meat products is a critical issue for the meat industry. In recent years consumers are increasingly conscious about healthy diet. However, most of the processed meat products contain high amounts of fat, which are related to chronic diseases such as obesity and cardiovascular heart diseases. Health organizations have suggested to reduce the intake of total dietary fat, particularly saturated fatty acids and cholesterol, as a mean to prevent cardiovascular heart diseases (NCEP, 1988). Consumers now want low or reduced-animal fat products with high palatability and nutritional quality (Pietrasik & Duda, 2000).

Animal fat is a major factor that determines the eating quality of meat products including texture, flavor and mouth-feel (Keeton, 1994). Therefore, reducing fat levels in meat products is not as simple as using less amounts of fat in the formulation. Twenty percent or higher reduction of fat content in meat products can lead to an unacceptable product texture, flavor and appearance (Miles, 1996). Total substitution of fat with water produces unacceptably soft and rubbery product with an increased moisture loss during processing (Claus & Hunt, 1991).

The problems caused by fat reduction in processed meat products can be minimized by replacing animal fat with fat replacers (Colmenero, 1996). Several studies have demonstrated that replacing animal fat with soy products or carbohydrate is successful in textural and sensory properties of low-fat products (Decker et al., 1986; Berry & Wergin, 1993; Yusof & Babji, 1996). Isolated soy proteins (ISP) were successfully incorporated into meat products to reduce fat, improve yields, and enhance emulsion stability. Carageenan increases yield, consistency, sliceability, and cohesiveness, while decreasing purge in low-fat products (Foegeding & Ramsey, 1986; Xiong et al., 1999; Lin & Mei, 2000). Maltodextrin, which is a hydrolysis by-product of starch, is widely used in foods as a funcitonal biopolymer that provides desirable texture, stability, appearance, and flavor (Wang & Wang, 2000).

Olive oil is a vegetable oil with the highest level of monounsaturated fatty acids (MUFA) and has attracted attention as a replcacer for animal fat in processed meat products. Olive oil

has a high biological value due to a favorable mix of predominantly MUFA and naturally occurring antioxidants including vitamin E, vitamin K, carotenoids and polyphenols such as hydroxytyrosol, tyrosol and oleuropein. Oleic acid makes up 92% of the MUFA in foods, and 60-80% of the oleic acid comes from olive oil (Pérez-Jiménez et al., 2007). Olive oil contains 56-87% monosaturated, 8-25% saturated and 3.6-21.5% polyunsaturated fatty acids (IOOC, 1984). The potential health benefits of olive oil include an improvement in lipoprotein profile, blood pressure, glucose metabolism and antithrombotic profile. It is also believed that olive oil has a positive influence in reducing inflammation and oxidative stress. Thus, intake of MUFA may protect against age-related cognitive decline and Alzheimer's disease. Olive oil is also reported to help prevent breast and colon cancer (Pérez-Jiménez et al., 2007, Waterman & Lockwood, 2007).

This chapter discusses the effect of olive oil on the quality of emulsion-type sausage (Moon et al., 2008) and pork patty (Hur et al., 2008) when used as an animal fat replacer in the products. The grade of olive oil used were extra virgin olive oil(defined by the European Union Commission reg. No. 1513/2001).

2. Fat replacers in processed meat products

Most efforts in developing low-fat meat products to satisfy concerned consumers have been focused on reducing fat and/or substituting animal fats in the formula with plant oils. Fat is an important determinant for the sensory properties of meat and meat products, and thus a simple reduction of animal fat content in the formulation can lead to a product with poor sensory quality. Therefore, strategies to reduce animal fat while retaining traditional flavor and texture of meat products.

Juiciness and mouthfeel are very closely related to the fat content in meat products. To a large extent these sensory quality can be retained by using binders in low-fat and/or healthy meat products. Binders have been added to meat products for many years for both technological reasons and cost savings. Many binders with a number of different properties are available, but all those used in value-added meat products are to improve water binding capacity. Among the binders, carrageenan is the most widely used in meat industry. According to Varnam & Sutherland(1995), iota-carrageenan with calcium ions forms a syneresis-free, clear plastic gel with good resetting properties after shear. It is particularly recommended for use in low-fat products. Iota-carrageenan has very good water retention properties, and enhance cold solubility and freeze-thaw characteristics of processed products. The presence of NaCl in solution inhibits swelling of carrageenan but this difficulty can be solved by using NaCl encapsulated with partially hydrogenated vegetable oil such as olive oil, soya oil, corn oil and palm oil. Hydrogenated corn oils or palm oils are particularly effective in replacing beef fat. Soya oil emulsion is also effective at levels up to 25%, especially when used in conjunction with isolated soya proteins (Varnam & Sutherland, 1995).

Olive oil can be used in processed meat products an an oil-in-water emulsion form (Hoogencamp, 1989). Briefly, water is heated to 60-65°C. This water is homogenized with the isolated soy protein (42.15%, w/w) and the mixture is cooled to 5°C and then placed in a chilled cutter. After homogenizing for 1 min, olive oil is added while homogenization is

continued. Finally, the mixture is homogenized for additional 3 min and then used for manufacturing sausages and patties.

The incorporation of olive oil has been studied in fermented sausages (Bloukas et al., 1997; Kayaardi & Gök, 2003; Koutsopoulos et al., 2008) and beef patties (Hur et al, 2008). Partial replacement of animal fats with olive oil has also been tested (ranging between 3–10 g of olive oil per 100 g of product) in frankfurter sausages and low-fat products. Previous studies (Jiménez-Colmenero, 2007; López-López et al., 2009b) indicated that partial replacement of pork backfat with olive oil increased MUFA contents without significantly altering the n-6/n-3 ratio.

3. Incorporation of olive oil in meat products

To develop healthier meat products, various technological options of replacing animal fat have been studied (Jiménez-Colmenero, 2007). Olive oil has been incorporated in meat emulsion systems such as frankfurters in liquid (Lurueña-Martinez et al., 2004; López-López et al., 2009a, 2009b) or interesterified form (Vural et al., 2004). However, oil-in-water emulsion is the most suitable technological option for stabilizing the non-meat fats added to meat derivatives as ingredients due to physicochemical properties (Bishop et al., 1993; Djordjevic et al., 2004). There are a number of procedures that can be used to produce a plant or marine oil-in-water emulsions (with an emulsifier, typically a protein of non-meat origin) for meat products (Jiménez-Colmenero, 2007), but only sodium caseinate has been used to stabilize olive oil for incorporation in frankfurter-type products (Paneras & Bloukas, 1994; Ambrosiadis et al., 1996; Paneras et al., 1998; Pappa et al., 2000; Choi et al., 2009).

Tables 1 and 2 are examples of fmomulas that use olive oil and different fat replacers in producing an emulsion-type sausage and pork patty.

Ingredients (%)		Control	ICM [1)]	ICMO [2)]
Pork ham		68.95	73.24	71.57
Pork backfat		19.25	-	-
Ice/water		9.75	7.71	9.38
Fat replacer	ICM[1)]	-	17.00	12.00
	Olive Oil	-	-	5.00
NPS [3)]		1.30	1.30	1.30
Phosphate		0.20	0.20	0.20
Sugar		0.50	0.50	0.50
Monosodium glutamate		0.05	0.05	0.05
Total		100	100	100

[1)] Isolated soy protein: carrageenan: maltodextrin: water = 2:1:1:20.

[2)] ICM+Olive Oil.

[3)] NaCl: NaNO2 = 99:1.

Table 1. Formulation of emulsion-type low-fat sausages manufactured with and without fat replacers.

	C	T 1	T 2	T 3
Lean pork	83.5	81.0	80.5	80.0
Pork back fat	10.0	5.0	5.0	5.0
Olive oil	-	5.0	5.0	5.0
ISP	-	0.5	0.5	0.5
Carageenan	-	-	0.5	0.5
Maltodextrin	-	-	-	0.5
Salt	1.2	1.2	1.2	1.2
Black pepper	0.3	0.3	0.3	0.3
Water	5.0	7.0	7.0	7.0
Total	100	100	100	100

[1)]C, 10 % backfat; T1, 5 % backfat + 5% olive oil + 0.5 % isolated soy protein; T2, 5% backfat + 5% olive oil + 0.5% isolated soy protein + 0.5% carageenan (T2). T3, 5% backfat + 5% olive oil + 0.5% isolated soy protein + 0.5% carageenan + 0.5% martodextrin.

Table 2. Formulation of pork patty with fat replacers

3.1 Chemical composition and nutritional value of meat products manufactured with olive oil

The chemical composition of emulsion-type sausages indicated that fat content was reduced by replacing the pork backfat with ICM, but increased with added olive oil (Table 3). Replacing backfat with fat replacers resulted in increased fat content at day 30 for ICM and day 15 and 30 for ICMO; however, the control was not differ. These results could be due to increased moisture loss (%) with longer storage time. ICM and ICMO had higher moisture content than control. When pork backfat is fully replaced by oil-in-water emulsion, which contains 52% olive oil, the sausage contains approximately 13 g of olive oil per 100 g of product. This means a considerable increase in the proportion of MUFA. Olive oil can make up almost 70% of the total fat content of the sausage. The caloric content of sausages was 225-245 kcal/100 g, and 70% of which were from fat. In traditional sausages, all are supplied by animal fat, whereas, in the sausage replaced with olive oil, the animal fat supplied only 20%. The other 50% is from the olive oil. It was suggested that meat products, strategically or naturally enriched with healthier fatty acids, can be used to achieve desired biochemical effects without dietary supplements or changing dietary habits (Jiménez-Colmenero et al., 2010).

Up to 7 – 13 g of olive oil could be added per 100 g sausages as an animal fat replacer. However, the purpose of replacing animal fat with olive oil is to produce low-fat products, and consequently such high proportion of olive oil is not desirable (Jiménez-Colmenero et al., 2007). One of the fundamental strategies in developing a healthier lipid formula is concentrating active components in target food products to enable the cosumption of recommended intake levels with normal portion sizes. Dietary models provided by the World Health Organization (2003) suggested that MUFA should be the major dietary fatty acids. If MUFAs are the predominant fatty acids in a product, the total fat intake would not be substantial (Pérez-Jiménez et al., 2007).

Protein content of the sausage (ICMO) containing ICM and olive oil was higher than that of the control. This could be attributed to higher lean content and ISP in the formulation of

ICMO. Therefore, the replacement of animal fat with olive oil may produce products with healthier lipid composition (higher MUFAs, mainly oleic acid) without substantial deterioration in nutritional quality.

In pork patty study, moisture content was significantly higher in the products with olive oil+ISP+carageenan (T2) and T2 with maltodextrin (T3) when compared with control and that with olive oil+ISP (T1) (Table 4). In contrast, control and T1 had significantly higher crude protein than T2 and T3. Crude fat content was higher in T1 and T2. The pork patty with olive oil treatment had higher ash content than control. Pietrasik and Duda (2000) reported that the increased weight losses when the reduction of fat is accompanied by an increase in the proportion of moisture, and protein levels remain essentially the same. However, substitution of backfat with olive oil produced pork patty not only with higher in moisture but also higher fat content than control in this study. Thus, it can be assumed that olive oil substitution for backfat may not induce weight loss of pork patty. These results agreed with Pappa et al. (2000) who reported no significant difference in yield when olive oil was replacing pork fat in low-fat frankfurters.

Treatment	Fat (%)	Protein (%)	Moisture (%)
Control			
1 day	19.72±1.56^{a}	15.06±0.71^{d}	61.96±1.78^{d}
15 days	19.36±1.34^{a}	15.16±0.49^{d}	61.34±1.40^{d}
30 days	19.62±1.44^{a}	15.34±0.70^{d}	61.15±1.46^{d}
ICM[1)]			
1 day	3.34±0.63^{e}	18.38±0.96^{a}	74.58±1.15^{a}
15 days	3.21±0.59^{e}	18.23±0.84^{a}	74.24±1.06^{a}
30 days	4.63±0.46^{d}	17.79±0.52^{ab}	72.77±0.58^{b}
ICM O[1)]			
1 day	7.35±0.19^{c}	16.70±0.75^{bc}	73.24±0.75^{ab}
15 days	8.65±0.29^{b}	17.27±0.50^{ab}	71.08±0.95^{c}
30 days	8.58±0.42^{b}	16.60±0.49^{bc}	71.12±1.06^{c}

[1)] See Table 1.
[a-e] Means ± S.E. with different letters in the same column indicate significant differences (p<0.05).

Table 3. Chemical composition of emulsion-type low-fat sausages with or without fat replacers

	C	T1	T2	T3
Moisture	60.42±0.65^{B}[2)]	60.32±1.05^{B}	62.15±0.22^{A}	61.63±0.37^{AB}
Crude Protein	23.37±0.44^{A}	20.28±0.62^{BC}	19.54±0.76^{C}	21.30±1.84^{B}
Crude fat	14.93±0.90^{B}	17.34±0.41^{A}	16.29±1.05^{A}	14.88±0.85^{B}
Ash	1.28±0.02^{B}	2.06±0.13^{A}	2.02±0.03^{A}	2.19±0.11^{A}

[1)]See Table 2.
[2)] [A-C] Means ± SD with different superscripts in the same row significantly differ at p<0.05.

Table 4. Proximate compositions in pork patty made by substituted olive oil for backfat

3.2 Physicochemical properties of meat products manufactured with olive oil

The water holding capacity (WHC) of meat products provide succulent texture and mouthfeel to consumers. A number of studies have proved that there are an inverse relationship between fat content and the amount of water released (Hughes et al., 1997). In Table 5, ICMO was not difference in WHC when compared with the control. It means that olive oil can be combined with other fat replacers such as ISP and carrageenan to improve WHC in meat products. In the case of ICMO, which was emulsified with ISP and carrageenan, the release of water seemed to be protected during storage days.

Cooking loss of meat products is usually influenced by fat content. The products with higher fat content lose less water after cooking ((Jiménez-Colmenero et al., 2007) because high-fat products contain less water. The cook losses of the low-fat sausages manufactured with olive oil and fat replacers (ICM and ICMO) were lower than those of the control (Table 5). However, when the reduction of fat contents in the sausages was considered, the increase of cook loss is not significant. Some fat replacers such as whey protein, carrageenan and tapioca starch could reduce the cook loss of low-fat sausages due to water retainability (Lyons et al., 1999).

Treatment		WHC (%)	Cook loss (%)
Control			
	1 day	71.02±1.17^{a}	13.30±0.37^{cd}
	15 days	69.52±0.89^{ab}	13.18±0.53^{d}
	30 days	68.33±0.93^{b}	13.86±0.52^{bcd}
ICM[1)]			
	1 day	68.32±0.59^{b}	14.37±0.82^{bc}
	15 days	67.95±0.95^{bc}	14.78±0.48^{a}
	30 days	66.77±0.59^{c}	14.90±0.40^{a}
ICMO[1)]			
	1 day	69.79±0.43^{ab}	13.13±0.54^{d}
	15 days	69.12±1.18^{ab}	14.01±0.34^{bc}
	30 days	68.28±0.82^{b}	14.61±0.52^{ab}

[1)] See Table 1., $^{a-d}$ Means ± S.E. with different letters in the same column indicate significant differences (p<0.05).

Table 5. Water holding capacity (WHC, %) and cook loss (%) of low-fat sausages with or without fat replacers

	C	T1	T2	T3
pH	5.82±0.03^{A}	5.75±0.02^{B}	5.78±0.01^{B}	5.78±0.02^{B}
WHC **(%)**	79.05±2.22$^{A2)}$	72.05±1.12^{B}	80.39±14.58^{B}	83.99±12.65^{A}
Fat retention (%)	79.31±0.02^{C}	83.97±0.01 ^{B}	84.64±1.06 ^{B}	86.61±1.28 ^{A}
Cooking loss (%)	28.05±0.70	27.30±0.69	27.72±1.10	26.95±1.61

[1)] See Table 2., [2)] $^{A-C}$ Means ± SD with different superscripts in the same row significantly differ at p<0.05.

Table 6. Changes of physical characteristics in pork patty made by substituted olive oil for backfat

On other hand, WHC of pork patty was significantly higher in control and T3 than T1 and T2. Control had higher pH than olive oil-added pork patties, but no significant differences

were found among the samples with 50% olive oil substitution for backfat. Fat retention was higher in the olive oil-substuted samples than control. Especially T3, the patty with olive oil+ISP+carageenan, showed the highest fat retention. However, cooking loss was not different among the treatments. In this present study, WHC was steadily decreased as olive oil substitution level increased. However, this does not mean that the quality of pork patty decreased, because fat retention was higher in olive oil-added pork patties, and cooking loss was not significantly different.

In other meat product studies, Kayaardi and Gok (2003) reported that replacing beef fat with olive oil had no effect on the pH value of the Soudjouks samples. Luruena-Martinez et al. (2004) and Muguerza et al. (2002) reported that the addition of olive oil did not produce significant differences in cooking losses of sausage but made the sausage lighter in color and more yellow (Muguerza et al., 2002). In contrast, Bloukas et al. (1997) reported that the higher the olive oil content, the higher the weight loss, probably due to higher amounts of water added. Hur et al. (2008) repoprted that WHC was decreased but fat retention was increased by olive oil substitution.

3.3 Color and lipid oxidation of meat products manufactured with olive oil

Color of meat products is an important quality parameter for purchase decision by consumers. The most common cause for changing color is the formation of metmyoglobin by oxygen-dependent meat enzymes. Aerobic micro-organisms are successfully competing with meat pigments for oxygen. Formation of metmyoglobin can vary, and occasionally discolored areas are present adjacent to and fully demarcated areas where coloration is bright pink. Use of low-quality fat containing high levels of peroxides can cause oxidation of meat pigments (Varnam & Sutherland, 1995).

Varnam & Sutherland(1995) reported that sausages can have a number of specific quality issues: 'Pressure marks' are the result of oxygen deficiency where packed sausages are in close contact to each other. Pigment is initially converted to reduced myoglobin and subsequently, as some diffusion of oxygen occurs, to metmyoglobin. 'White spot' appears to be an oxidative defect, which involves formation of circular grey or white areas that increase in size with continuing storage. It could be associated with low SO_2 levels and use of fats with a high peroxide content.

The sausage incorporated with ICM and olive oil as fat replacers showed higher yellowness and redness (Table 7). Yellower color could be from the original color of olive oil and redder color from higher lean ratio, which includes higher myoglobin content, compared to traditional sausages (control).

Olive oil and ISP are known to have antioxidant properties. The sausages emulsified with ISP and olive oil (ICMO) inhibited lipid oxidation (Table 7). The progress of lipid oxidation can cause changes of meat quality including color, flavor, odor, texture and even the nutritional value in meat products (Fernandez et al., 1997). The stability of fat often limits the shelf life of meat products. The incorporation of olive oil and ISP into meat products may improve the shelf life of the products due to their antioxidant properties. In our study, TBARS values of ICMO were lower than those of the control on days 15 and 30. The TBARS of ICMO sample remained constant throughout the 30 days of storagebut those of the control and ICM increased ($p< 0.05$) from days 15 to 30. The higher TBARS value for the control on each storage day might be due to high fat content in control sausages.

Treatment	Lightness (L^*)	Redness (a^*)	Yellowness (b^*)	TBARS (mg malonaldehyde/kg sample)
Control				
1 day	78.39±0.37^{a}	11.06±0.21^{b}	3.45±0.17^{b}	0.16±0.03^{c}
15 day	77.25±0.64^{ab}	10.41±0.19^{b}	2.34±0.24^{c}	0.22±0.03^{b}
30 day	76.41±0.88^{b}	10.22±0.09^{b}	2.49±0.61^{bc}	0.32±0.05^{a}
ICM[1)]				
1 day	74.95±0.69^{c}	12.13±0.40^{a}	3.30±0.16^{b}	0.16±0.02^{c}
15 day	73.48±0.98^{cde}	10.42±0.07^{b}	2.42±0.24^{bc}	0.14±0.04^{cd}
30 day	71.69±1.31^{e}	10.29±0.13^{b}	2.20±0.05^{c}	0.24±0.02^{b}
ICMO[1)]				
1 day	73.45±0.18^{de}	11.80±0.64^{ab}	4.04±0.13^{a}	0.17±0.02^{c}
15 day	72.49±0.17^{e}	10.46±0.25^{b}	2.44±0.15^{bc}	0.15±0.04^{cd}
30 day	72.01±0.65^{e}	10.31±0.06^{b}	2.79±0.13^{bc}	0.20±0.03^{bc}

[1)] See Table 1., $^{a-e}$ Means ± S.E. with different letters in the same column indicate significant differences (p<0.05).

Table 7. Color and lipid oxidation of low-fat sausages with or without fat replacers

L*-value of raw pork patty was higher in control and T1 than other samples, but no significant difference were found after cooking (Table 8). a*-value was significantly higher in control than the samples with olive oil-added products in both raw and cooked states. It can be assumed that redness may be higher in control than olive oil-added pork patties, but lightness and yellowness may not be much different. Paneras et al. (1998) also reported differences in color when low fat frankfurters were produced with different levels of vegetable oils. Low-fat frankfurters were darker, redder and more yellow than high fat frankfurters. However, Marquez et al. (1989) found no differences in color parameters by oil treatments in beef frankfurters. These studies indicated that the change of meat color by oil treatment can vary depending upon the meat products.

	Color	C	T1	T2	T3
Raw sample	L*	55.89±1.46^{A}[2)]	55.31±0.96^{A}	52.00±0.62^{B}	52.58±1.32^{B}
	a*	13.86±0.35^{A}	11.75±0.63^{B}	11.75±0.45^{B}	11.84±0.52^{B}
	b*	9.46±0.09	9.77±0.48	9.04±0.70	9.48±0.49
Cooked sample	L*	62.11±5.90	63.98±3.58	66.71±0.40	66.26±1.94
	a*	7.60±0.30^{A}	7.02±0.33^{B}	6.67±0.13^{BC}	6.07±0.24^{C}
	b*	9.37±0.73^{B}	11.06±0.08^{A}	8.57±0.56^{C}	9.80±0.93^{AB}

[1)] See Table 2.,
[2)] $^{A-C}$ Means ± SD with different superscripts in the same row significantly differ at p<0.05.

Table 8. Changes of meat color in pork patty by substituting backfat with olive oil

Chin et al. (1999) and Claus et al. (1990) found that redness and lightness values were more affected by fat/lean ratio and myoglobin concentration of the lean part. Muguerzaet al. (2002) and Bloukas et al. (1997) also found that replacing, in part, backfat with olive oil produced yellower sausages than controls. Muguerza et al. (2002) reported that antioxidant present in olive oil and ISP helped maintaining color by minimizing color oxidation. The present study is in agreement with the findings of other researchers (Kayaardi & Gök, 2003; Ansorena & Astiasarán, 2004; Bloukas et al., 1997) who reported increase of lipid oxidation in meat products during fermentation and ripening period. They found that replacing animal fat with olive oil was effective for inhibiting the lipid oxidation during storage. Our previous and present results indicated that replacing animal fat with olive oil can be effective in inhibiting lipid oxidation in meat products during storage.

3.4 Texture and sensory properties of meat products manufactured with olive oil

Textural properties of the emulsion-type sausages are affected by the replacement of backfat with olive oil emulsion (Table 9). In general, frankfurters made with oil-in-water emulsions presented higher hardness, cohesiveness and chewiness and lower adhesiveness than traditional frankfurters. The textural properties of frankfurters manufactured with olive oil are influenced by the characteristics of oil-in-water emulsion and its role in the meat protein matrix. Frankfurters with olive oil emulsion containing caseinate or soy protein presented similar hardness and chewiness to control, but those with soy protein presents higher springiness and cohesiveness (Jiménez-Colmenero et al. 2010) (Table 9).

The frankfurters containing olive oil emulsion with caseinate or soy protein had higher hardness, cohesiveness, gumminess and chewiness values than the traditional sausages. The result of texture might be due to the reduced fat content sausages. In high fat frankfurters, in which pork backfat is replaced by olive oil, generally have less flavor intensity and are harder and less juicy (Jiménez-Colmenero et al., 2010). However, these differences are marginal, and the frankfurters received similar scores for general appearance and acceptability (Jiménez-Colmenero et al., 2010). Partial substitution of animal fat with olive oil reduced juiciness scores.

Parameter	Control	ICM[1)]	ICMO[1)]
Hardness (kg)	0.33±0.04b	0.42±0.02a	0.40±0.03a
Cohesiveness	60.85±1.52[b]	66.47±0.90[a]	66.09±0.54[a]
Springiness	13.11±0.27	13.53±0.04	13.23±0.24
Gumminess (g)	19.26±0.88[b]	22.09±0.65[a]	21.74±0.30[a]
Chewiness (g)	228.70±6.02[b]	271.28±6.30[a]	268.11±8.55[a]

[1)] See Table 1.
[a-b] Means ± S.E. with different letters in the same row indicate significant differences (p<0.05).¶(9pt)

Table 9. Textural attributes of low-fat sausages with or without fat replacers

The textural properties of pork patties are presented in Table 10. Brittleness and hardness were significantly higher in the patties with olive oil than control, whereas springiness was the lowest in T1. Cohesiveness, gumminess and chewiness were significantly higher in T2 and T3 than control and T1. Chin et al. (1999) found higher hardness values when animal fat was replaced with a mixture of ISP and carrageenan in 30% fat bologna sausages. These results are similar to the findings of Crehan et al. (2000), who reported that added maltodextrin treatment as a fat replacer had higher hardness, gumminess and chewiness than control in 12% fat sausages. The present study was also supported by the findings of Pietrasik and Duda (2000) who reported that replacing backfat with the mixture of carrageenan and ISP was positively correlated with hardness, cohesiveness, gumminess and chewiness. Bloukas et al. (1997) found that fermented sausages with direct incorporation of olive oil in liquid form were softer than control sausages. Luruena-Martinez et al. (2004) also reported that olive oil addition together with fat reduction caused a significant decrease in hardness and the related parameters such as chewiness and gumminess due to high monounsaturated fat in the product. In contrast, we found that pork patties made with olive oil were not only harder but also higher in other mastication power compared with control. Usually, a decrease in textural properties with the increase in olive oil are expected because a solid fat is replaced with a liquid oil. (, the changes of mechanical texture should be influenced by other ingredients such as a carageenan and maltodextrin used in this study.

	C	T1	T2	T3
Brittleness (g)	0.42±0.11$^{B2)}$	0.72±0.17^{A}	0.72±0.03^{A}	0.60±0.17^{AB}
Hardness (g)	470±40.0^{B}	720±16.0^{A}	730±40.0^{A}	600±17.0^{A}
Cohesiveness (%)	49.44±6.49^{AB}	37.53±10.17^{B}	52.04±1.74^{A}	54.09±6.34^{A}
Springiness (%)	13.64±0.08^{A}	11.83±1.67^{B}	13.66±0.31^{A}	13.69±0.15^{A}
Gumminess (g)	23.08±2.09^{B}	27.58±12.44^{AB}	37.84±2.74^{A}	31.75±5.72^{AB}
Chewiness (g)	314.87±27.14^{B}	312.43±90.27^{B}	517.31±47.06^{A}	434.42±75.27^{A}

1) See Table 2.
2) $^{A-B}$ Means ± SD with different superscripts in the same row significantly differ at $p<0.05$.

Table 10. Changes in the textural properties of pork patties by substituting backfat with olive

In sensory evaluation, ICMO was rated the lowest for color and overall acceptability when compared with the control, traditional sausages (Table 9). Muguerza et al. (2002) reported that sausages, which replaced 30 or 20% backfat with 20% olive oil, were rated worse for color, odor and taste than without added olive oil. However, panels did not recognize the differences in flavor and juiciness between ICMO and traditional sausages in the present study. Bloukas and Paneras (1993) found that low-fat frankfurters (11% fat content) with olive oil had similar flavor but were less palatable than the traditional frankfurters (28% fat content). Lyons et al. (1999) also found that the combination of whey protein concentrate, carrageenan and starch resulted in a low-fat sausage with similar mechanical and sensory characteristics to 20% full-fat sausages. High fat sausages (26%) are less firm and juicy than

low-fat sausages (10%) made with a combination of olive, cottonseed and soybean oils but it is difficult to realize the differences in overall acceptability (Jiménez-Colmenero et al., 2010).

Sensory attributes	Control	ICM[1)]	ICMO[1)]
Color	6.10±0.88[a]	6.50±0.97[a]	4.60±0.70[b]
Aroma	5.60±0.70	5.90±0.48	5.50±0.53
Flavor	5.90±0.88	6.10±0.74	5.50±1.08
Tenderness	5.36±0.42[b]	6.10±0.37[a]	5.87±0.64[ab]
Juiciness	5.90±0.74	6.00±0.94	6.00±1.05
Overall acceptability	6.10±0.74[ab]	6.25±0.79[a]	5.50±0.85[b]

[1)] See Table 1.
[a-b] Means ± S.E. with different letters in the same row indicate significant differences (p<0.05).

Table 11. Sensory attributes of low-fat sausages with or without fat replacers

The sensory evaluation of pork patties (Table 12) indicated that color, aroma and flavor of control were higher than those of the olive oil-added ones, whereas tenderness was higher in olive oil-added samples.

	C	T1	T2	T3
Color	6.90±0.32[A]	6.40±0.52[AB]	6.30±0.67[B]	6.50±0.53[AB]
Aroma	6.90±0.88[A]	5.70±0.48[B]	5.70±0.48[B]	5.40±0.52[B]
Flavor	6.40±0.52[A]	5.60±0.70[B]	5.60±0.52[B]	5.60±0.70[B]
Tenderness	5.20±0.42[B]	5.70±0.67[AB]	5.50±0.53[AB]	5.90±0.74[A]
Juiciness	5.00±0.82	4.70±0.67	4.80±0.63	4.90±0.74
Overall acceptability	7.20±0.42[A]	6.40±0.84[B]	6.50±0.71[B]	6.80±0.63[AB]

[1)] See Table 2.,
[2)] [A-B] Means ± SD with different superscripts in the same row significantly differ at p<0.05.

Table 12. Changes of sensory evaluation value in pork patty made by substituted olive oil for backfat

Control was significantly higher in overall acceptability than olive oil-added pork patties. The substitution of pork backfat with olive oil is limited as it may affect the taste of the pork patty. Pappa et al. (2000) reported that the replacing pork backfat with olive oil positively affected the overall acceptability of the low-fat frankfurters. In contrast, Bloukas and Paneras (1993) reported that low-fat frankfurters produced by total replacement of pork

backfat with olive oil had lower overall palatability than high-fat frankfurters produced with pork backfat. The ingredients used or the amount of olive oil added in the formula could have influenced this difference in sensory scores. Also, the effect of olive oil substitution of backfat on quality can vary depending upon meat products. The patties with olive oil had lower sensory evaluation scores. Meanwhile, tenderness was higher in the sample with olive oil than the control. Paneras et al. (1998) reported that low-fat frankfurters produced with vegetable oils were firmer and less juicy than high-fat controls. A possibility of reducing the negative effects due to the high fat content of these products is partially substituting pork backfat with other ingredients (Muguerza et al., 2001). Fat is very important for the rheological and structural properties of meat products and the formation of a stable emulsion (Luruena-Martinez et al., 2004). The tenderness of olive oil-added pork patties were higher than control because olive oil is more fluid than backfat in sensory evaluation.

4. Conclusion

The addition of olive oil to a mixture of fat replacer resulted in somewhat undesirable color and overall acceptability, but lipid oxidation was inhibited. Soem quality problems including color of sausages can be minimized by combining carrageenan, maltodextrin and isolated soy protein with olive oil. The physical properties of pork patties made with olive oil emulsions were stable when compared with commercial pork patties, but they were significantly influenced by other ingredients in the oil emulsions. In conclusion, the use of olive oil in meat products to replace backfat may have a beneficial effect to human health. However, sensory quality of the products needs further improvment so that the product is compatible to conventional products

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Meat Fat Replacement with Olive Oil

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1. Introduction

The consumption of convenience foods in the restaurants such as beef or chicken burgers is increasing in Jordan. Burger is a meat product prepared from minced lean meat, with or without addition of other ingredients. The total fat content must not exceed 15% (JS: 1334/2002). In Jordan, burgers are prepared from two main meat sources: beef or chicken. Many efforts have been made to improve the quality and stability of burgers because consumer demand for healthy fast food has rapidly increased in the recent years.

Complete or partial replacement of burger fat with oil rich in monounsaturated fatty acids, such as olive oil may improve the oxidative stability of chicken burger and the nutritional value of beef burger. Another approach that can be followed to improve the quality of beef burgers is the partial replacement of beef meat with chicken meat.

This study aimed at:

1. Studying the effect of partial replacement of beef tallow and chicken fat with olive oil on some chemical and sensory properties of a freshly prepared and stored burger.
2. Studying the effect of partial replacement of beef tallow and meat with chicken meat and fat (50:50) on some chemical and sensory properties of a freshly prepared and stored burger.
3. Studying the effect of grilling on some chemical and sensory properties of a freshly prepared and stored burger formulations.

Five burger formulations were prepared and studied during storage and after grilling at 75°C for 20 minutes. These formulations were: beef, chicken, mixed beef and chicken (50:50), beef with olive oil and chicken with olive oil.

The effect of storage and grilling was evaluated by determining cooking loss by using weight differences between raw and cooked burgers, thiobarbituric acid reactive substances (TBARS) (Faustman, *et al.*, 1992), fatty acid profile using GLC analysis; fatty acid methyl esters (FAMEs) of the burger samples were prepared according to Chritopherson and Giass (1969) method, cholesterol and 7-ketocholesterol; cold saponification and extraction was

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carried out according to the method used by Sander, *et al.* (1988) and the trimethylsilyl derivatives (TMS) of cholesterol and cholesterol oxides were carried out according to the method used by Pie, *et al.* (1990).

2. Moisture, fat and protein content

The moisture, fat and protein contents for both beef burger treatments before grilling, were about 65.50%, 15.11%, and 18.20%, respectively. The moisture, fat and protein contents of both chicken burger samples before grilling were about 66.50%, 15%, and 17.50%, respectively.

The moisture loss percentage of the freshly prepared treatments due to grilling was between 20.37-25.62%, and fat loss was between 18.85-21.51%. On the other hand, the increase in protein contents was (96-116%) of the burger samples. Moisture and fat contents of the grilled samples were lower than those of raw samples, while protein content was higher. This is mainly due to the loss of water and fat.

3. Oxidative rancidity measured by TBARS test

The initial TBARS values of the beef sample expressed as mg malondialdehyde/kg meat, were about two times greater than those of chicken sample. These results reflect the quality of the raw materials, which in the case of beef, it already had a high initial degree of peroxidation. Inappropriate storage conditions of meat, together with the action of light, oxygen and the presence of myoglobine probably accelerated oxidation.

Characterisic	Time of storage (month)	*Treatment**				
		Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Raw	0	$_{b}2.26^{a}$	$_{b}1.21^{c}$	$_{b}2.09^{b}$	$_{b}2.27^{a}$	$_{b}0.74^{d}$
	1	$_{a}2.71^{d}$	$_{a}3.00^{c}$	$_{a}2.59^{e}$	$_{a}5.07^{b}$	$_{a}5.23^{a}$
	3	$_{a}2.62^{a}$	$_{b}1.20^{c}$	$_{b}2.13^{b}$	$_{b}2.59^{a}$	$_{b}0.77^{d}$
Grilled	0	$_{b}0.57^{d}$	$_{b}5.09^{a}$	$_{b}2.10^{c}$	$_{c}0.38^{e}$	$_{b}3.76^{b}$
	1	$_{a}1.09^{d}$	$_{a}7.04^{b}$	$_{a}3.68^{c}$	$_{a}0.91^{e}$	$_{a}7.99^{a}$
	3	$_{a}1.03^{d}$	$_{c}4.53^{a}$	$_{c}1.53^{c}$	$_{b}0.79^{e}$	$_{c}3.12^{b}$

Each value is the mean of three replicates.
* Values within the same column with different subscripts denote significant differences ($p \leq 0.05$) between storage times according to LSD.
** Values within the same row with different superscripts denote significant differences ($p \leq 0.05$) between the treatments according to LSD.

Table 1. Thiobarbituric acid reactive substances values (TBARS) expressed as mg malondialdehyde /kg meat for the raw and grilled burger samples during storage time.

It can be observed that TBARS values increased during the first month.The increase was higher in the chicken sample and those with olive oil than those of beef sample.These results might be explained by the fact that the fatty acids of these samples have higher degree of unsaturation when compared with those of beef.

Melton (1985) reported that oxidized flavors were detectable at TBARS numbers of 0.3-1.0 in beef or pork, 1or 2 in chicken, and higher than 3 in turkey. The TBARS values obtained in this study, remarkably exceeded these ranges. So it can be assumed that these high values of TBARS could be attributed to oxidation as well as other interferences.

On the other hand, decrease in TBARS values noticed at the end of storage period were 85, 60, 47, 18 and 3% for chicken with olive oil, chicken, beef with olive oil, mixed and beef treatments, respectively. This behavior may be ascribed to the combination of aldehydes with other compounds and to the loss of volatile aldehydes (Severini, *et al*., 2003).

Different trends were observed on the effect of grilling on TBARS values, since TBARS values decreased in both beef samples, whereas they increased in both chicken samples. This finding may be attributed to the fact that chicken fat contains higher levels of PUFA, which are prone to higher level of oxidation.

4. Cholesterol and cholesterol oxides

It was evident that cholesterol content of the raw and grilled chicken sample was about 39% higher than those of beef sample. This is due to the use of chicken skin which contains high level of cholesterol in chicken burger. Mixed meat samples had cholesterol content which was about 15% lower than chicken and 18% higher than those of beef.

Substitution of the added beef and chicken fat with olive oil resulted in a considerable decrease in cholesterol contents. The reduction in beef and chicken samples was about 53% and 58%, respectively

Characteristic	Time of storage (month)	*Treatment				
		Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Raw	0	$_{a}333.87$	$_{a}462.10$	$_{a}391.67$	$_{a}157.70$	$_{a}193.43$
	1	$_{a}331.27$	$_{a}461.67$	$_{a}390.66$	$_{a}156.61$	$_{a}193.03$
	3	$_{a}331.30$	$_{a}460.27$	$_{a}390.47$	$_{a}155.73$	$_{a}192.00$
	**Means	$^{c}332.15_{a}$	$^{a}461.35_{a}$	$^{b}390.93_{a}$	$^{e}156.68_{a}$	$^{d}192.82_{a}$
Grilled	0	$_{a}331.73$	$_{a}460.13$	$_{a}390.27$	$_{a}156.47$	$_{a}191.13$
	1	$_{a}330.23$	$_{a}459.37$	$_{a}389.30$	$_{a}154.92$	$_{a}191.07$
	3	$_{a}330.93$	$_{a}459.11$	$_{a}389.13$	$_{a}154.67$	$_{a}190.28$
	**Means	$^{c}330.96_{a}$	$^{a}459.54_{a}$	$^{b}389.57_{a}$	$^{e}155.35_{a}$	$^{d}190.83_{a}$

Each value is the mean of three replicates.

* Values within the same column with same subscripts are not significantly ($p > 0.05$) different according to LSD.

** Values within the same row with different superscripts denote significant differences ($p \leq 0.05$) between treatments according to LSD, whereas values within the same column with same subscripts denote no significant ($p > 0.05$) differences among raw and grilled samples according to LSD.

Table 2. Cholesterol content (mg/100 g fat) for the raw and grilled burger samples during storage.

Characteristic	*Treatment**				
	Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Raw	$_{c}50.12^{a}$	$_{a}70.82^{a}$	$_{b}59^{a}$	$_{e}23.78^{a}$	$_{d}29^{a}$
Grilled	$_{c}38.76^{b}$	$_{a}56^{b}$	$_{b}45.42^{b}$	$_{e}17.77^{b}$	$_{d}23^{b}$

Each value is the mean of three replicates.
* Means in the same row with the different subscripts denote significant differences among treatments of burger ($p \leq 0.05$) according to LSD.
** Means in the same column with different superscripts denote significant differences among raw and grilled burger samples ($p \leq 0.05$) according to LSD.

Table 3. Cholesterol values (mg/100g burger) for the raw and grilled burger samples.

Storage time and grilling did not affect cholesterol contents of all treatments, calculated on the fat basis (mg cholesterol/100g fat).

However, cholesterol content calculated on the burgers basis (mg cholesterol/100g burger) showed lower cholesterol in grilled samples compared to the raw one. The reduction was about 23, 21, 23, 25 and 21% for beef, chicken, mixed, beef with olive oil and chicken with olive oil samples, respectively. This reduction might be due to the loss of fat during cooking.

7-ketocholesterol was used in this study as a tracer of the degree of cholesterol oxidation, because of its fast and continuous formation at levels relatively high with respect to the other oxidation products (Park and Addis, 1985). moreover, the chromatographic peak of 7-ketocholesterol does not overlap with other peaks of cholesterol oxides products and components of food matrices (Rodriguez-Estrada, *et al.,* 1997).

In this study, there was no detectable amount of 7-ketocholesterol in all raw and grilled samples, indicating that storage and grilling did not affect the stability of cholesterol against oxidation. This could be explained by the fact that grilling conditions were not severe, since the maximum temperature of grilling was about 75°C and the time of grilling did not exceed 20 minutes. Cholesterol shows high oxidation stability at temperature below 100°C (Kyoichi, *et al.,* 1993). Furthermore, the grilling machine permitted low oxygen level to be in contact with burger during grilling because the upper part of the grill was closed and directly came into contact with the burgers.

5. Fatty acids profile

The effect of formulation, grilling and storage period on SFA, MUFA and PUFA contents of the burgers was observed. As expected, fatty acid composition of burgers reflected the fatty acid composition of the tissues and the fat used for their manufacturing.

It is well known that SFA are considered as a primary cause of hypercholesterolemia, and MUFA provide the body of essential fatty acids and decrease LDL cholesterol in the body (Mattson and Grundy, 1985). On the other hand, the addition of beef meat and fat to chicken burger enhanced its oxidative stability by increasing SFA by 32% and decreasing PUFA content by 34%, approximately. PUFA are easily prone to oxidation generating short chain compounds that deteriorate the sensory properties of the meat products.

Fatty acid	Treatment									
	Beef		Chicken		Mixed		Beef with olive oil		Chicken with olive oil	
	Raw	Grilled	Raw	Grilled	Raw	Grilled	Raw	Grilled	Raw	Grilled
Myristic C14:0	1.36	1.34	0.58	0.53	0.88	0.76	0.25	0.24	0.29	0.22
Palmitic C16:0	34.78	31.79	26.71	26.58	30.69	28.87	16.71	15.42	17.25	14.45
Palmitoleic C16:1	1.01	1.48	4.72	4.62	3.02	3.73	0.81	1.11	2.68	3.34
Stearic C18:0	22.36	20.57	6.13	6.00	12.61	10.48	10.21	8.93	5.86	4.81
Oleic C18:1	37.72	39.65	42.84	42.88	39.93	42.88	58.84	63.32	59.92	65.37
Linoleic C18:2	1.81	3.1	17.82	17.81	11.60	12.03	8.63	8.94	11.97	11.94
Linolenic C18:3	0.35	0.96	1.10	0.88	0.79	0.83	0.86	0.89	1.26	0.93
Arachidic C20:0	0.04	traces	0.02	traces	0.02	traces	0.03	traces	0.01	traces

Each value is the mean of three readings of fatty acids after samples formulation.

Table 4. Means values of fatty acids profile (g/100g fat) for the raw and grilled burger samples after formulation.

Another strategy for changing fatty acid profile of meat products rather than meat mixing is the replacement of animal fats by vegetable oils. Olive oil is a vegetable oil whose MUFA content is high. The MUFA, PUFA and SFA contents were about 72%, 10% and 13%, respectively. The addition of olive oil in place of beef and chicken fat changed the fatty acids composition of the beef and chicken burgers. The decrease in SFA of beef sample was about 54%, whereas the increase in MUFA and PUFA contents was about 54% and 33.9%, respectively, of their original contents in beef fat. On the other hand, the increase in MUFA was about 32%, whereas the decrease in SFA and PUFA contents was about 30% of their original contents in chicken fat. The decrease in SFA contents in these burger samples was due to the decrease in myristic, palmitic and stearic acid contents, while the increase in MUFA was due mainly to oleic acid, since the addition of olive oil decreased the palmitoleic acid contents. The increase in PUFA content of beef sample was mainly due to the increase in linoleic and to a less extent to the increase in linolenic content.

MUFA and PUFA contents showed gradual and significant decrease for all treatments during storage period, especially at the end of storage. This may be due to the oxidation of unsaturated fatty acids.

In the case of PUFA, the decrease in their contents of beef with olive oil was lower than in beef with tallow (≈ 47%), while chicken samples showed reverse trend, since the decline in PUFA contents of chicken was about 8% compared to 22% in chicken with olive oil.

Characteristic	Time of storage (month)	*Treatment**									
		Beef		Chicken		Mixed		Beef with olive oil		Chicken with olive oil	
		Raw	Grilled	Raw	Grilled	Raw	Grilled	Raw	Grilled	Raw	Grilled
SFA	0	$_{a}58.54^{a}$	$_{a}53.70^{b}$	$_{a}33.42^{a}$	$_{a}33.11^{b}$	$_{a}44.20^{a}$	$_{a}40.11^{b}$	$_{a}27.20^{a}$	$_{a}24.61^{b}$	$_{a}23.40^{a}$	$_{a}19.48^{b}$
	1	$_{a}58.28^{a}$	$_{a}53.75^{b}$	$_{a}33.50^{a}$	$_{a}33.25^{b}$	$_{a}44.57^{a}$	$_{a}40.24^{b}$	$_{a}26.93^{a}$	$_{a}24.90^{b}$	$_{a}23.61^{a}$	$_{a}19.50^{b}$
	3	$_{a}58.89^{a}$	$_{a}53.63^{b}$	$_{a}33.76^{a}$	$_{a}33.57^{b}$	$_{a}44.61^{a}$	$_{a}40.49^{b}$	$_{a}27.13^{a}$	$_{a}24.92^{b}$	$_{a}23.72^{a}$	$_{a}19.73^{b}$
MUFA	0	$_{a}38.73^{b}$	$_{a}41.13^{a}$	$_{a}47.56^{a}$	$_{a}47.50^{a}$	$_{a}42.95^{b}$	$_{a}46.69^{a}$	$_{a}59.65^{b}$	$_{a}64.43^{a}$	$_{a}62.60^{b}$	$_{a}68.71^{a}$
	1	$_{b}38.11^{b}$	$_{b}39.99^{a}$	$_{b}45.87^{a}$	$_{b}45.76^{a}$	$_{b}41.70^{b}$	$_{b}46.02^{a}$	$_{b}56.90^{b}$	$_{b}63.46^{a}$	$_{b}60.19^{b}$	$_{b}67.72^{a}$
	3	$_{c}35.35^{b}$	$_{c}36.50^{a}$	$_{c}35.17^{b}$	$_{c}40.60^{a}$	$_{c}36.58^{b}$	$_{c}38.72^{a}$	$_{c}47.22^{b}$	$_{c}54.50^{a}$	$_{c}50.59^{b}$	$_{c}57.52^{a}$
PUFA	0	$_{a}2.16^{b}$	$_{a}4.06^{a}$	$_{a}18.92^{a}$	$_{a}18.69^{a}$	$_{a}12.39^{b}$	$_{a}12.86^{a}$	$_{a}9.49^{b}$	$_{a}9.82^{a}$	$_{a}13.26^{a}$	$_{a}12.87^{a}$
	1	$_{b}1.53^{b}$	$_{b}2.47^{a}$	$_{b}17.82^{b}$	$_{a}18.55^{a}$	$_{b}11.93^{a}$	$_{b}11.42^{b}$	$_{b}7.29^{a}$	$_{b}7.93^{a}$	$_{b}12.59^{b}$	$_{a}12.77^{a}$
	3	$_{c}1.15^{b}$	$_{b}2.69^{a}$	$_{b}17.64^{b}$	$_{a}18.42^{a}$	$_{c}11.29^{a}$	$_{c}10.79^{b}$	$_{c}5.94^{b}$	$_{b}7.77^{a}$	$_{c}10.34^{b}$	$_{b}12.02^{a}$

Each value is the mean of three replicates.
* Values within the same column with different subscripts are significantly ($p \leq 0.05$) different according to LSD.
** Values within the same row with different superscripts denote significance different ($p \leq 0.05$) among raw and grilled sample according to LSD

Table 5. Effect of formulation, storage time and grilling on fatty acids profile (g/100g fat) of the burger samples.

Grilling significantly decreased SFA, and increased MUFA contents of all samples, except for MUFA contents of chicken sample which remained constant. PUFA contents, in general, increased in most samples, but in some cases there was no clear trend.

6. Cooking loss

Chicken sample with olive oil showed lower cooking loss in weight due to grilling when compared to the corresponding samples without olive oil. This result showed the ability of protein matrix to bind monounsaturated fat. Chicken samples with olive oil had lower cooking loss in weight when compared to beef samples which was due to the highest water holding capacity, lipid capacity and lipid stability of chicken meat rather than beef meat.

Characteristic	Time of storage (month)	*Treatment**				
		Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Cooking loss%	0	$_{b}49.69^{c}$	$_{b}50.22^{b}$	$_{b}51.30^{a}$	$_{b}50.26^{b}$	$_{b}43.28^{d}$
	1	$_{b}49.86^{c}$	$_{b}50.48^{b}$	$_{b}51.53^{a}$	$_{b}50.21^{b}$	$_{b}43.02^{d}$
	3	$_{a}51.70^{c}$	$_{a}52.63^{b}$	$_{a}53.17^{a}$	$_{a}52.78^{b}$	$_{a}47.39^{d}$

Each value is the mean of three replicates.
* Values within the same column with different subscripts are significantly ($p \leq 0.05$) different according to LSD.
** Values within the same row with different superscripts denote significant differences ($p \leq 0.05$) according to LSD.

Table 6. Percentage cooking loss in weight of burger samples during storage period.

In the mixed treatment we expected that cooking loss value will be between beef and chicken sample values, but unexpected result was obtained, the outcome showed that mixed treatment had the highest cooking loss in weight. More investigation is needed to explain the results.

The highest cooking loss was found after three months of storage which might be due to the weakness of protein matrix to entrap moisture and fat during storage, moreover, this weakness of protein matrix results in decrease of water and lipid holding capacity and stability, which might be due to denaturation of protein during frozen storage.

7. Sensory evaluation

Cooked burgers from each treatment were evaluated by 18 panelists from the sensory evaluation team at the Department of Nutrition and Food Technology. The panelists were both male and female, and were of different ages; they were requested to taste each sample separately without comparing it with other samples. Panelists were familiarized with the questionnaire form used. The samples were evaluated for desirability in appearance, color, tenderness, flavor, juiciness and overall acceptability using a 9-hedonic scale test as described by LARMOND (1991), varying from 9 (like extremely) to 1 (dislike extremely). Pieces of bread and water were used to neutralize the taste between samples.

The sensory evaluation results showed that all the sensory characteristics did not exceed the range like moderately, or fell to dislike slightly. This low score given by the panelists for all samples might be attributed to the fact that the prepared burgers were free of any added ingredients or additives that are usually added to these type of products such as spices, salt, protein derivatives of vegetable origin, dietary fibers, antioxidants, flavor enhancers and other additives which result in enhancing the sensory characteristics and the stability of the meat products.

Since the fat content of all burger treatments was about 15%, these products might contain up to 20-30% of fat to give the desirable succulence and texture.

Mixing of chicken with beef meat enhanced the sensory characteristics of the beef. In general, mixed sample had sensory scores higher than beef sample, and were close to the chicken sample. Mixed formulation was the most stable with respect to the sensory characteristics during the storage period. Freshly prepared mixed formulation samples had appearance and color scores (6.94 and 6.89, respectively) higher than those of the beef and chicken samples.(6.11 and 6.83, respectively for appearance) and (5.67 and 6.61, respectively for color). This may be due to the dilution of the redness color of beef meat as well as the dilution of the yellowness of the chicken meat which resulted in moderate appearance and color between beef and chicken meats (between redness and yellowness), since beef meat contains more myoglobin than chicken.

Appearance and color are related sensory qualities, so this modification in color of the mixed treatment affected the appearance, which in role affected the panelist's evaluation.

Tenderness evaluation of meat and meat products by panelists is correlated mainly with juiciness. Therefore, close scores of tenderness and juiciness of beef chicken and mixed treatments were observed. Tenderness and juiciness scores of the mixed formulations were significantly higher than those of beef, and very close to those of chicken. This indicated that tenderness and juiciness are strongly related to the type of meat more than to other factors.

Characteristic	Time of storage (month)	Treatment				
		Beef	Chicken	Mixed	Beef with olive oil	Chicken with olive oil
Appearance	0	$_{a}6.11^{a}$	$_{a}6.83^{a}$	$_{a}6.94^{a}$	$_{a}6.00^{a}$	$_{a}6.56^{a}$
	1	$_{a}6.06^{ab}$	$_{ab}6.22^{ab}$	$_{a}7.00^{a}$	$_{a}5.33^{b}$	$_{a}5.61^{b}$
	3	$_{a}5.94^{ab}$	$_{b}5.50^{b}$	$_{a}6.83^{a}$	$_{b}4.16^{c}$	$_{a}5.72^{b}$
Color	0	$_{a}5.67^{b}$	$_{a}6.61^{ab}$	$_{a}6.89^{a}$	$_{a}5.88^{b}$	$_{a}6.67^{ab}$
	1	$_{a}5.56^{bc}$	$_{ab}6.00^{ab}$	$_{a}7.00^{a}$	$_{a}5.39^{c}$	$_{a}5.61^{bc}$
	3	$_{a}6.33^{a}$	$_{b}5.11^{b}$	$_{a}6.44^{a}$	$_{b}4.00^{c}$	$_{a}5.61^{ab}$
Tenderness	0	$_{a}4.44^{b}$	$_{a}6.56^{a}$	$_{a}6.10^{a}$	$_{a}4.27^{b}$	$_{a}6.44^{a}$
	1	$_{a}4.55^{b}$	$_{a}6.33^{a}$	$_{a}6.72^{a}$	$_{a}4.50^{b}$	$_{a}6.50^{a}$
	3	$_{a}4.60^{b}$	$_{a}6.22^{a}$	$_{a}6.67^{a}$	$_{a}4.33^{b}$	$_{a}6.17^{a}$
Flavor	0	$_{a}4.94^{b}$	$_{a}6.33^{a}$	$_{a}5.78^{ab}$	$_{a}5.06^{b}$	$_{a}6.06^{a}$
	1	$_{a}5.06^{bc}$	$_{a}5.88^{ab}$	$_{a}6.28^{a}$	$_{ab}4.72^{c}$	$_{a}5.12^{bc}$
	3	$_{a}4.83^{bc}$	$_{a}5.50^{ab}$	$_{a}5.94^{a}$	$_{b}3.94^{c}$	$_{a}5.63^{ab}$
Juiciness	0	$_{a}4.17^{b}$	$_{a}6.44^{a}$	$_{a}6.11^{a}$	$_{a}4.44^{b}$	$_{a}6.17^{a}$
	1	$_{a}4.27^{b}$	$_{a}5.61^{a}$	$_{a}5.67^{a}$	$_{a}4.22^{b}$	$_{a}5.44^{a}$
	3	$_{a}4.44^{b}$	$_{a}5.61^{a}$	$_{a}5.83^{a}$	$_{a}3.56^{c}$	$_{a}5.50^{ab}$
Overall acceptability	0	$_{a}5.38^{b}$	$_{a}6.52^{a}$	$_{a}6.39^{ab}$	$_{a}5.39^{b}$	$_{a}6.10^{ab}$
	1	$_{a}5.00^{c}$	$_{ab}6.06^{ab}$	$_{a}6.50^{a}$	$_{ab}4.44^{c}$	$_{a}5.17^{bc}$
	3	$_{a}5.22^{a}$	$_{b}5.51^{a}$	$_{a}6.00^{a}$	$_{b}3.72^{b}$	$_{a}5.83^{a}$

Means in the same column with the same subscripts denote no significant differences among treatments of burger ($p > 0.05$) according to LSD.
Means in the same row with different superscripts denote significant differences among treatments of burger ($p \leq 0.05$) according to LSD.
Means are the average of 18 reading.

Table 7. Effect of formulation and storage time on sensory evaluation scores for the burger samples.

Sensory scores	Appearance	Color	Tenderness	Flavor	Juiciness	Overall acceptability
Appearance	1.00	0.95*	0.60*	0.79*	0.72*	0.90*
Color	0.95*	1.00	0.59*	0.71*	0.70*	0.85*
Tenderness	0.60*	0.59*	1.00	0.78*	0.96*	0.79*
Flavor	0.79*	0.71*	0.78*	1.00	0.84*	0.92*
Juiciness	0.72*	0.70*	0.96*	0.84*	1.00	0.88*
Overall acceptability	0.90*	0.85*	0.79*	0.92*	0.88*	1.00

* Correlation is significant at the 0.05 level

Table 8. Pearson's correlation coefficients between the sensory scores for the burger formulations.

Substitution of meat fat in beef and chicken samples with olive oil, in general, did not affect the sensory characteristics, since no significant differences were found between the sensory scores of the samples with and without olive oil. Beef with olive oil showed lower sensory scores after three months of storage compared to the beef sample with tallow, whereas the sensory characteristics of the chicken with olive oil remained stable during the storage period.

Although chicken with olive oil treatment showed lower cooking loss compared with the chicken treatment The tenderness and juiciness scores of these two treatments were not significantly different.

Storage time did not significantly affect the sensory evaluation scores of each treatment, except for chicken in which the appearance, color and overall acceptability at the end of storage were lower than the initial values. Appearance, color, flavor and overall acceptability of beef with olive oil also were affected by storage time. This decline in sensory parameters of these samples should be attributed to oxidation

In conclusion, it could be observed that the addition of olive oil did not affect the sensory properties of chicken burger, but it had a slight negative effect on these properties of beef burger, and addition of chicken meat to beef burger improved their sensory properties, which was very close to those of chicken sample. In addition, although, the fatty acid oxidation measured by TBARS of all treatments during storage and by grilling was relatively high, but it didn't affect significantly the sensory properties of their samples.

As a result of this research, it is recommended to introduce olive oil in burgers and other potential meat products to improve their nutritional value and to reduce their cholesterol content, and also to produce burger by mixing chicken and beef meat to enhance the sensory properties of the beef and to improve the oxidative stability of the chicken. However, Further studies are needed to determine the most suitable ratio of chicken/beef meat and fat to be used in burger formulas which give the best chemical and sensory properties.

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Biocatalyzed Production of Structured Olive Oil Triacylglycerols

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1. Introduction

Functional properties of fats and oils do not depend only on their fatty acid composition but also on the distribution of these fatty acids in the three positions of the glycerol backbone. This gives the fat or oil its commercial value. (Zhao,2005) There is a growing demand for lipids with desired characteristics ,thus researches have given way to these demands by the development of structured lipids with triacylglycerols that have predetermined composition and distribution of fatty acids. Structured lipids are now considered as alternatives to conventional fats not on the basis of saturate/polyunsaturate ratios but rather on their impact on cholesterol deposition. With the advances in the biotechnology and chemistry of fats and oils it is now possible to design fats and oils with properties that are desired. Recent years have seen great interest in the biotechnological modification and synthesis of structured triacylglycerols. Modification of fats and oil triacylglycerols to improve functionality have been carried out with various oils including olive oil. Olive oil enjoys a privileged position amongst edible oils and is still a buoyant commerce because of the large consumption of Mediterranean inhabitants (Oh et al,2009). It is one of the most expensive vegetable oils and of all the vegetable oils, olive oil is the best source of the monounsaturated fatty acid, oleic acid (72-83%)Risk factors for cardiovascular disease such as the level of homocysteine and total and low density lipoprotein (LDL) cholesterol in plasma have been reported to be reduced by oleic acid (Baro et al,2003). Olive oil is more than just oleic acid and because of its properties and qualities, it is used almost entirely in dietary consumption and even new markets have been created for this oil.

2. Triacyglycerol structure and characteristics

Triacylglycerols are by far the most abundant single lipid class and virtually all important fats and oils of plant or fat origin and most animal depot fats consist almost entirely of this lipid.

2.1 Triacylglycerol structure

Glycerol is a trihydric alcohol (containing three **-OH** hydroxyl groups) that can combine with up to three fatty acids to form monoacylglycerols, diacylglycerols, and triacylglycerols.

Fatty acids may combine with any of the three hydroxyl groups to create a wide diversity of compounds. A triacylglycerol (TAG)(Fig.1) consists of three fatty acids(R) to one glycerol molecule.

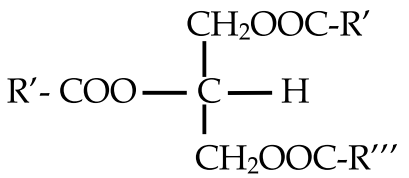

Fig. 1. Stucture of a triacylglycerol

If all three fatty acids are identical,it is a simple triacylglycerol. The more common forms however are the "mixed" triacylglycerols in which two or three kinds of fatty acids are present in the molecule. The positions occupied by these fatty acids are numbered relative to their stereospecificity or stereospecific numbering (sn) as sn-1,sn-2 and sn-3.The orientation of the triacylglycerol structure specificity is as follows: if the fatty acid esterified to the middle carbon of the glycerol backbone is considered to the left (on the plane of the page) , then the top carbon is sn-1,the bottom carbon is numbered sn-3 (below or behind the plane of the page) and the middle carbon is subsequently numbered as sn-2. The fatty acids in the triacylglycerol define the characteristics and properties of the triacylglycerol molecule. Both the physical and chemical characteristics of fats are influenced greatly by the kinds and proportions of the component fatty acids and the way in which these are positioned in the glycerol molecule (Breckenridge, W.C. 1978; Christie, W.W.1982; Karupiah, T. & Sundram, K.2007).

2.2 Triacylglycerol species of olive oil

The triacylglycerol composition is a relevant information for the restructuring of lipids. Most often this defines the properties being sought to make them more suitable for their end use. These are mainly nutritional or physical. Nutritional properties are important in structured lipids as there is a growing appreciation for this information because metabolism is intimately linked to triacylglycerol composition. Triacylglycerol composition by HPLC of olive oil as reported in literature is given in Table 1(Christie,1982;Uzzan,1996;Aranda et al,2004). Most prevalent triacylglycerols in olive oil is the oleic-oleic-oleic (OOO) triacylglycerol, followed, in order of incidence, by palmitic-oleic-oleic (POO), then oleic-oleic-linoleic (OOL), then palmitic-oleic-linoleic (POL), then by stearic-oleic-oleic (SOO).The triacylglycerol species show a small degree of asymmetry in the distribution of fatty acids among the three positions of the glycerol moiety.

However, a single symmetric triacylglycerol specie (OOO) represents almost half of the total triacylglycerols. New developments in analytical methodology have allowed the evaluation of the degree of asymmetry in other fractions. The information on the individual triacylglycerols would be very useful in the structured lipid production.

Triacylglycerol (TG)specie	% of Total TG (Range)
LLL	0 - 0.8
OLL	0.3 - 5.8
OLLn	0.9 – 0.6
OOLn	1.0 – 1.5
PLL	0.5 – 2.8
POLn	0.3 - 1.1
OOL	10.4 - 18.2
PoOO	0 - 1.1
POL	4.5 - 12.3
PPoO	0.4 - 1.2
PPL	0.7 - 2.1
OOO	21.8– 43.1
POO	20 - 23.1
PPO	2.9 - 5.3
PSPo	0 - 0.8
PPP	0 - 0.5
SOO	3.6 - 3.7
PSO	0.4 - 1.2
PPS	0 - 0.6

Table 1. Triacylglycerol Composition as Analyzed by HPLC

2.3 Fatty acid profile and distribution intriacylglycerols

The fatty acid composition of olive oil varies widely depending on the cultivar, maturity of the fruit, altitude, climate, and several other factors(Galtier et al,2008). The major fatty acids in olive oil triacylglycerols are oleic acid ($C_{18:1}$), a monounsaturated omega-9 fatty acid which makes up 55 to 83% of olive oil. Another fatty acid is linoleic acid ($C_{18:2}$), a polyunsaturated omega-6 fatty acid that makes up about 3.5 to 21% of olive oil. Palmitic Acid ($C_{16:0}$), a saturated fatty acid that makes up 7.5 to 20% of olive oil, stearic Acid (C18:0), a saturated fatty acid that makes up 0.5 to 5% of olive oil and linolenic acid ($C_{18:3}$) (specifically alpha-Linolenic Acid), a polyunsaturated omega-3 fatty acid that makes up 0 to 1.5% of olive oil .Olive oil contains more oleic acid and less linoleic and linolenic acids than other vegetable oils, that is, more monounsaturated than polyunsaturated fatty acids . This renders olive oil more resistant to oxidation. The different fatty acids have stereospecific distribution on the glycerol backbone rather than a completely random or "restricted random" distribution. In most vegetable oils either 18:1 or 18:2 are exclusively at the sn-2 position in the triacylglycerol species like OOO,LLL,POL and LLO. Linolenic acid (C18:3) occurs less commonly,but when present , is at the sn-3 position as seen for OOLn in canola oil. Oleic acid is commonly at the sn-2 position of the olive oil triacylglycerols. (Karupiah & Sundram, 2007).Table 2 shows the fatty acid distribution in the three positions

of the glycerol molecule as reported by Uzzan (1996). In esterified olive oil, the content of saturated acids palmitic and stearic in position 2 is higher, with values of approximately 13-15% compared to the normal 1.5-2%.

Nature of FA	% total FA in 2	% total FA in 1+3
C14:0		
C16:	1.4	15.0
C18:0	-	3.4
C18:1	82.9	72.8
C18.2	14.0	7.4
C18:3	0.8	0.9

Table 2. Fatty Acid Distribution in the three positions of olive oil triacylglycerol

3. Structured triacylglycerols (sTAGS)

Structured lipids may be defined as triacylglycerols restructured or modified to change the fatty acid composition or their positional distribution in glycerol molecules by a chemical or enzymatic process The term "structured triacylglycerol" was first introduced by Babayan (1987) to describe fats and oils that have been modified to change the fatty acid composition and the structure of triacylglycerols after the application of modification technologies. According to Hoy and Xu (2003) structured triacylglycerols (ST) generally are any fats that are modified or restructured from natural oils and fats, or fatty acids there from, having functionalities or nutritional properties for edible or pharmaceutical purposes. This definition covers any fats produced by either chemical or enzymatic methods for special functionality or nutritional use, including cocoa butter equivalents, breast milkfat substitutes,some low calorie fats, oils enriched in essential fatty acids γ-linolenic, arachidic, α-linolenic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, margarines or plastic fats and structured triacylglycerols containing both long chain (essential) and medium/short chain fatty acids. Adamczak (2004) defined structured triacylglycerols as triacylglycerols with a precisely defined composition and position of fatty acids esterified with glycerol possible only with the use of lipases or enzymatic modification. Structured triacylglycerols are often referred to as a new generation of lipids that are considered as nutraceuticals or functional foods or functional lipids. Regardless of the definition restructuring of triacylglycerols can be designed for use as medical or functional food as well as nutraceuticals depending on the type of application.

3.1 Synthesis and production of sTAGS

Biotechnology has experienced considerable advances in the past years via the use of fats and oils. The enzymatic process of modification is one of the advantages of fats and oils biotechnology which gives additional levels of flexibility in controlling and designing structured triacylglycerols.

3.1.1 Lipases

In recent years, the use of lipases to modify the properties of triacylglycerols has received considerable interest and has been the subject of extensive research worldwide. Lipases catalyze three types of reactions and the catalytic action of lipases is reversible. They catalyze hydrolysis in an aqueous system, but also esterification (reverse reaction of hydrolysis) in a microaqueous system, where water content is very low. Transesterification is categorized into four subclasses according to the chemical species which react with the ester. Alcoholysis is the reaction with an ester and an alcohol, while acidolysis is the one with an ester and an acid. Interesterification is a reaction between two different esters, where alcohol and acid moiety is swapped. Lipases can be classified according to their positional specificity into two groups: 1,3-positional-specific and non-positional-specific. Usually, pancreatic and fungal lipases are 1,3-positional-specific, while yeast and bacterial ones are non-positional specific or weakly 1,3-positional-specific. It should however be noted that the positional specificity of lipases is not strictly divided into the two categories, but it varies widely in the range of very distinctly 1,3-positional-specific to very weakly specific or completely non-positional-specific By exploitation of the specificity of lipases it is possible to produce acylglycerol mixtures which cannot be obtained by conventional chemical modification processes. Specificity of lipases can be utilized to produce products that cannot be produced otherwise which means that with 1,3 specific lipases, reactions involving triacylglycerol changes are confined to the sn-1 and sn-3 positions and the sn-2 acyl groups remain unaltered. There are several advantages connected to the use of lipases. The relatively mild reaction conditions for lipases reduce the amount of by products formed in a reaction. The use of lipases also renders it possible to process substances such as polyunsaturated fatty acids which cannot be processed by the conventional high temperature/high pressure processes.(Kennedy,1991Adamczack,2004) With the application of new biotechnological techniques ,companies are now able to produce lipases at lower costs. This will make the enzymatic processes far more competitive to the existing processes for the production of structured triacylglycerols.

3.1.2 Enzymatic processes of modification

Structured triacylglycerols may be prepared by hydrolysis of fatty acyl groups from a mixture of TAGs and random re-esterification follows onto the glycerol backbone. Depending on the desired metabolic effect , a variety of fatty acids are used in this process, including different classes of saturated, monounsaturated, and polyunsaturated fatty acids.Thus, a mixture of fatty acids is incorporated onto the same glycerol molecule. These manufactured lipids are structurally and different metabolically from the more simple, random physical mixtures of medium-chain triacylglycerols (MCTs) and long-chain triacylglycerol (LCT). Six possible fatty acid combinations could result for structured triacylglycerols prepared with an MCT and LCT and these are two MCFAs and one LCFA; one MCFA and two LCFAs; the two positional isomers; and small amounts of the starting MCT and LCT (Fig.2).Based on their high regiospecificity, lipases are effective biocatalysts for the manufacture of structured lipids that have a predetermined composition and distribution of fatty acids on the glycerol backbone. Structured lipids resembling TAGs of human milk have been produced by trans-esterification of tripalmitin, depending on the desired metabolic effect from plant oil, with oleic acid or PUFAs, obtained from plant oils,

using sn-1,3-specific lipases as biocatalysts . Such TAGs were found to closely mimic the fatty acid distribution of human milk and may be used in infant food formulations. Apart from imitating the human milk more closely, the occurrence of palmitic acid lipase catalyzed esterification has been used in fat modification to improve absorption properties and the nutritional value of lipids. The most commonly used method is acidolysis for the production of MLM type (M-medium chain fatty acid; L-long chain fatty acid using a regiospecific lipase to incorporate the medium chain fatty acids into the sn-1 and sn-3 positions of the triacyglycerol molecule. Currently interesterification is viewed as an alternate process to the partial hydrogenation of oils and fats. The process involves randomization among all three stereospecific positions of fatty acids in native edible oils and fats by enzymatic catalysis at low temperatures. The positional distribution of the fatty acids on the glycerol backbone is altered either through fatty acids switching positions within a triacylglycerol molecule or between triacylglycerols. If interesterification involves triacylglycerol species within the dietary fat, the fatty acid composition remains the same. There are many applications of interesterification. It is not only the management of the fatty acid mixtures which could lead to the improvement of physical properties such as in the case of cocoa butter equivalents or substitutes but it is used in the production of structured lipids which can provide specific metabolic effects for nutritive and therapeutic purposes (Kennedy,1991; Marangoni, 1993; Klemann,1994).

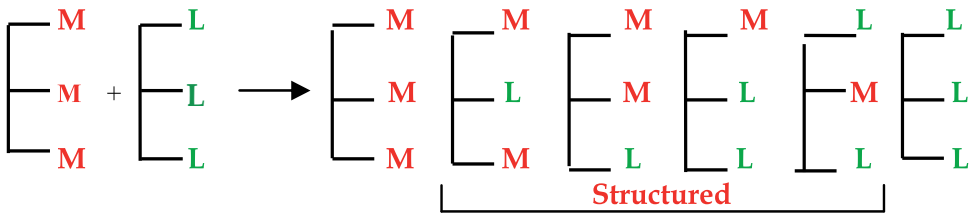

Fig. 2. Production of Structured Triacylglycerols

4. Structured olive oil triacylglycerols

Several researches have reported of the use of lipase catalyzed modification as the process for the production of structured olive oil triacylglycerols. In a study by Lee et al., 2006, olive oil triacylglycerols was used as a delivery medium for enrichment of conjugated linoleic acid in a dietary oil. Conjugated linoleic acid (CLA) are positional isomers of conjugated octadienoic acids two of which are cis-9,trans 11 and trans 10 cis-12 and are known to possess biological activity. The consumption of dietary CLA has decreased in recent years due to the replacement of animal lipids that contain little CLA .To consume more CLA and derive more health benefits, the enrichment of CLA in food has been attempted through modification of lipids in which synthesis of structured lipids is the most desirable method. Commercially produced CLA isomers were incorporated into extra virgin olive oil through a I,3 specific lipase from *Rhizomucor miehie* that catalyzed acidolysis to produce the structured olive oil triacylglycerols. The olive oil synthesized structured olive oil contained reduced content of oleic acid which was 43.1 mol % from the original value of 75.7 mol% of the total fatty acids. The decrease was compensated by the increase of CLA content at 42.5 mol %.Major CLA isomers incorporated into the triacylglycerol molecules were cis-9,trans-

11 at 16.9 mol % and trans-10, cis-12 at 24.2%.The study suggests that restructuring olive oil may be a suitable way to incorporate or deliver CLA into human diets.

Structured triacylglycerols synthesized by the acidolysis of olive oil and capric acid was carried out with immobilized lipase derived from *Thermomyces lanuginosus* to produce olive oil triacylglycerols with medium chain fatty acids in its glycerol moiety.(Oh et al,2009).

Medium chain triacylglycerols (MCTs) also offer numerous health benefits and have been widely studied for medical, nutritional and food applications. Structured lipids containing medium chain fatty acids at sn 1,3 positions and long chain fatty acids at the sn-2 position of triacylglycerols are more readily absorbed and oxidized for energy as compared to long chain triacylglycerols (LCT). Results of the study showed that the fatty acid composition of the olive oil triacylglycerols was significantly changed. The major fatty acid in the triacylglycerols was oleic acid originally, but after restructuring capric and oleic acids became the major fatty acids of the triacylglycerols. The study carried out by Fomuso and AKoh (2002) performed the lipase (1,3 specific lipase from *Rhizomucor miehie*) catalyzed acidolysis of olive oil in a bench scale packed bed reactor. Findings showed olive oil to be characterized by four major clusters of triacylglycerol species with Equivalent carbon number (ECN) ,$C_{44,}C_{46}$, C_{48}, and C_{50}.Three monosubstituted products and two disubstituted products were detected after the reaction Monosubstituted products have ECN of C_{36}, C_{38}, and $C_{40.}$ And the disubstituted products had ECN of, C_{30}, and $C_{32.}$ Fatty acid distribution of the sn-2 position of olive oil was ˛74.8% oleic acid and 25.2 % linoleic acid .The structured olive oil had 7.2% capryllic acid, 69.6% oleic acid, 21.7% linoleic acid and 1.5% palmitic acid at the sn-2 position. The results showed a structured olive oil that would have improved properties and nutritional value.

The production of cocoa butter equivalents is a promising application of the biotechnological production of structured lipids. Due to the high cost and fluctuations in supply and demand of cocoa butter, the industry has looked into the production of cocoa butter equivalents from other oil sources. Cocoa butter equivalents can be produced by the enzymatic acidolysis using sn 1,3 specific lipases that can catalyze the incorporation of palmitic acid (C_{16}) and stearic acid (C_{18}) to the sn-1,3 positions of a source containing oleic acid at sn-2 position until a similar composition of cocoa butter is obtained. The three main triacylglycerols are the 1,3 dipalmitoyl-2- oleoyl-glycerol (POP); 1(3)-palmitoyl-3(1)stearoyl-2-oleoyl glycerol (POS) and 1,3-distearoyl-2-oleoyl glycerol (SOS) with oleic acid at the sn-2 position of the glycerol backbone (Lipp,et al,2001).Using olive pomace oil these three major triacylglycerols can be achieved to produce a cocoa butter like fat. Olive pomace oil's chemical composition does not differ from refined olive oil. It has the same triacylglycerol profile of olive oil because it is olive oil extracted via solvent. In a study by Ciftci and Fadiloglu(2009) utilizing the olive pomace oil for the production of a cocoa butter like fat, findings showed that the triacylglycerol composition of the prepared product was similar to that of the commercial cocoa butter which contained 18.9% POP, 33.1% POS, and 24.7% SOS..The triacylglycerol composition of refined olive pomace oils was redesigned so that properties such as the melting point, solid fat content and fat crystal network microstructures of the structured olive pomace oil and cocoa butter were very much similar.

In another study of Olive oil triacylglycerol restructuring, olive oil was blended with coconut oil to get a balanced proportion of saturated to unsaturated fatty acids and was subjected to lipase catalyzed interesterification to rearrange the fatty acids in the triacylglycerol molecule that would have both a short chain fatty acid and long chain fatty acid in one triacylglycerol molecule (Nagaraju & Lokesh, 2007). Results showed there were no significant differences between the blended and interesterified oils in terms of the fatty acid composition but HPLC analysis showed that there were new triacylglycerol molecular species formed. Studies have shown that structured lipids have a unique metabolism and exhibit better benefits when compared with the blended oils having similar fatty acid composition. The study of Nagaraju and Lokesh (2007) showed a reduction of serum cholesterol levels by 25% as compared to the oil blend. Cholesterol levels in rat liver was also reduced by 32% as compared with results using physical blending The effect was certainly significant.

5. Conclusion

Through enzyme biotechnology, olive oil triacylglycerols can be structured to contain medium chain fatty acids or functional fatty acids like the conjugated linoleic acid for the production of a structured olive oil that would have improved biological and nutritional properties. Structured lipids provide attributes that consumers will find valuable whether for demands of healthier oils or for physical requirements to give appropriate properties. There is also a need for more researches with olive oil restructuring that will allow for better understanding and more control over the various interesterification processes and reduction in costs associated with large-scale production of structured olive oil triacylglycerols.

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Olive Oil as Inductor of Microbial Lipase

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1. Introduction

Increasing interest in lipases has been observed at the end of the last century, due to their potential application, in (bio)degradation as well as in (bio)synthesis of glycerides. The advantages of the enzymatic hydrolysis over the chemical process consist of less energy requirements and higher quality of the obtained products. Beside this, lipases are also efficient in various reactions such as esterification, transesterification and aminolysis in organic solvents. Examples in the literature are numerous. Lipases are used in different fields such as resolution of racemic mixtures, synthesis of new surfactants and pharmaceuticals, bioconversion of oils and fats and detergency applications.

Lipase activity has been found in different moulds, yeasts and bacteria. Numerous papers have been published on selection of lipase producers and on fermentation process. This kind of information is important in order to identify optimal operation conditions for enzyme production. Previous studies on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms. Obtained results showed that lipase production seems to be constitutive and independent of the addition of lipid substrates to the culture medium. However, their presence can enhance the level of produced lipase activity. On the other hand, it is well known that, in other microorganisms, lipid substrates are necessary for lipase production. These enzymes are generally produced in the presence of a lipid such as oil or triacylglycerols or any other inductor, such as fatty acids. Lipidic carbon sources seem to be essential for obtaining a high lipase yield. The review is focused on the olive oil as lipase inductor.

1.1 Lipases

Lipases, (triacylglycerol acylhydrolases; EC 3.1.1.3.) are one of the most important classes of hydrolytic enzymes that catalyse both hydrolysis and synthesis of esters. Hydrolysis of a triacylglycerols by lipases can yield di- and monoacylglycerols, glycerol and free fatty acids. Lipases are valuable biocatalysts with diverse applications. Although lipases share only 5% of the industrial enzyme market, they have gained focus as biotechnologically valuable enzymes. They play vital roles in food, detergent and pharmaceutical industries.

Commercial microbial lipases are produced from bacteria, fungi and actinomycetes (Babu & Rao, 2007). Their industrial importance arises from the fact that they act on a variety of substrates promoting a broad range of biocatalytic reactions. Lipases from different sources

show different substrate specificities and they are widely used in industrial applications for biosynthesis (Jaeger & Eggert, 2002).

Most of the lipases, which are used in laboratory investigations and/or in industrial production, are substrate tolerant enzymes, which accept a large variety of natural and synthetic substrates for biotransformation. Microbial lipases are mostly inducible extracellular enzymes, synthesized within the cell and exported to its external surface or environment. Lipases are ubiquitous enzymes which are widely distributed in plants, microbes and higher animals. Microbial sources are superiour to plants and animals for enzyme production and this can be attributed to easy cultivation and genetic manipulation (Hasan et al., 2006). Each microorganism requires a different carbon source to produce lipase at its maximum level.

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors such as temperature, pH, and dissolved oxygen. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or triacylglycerol or any other inductor, such as fatty acids, hydrolysable esters, Tweens, bile salts, and glycerol. Lipidic carbon sources seem to be essential for obtaining a high lipase yield. However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization. These nutritional requirements for microbial growth are fulfilled by several alternative media as those based on defined compounds like sugars, oils, and complex components such as peptone, yeast extract, malt extract media, and also agroindustrial residues containing all the components necessary for microorganism development. A mix of these two kinds of media can also be used for the purpose of lipase production. The main studies available in the literature since 2000 covering these subjects are presented below, divided by the kind of microorganisms used (Fernandes et al., 2007; Li et al., 2004; Tan et al., 2003).

1.2 Lipase catalytic properties

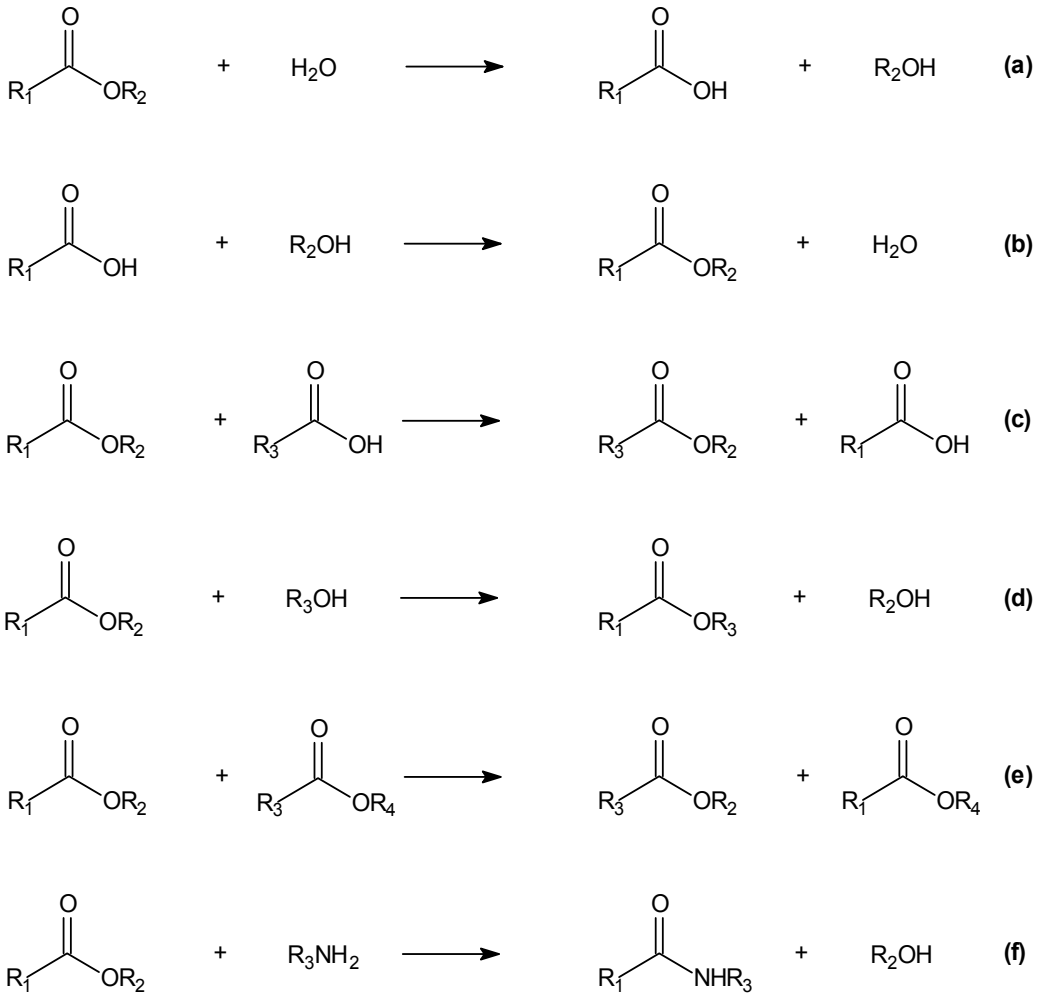
Lipase hydrolysis of water-insoluble substrates results from adsorption of the enzyme to the substrate-water interface, which can induce a conformational change in the enzyme structure, causing reaction rates to be influenced by both this adsorptive interaction as well as interaction with substrates. When lipases are active in organic solvents in which substrates are soluble, reactions follow normal enzyme kinetic models (Martinelle & Hult, 1995).

Reactions in which lipases may be involved, both in nature and in laboratory or industrial application, are: (a) enzyme-catalyzed hydrolysis, (b) enzyme-catalyzed esterification, (c) enzyme-catalyzed transesterification by acidolysis, (d) enzyme-catalyzed transesterification by alcoholysis, (e) enzyme-catalyzed interesterification and (f) enzyme-catalyzed aminolysis (Scheme 1).

1.3 Plant oils

The major components of fats and vegetable oils (98%) are triacylglycerols, which consist of glycerol molecules esterified with three long-chain fatty acids. The remainder of the oil,

although only a small part in proportion to triacylglycerols, includes a very large number of minor compounds, including the phenolics and the sterols. These compounds give olive oil its unique flavour and contribute greatly to the nutritional benefits.



Scheme 1. Processes catalyzed by lipases: (a) enzyme-catalyzed hydrolysis, (b) enzyme-catalyzed esterification, (c) enzyme-catalyzed transesterification by acidolysis, (d) enzyme-catalyzed transesterification by alcoholysis, (e) enzyme-catalyzed interesterification and (f) enzyme-catalyzed aminolysis.

The structure of triacylglycerol molecule is depicted in Fig. 1. The seed triacylglycerols are usually characterized by predominance of C_{18}-unsaturated and polyunsaturated fatty acids, and this distinguishes them from animals fats, which are generally of a more saturated nature. The C_{18}-unsaturated fatty acids (oleic, linoleic, and linolenic) are particularly important and govern, to a large degree, the physical properties of the oil and hence its use and commercial value.

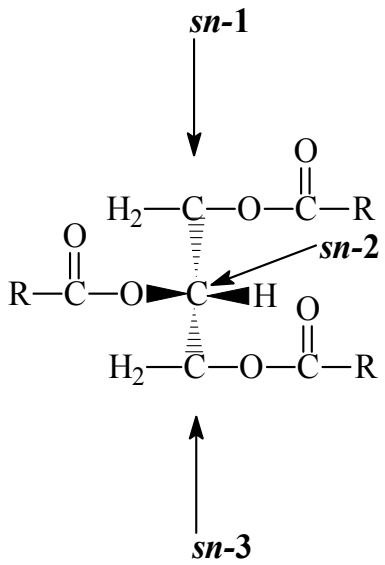


Fig. 1. The particular fatty acids in the plant triacylglycerols are not distributed randomly between the different *sn*-carbon atoms. It is a general rule that saturated species of fatty acids are confined to the positions *sn*-1 and *sn*-3 with some enrichment in the first position, and that the polyunsaturated C_{18} fatty acids are located mainly at position *sn*-2 (Gunstone & Ilyas-Qureshi, 1965; Gunstone et al., 1965)

2. Lipase biosynthesis

Lipase production requires carbon and nitrogen sources as required by any fermentation process. Most of the lipases production studies do not use simple sugars as carbon sources. They rather use lipid substrates as sole carbon sources (Zhang et al., 2009a; Zhang et al., 2009b; Hun et al., 2003). Lipase production is rarely constitutive and the quantity of the extracellular lipase produced is meagre (Lee et al., 2001). Hence inductors like vegetables oils (Kumar et al., 2005), Tween 20, Tween 80 (Li et al., 2004), hexadecane (Boekema et al., 2007), and synthetic like tributyrin and tripalmitin, are used. Generally, production of lipases increases when the relative percentage of C18:n fatty acid esters in the respective vegetable oil is increased; this indicates the importance of such substances in the synthesis and secretion of the enzyme (Lakshmi et al., 1999). Among vegetable oils, olive oil has also been referred as one of the best inductors of lipase production (Table 1; Sokolovska et al., 1998). To elucidate some aspects of the induction effect of lipid related substrates on lipase production, mixed carbon sources consisting of a soluble compound selected for its growth-promoting capacity and a fatty acid selected as inductor of the enzyme production were used (Dalmau et al., 2000). This strategy allows for the microorganism to use both substrates in a sequential or simultaneous way, depending on its metabolism. Biomass production can be higher in most cases, no increase in lipolytic activity can be observed. This suggested a possible competing effect of some soluble carbon sources or a close relation between extracellular lipase activity production and consumption of fatty acids.

Source of lipase	References
Aspergillus sp.	Cihangir & Sarikaya, 2004; Papanikolaou et al., 2011
Aspergillus niger	Pokorny et al., 1994
Aspergillus niger MYA 135	Colin et al., 2011
Bacillus sp.	Sugihara et al., 1991; Eltaweel et al., 2005
Bacillus subtilis NS 8	Olusean et al., 2011
Burkholderia cepacia LTEB11	Baron et al., 2011
Candida sp.	Annibale et al., 2006; Brozzoli et al., 2009
Candida cylindracea (ATCC 14830)	Salihu et al., 2011
Candida rugosa (DSM 2031)	Lakshmi et al., 1999
Geotrichum candidum 4013	Stránsky et al., 2007; Brabcová et al., 2010
Mycotorula sp.	Peters & Nelson, 1948
Penicillium sp.	Lima et al., 2003; Papanikolaou et al., 2011
Penicillium aurantiogriseum	Lima et al., 2003
Penicillium cyclopium	Chahinian et al., 2000
Pseudomonas aeruginosa KKA-5	Sharon et al., 1998
Rhizopus arrhizus	Elibol & Oyer, 2000
Rhizopus delemar	Acikel et al., 2011
Rhizopus oryzae	Hiol et al., 2000; Salleh et al., 1993; Nunes et al., 2011
Rhodotorula glutinis	Papaparaskevas et al., 1992
Serratia rubidaea	Immanuel et al., 2008
Yarrowia lipolytica	Dominguez et al., 2003; Pignčde et al., 2000; Najjar et al., 2011

Table 1. Sources of lipases induced by olive oil

The induction process can be accomplished by adding edible oils such as butter fat, olive, canola and fish oils to the fermentation medium. It is well known that certain lipids in the culture medium can influence the production and activity of lipases from microorganisms. Generally, the activity of intra and extracellular lipases increases with increasing lipid concentrations, although excessive levels in the growth medium may be cytotoxic. The mechanisms regulating lipase biosynthesis vary widely in different microorganisms.

2.1 *Aspergillus niger*

Lipases from *Aspergillus niger* were induced by solid-state fermentation using, as substrate, agroindustrial residue supplemented with by-products from corn oil refining process or olive oil. Based on the values of lipase activity obtained after 48 hour fermentation by-products from corn oil refining were tested as inductors in the preparation of fermentation

medium. The best results were achieved with soapstock and stearin, reaching values of 62.7 and 37.7 U/gds, respectively, which are higher than the value for olive oil (34.1 U/gds). The use of fatty acids residue inhibited lipase production. This kind of inhibition has already been reported by other authors (Corzo & Revah, 1999; Li et al., 2004). The inhibition effect was not observed for low fatty acid concentrations using palmitic and oleic acid during lipase production by *Candida rugosa* (Dalmau et al., 2000) and *Rhyzopus arrhizus* (Li et al., 2006), respectively.

2.2 *Candida rugosa*

The synthesis and secretion of lipases in *C. rugosa* have been studied with carbon sources that are known to affect the production of lipase in two opposite ways: glucose (repressor) and oleic acid (inductor; Ferrer et al., 2001). In these studies, lipase production was monitored both by enzyme activity and by immunodetection with specific antibodies. These studies showed that, according to their regulation, lipase-encoding genes might be grouped in two classes, one of which is constitutively expressed and the other is induced by fatty acids. The synthesis of inducible enzymes is inhibited at the level of transcription by the addition of glucose, and, conversely, oleic acid appears to hinder the synthesis of the constitutive lipase (Lotti et al., 1998).

The studies clearly show that different inductors may change the expression profile of individual lipase genes. A differential transcriptional control of *lip* genes had been previously suggested from several studies on the relationship between culture conditions of *C. rugosa* and the lipase/esterase profiles secreted by this organism (Gordillo et al., 1995; Lotti et al., 1998; Linko & Wu, 1996). *Lip* isoenzymes have differences in their catalytic properties (Rua et al., 1993; Diczfalusy et al., 1997; Tang et al., 2000).

Del Río et al. (Del Río et al., 1990) demonstrated the diauxic growth of *C. rugosa* on olive oil. Two stages could be observed in the consumption of the olive oil: the first one was related to the glycerol depletion without lipase production, and the second one was associated with the fatty acids consumption when the enzyme appeared in the medium. According to this observation, the initial presence of a small quantity of lipase would be sufficient to hydrolyze the triacylglycerol to glycerol and fatty acids. Therefore, production of high levels of lipase would be associated with the consumption of fatty acids. Similar results have been obtained by Sokolovska et al. (Sokolovska et al., 1998), who used olive oil and oleic acid for lipase production. It has been observed that the uptake of oleic acid by *C. rugosa* is favored by the presence of extracellular lipases (Montesinos et al., 1996). Based on the observations and hypothesis just described, Serra et al. (Serra et al., 1992) calibrated and validated a model for lipase production on olive oil and free fatty acids in batch fermentation. Sokolovska et al. (Sokolovska et al., 1998) did not observe significant differences in lipase production using these substrates. Montesinos et al. (Montesinos et al., 1996) developed a simple structured mathematical model for lipase production by *C. rugosa* in batch fermentation. Lipase production is induced by extracellular oleic acid present in the medium. The acid is transported into the cell, where it is consumed, transformed, and stored. Lipase is then excreted to the medium, where it is distributed between the available oil-water interface and the aqueous phase. Cell growth is modulated by the intracellular substráte concentration. Model parameters were determined in a calibration step, and then

the whole model was experimentally validated with good results. This model was later modified to be applied from batch to fed-batch and continuous lipase production (Montesinos et al., 1997). Finally, it was exploited in simulations and for the design of new operational conditions as discussed next.

Annibale et al. (Annibale et al., 2006) and Brozzoli et al. (Brozzoli et al., 2009) confirmed that lipase production by *Candida* sp. was found to be completely repressed by the presence of simple sugars and induced by using natural oils.

2.3 *Pseudomonas* sp

An extracellular lipase was isolated and purified from the culture broth of *Pseudomonas aeruginosa* SRT 9 (Borkar et al., 2009). Production medium was prepared containing olive oil (1% w/v) as inductor. Marked stability and activity of induced lipase in organic solvents suggest that this lipase is highly suitable as a biotechnological tool with a variety of applications including organo synthetic reactions and preparation of enantiomerically pure pharmaceuticals. A strain of *Pseudomonas mendocina* producing extracellular lipase was isolated from soil (Dahiya et al., 2010). The bacterium accumulates lipase in culture fluid when grown aerobically at 30 °C for 24 h in a medium composed of olive oil (1%) as substrate. This lipase was capable of hydrolyzing a variety of lipidic substrates and is mainly active towards synthetic triglycerides and fatty acid esters that possess a butyryl group. The medium for lipase production from *Pseudomonas fluorescens* P21 had glucose as carbon source (Cadirci & Yasa, 2010). When glucose was replaced by various lipids, olive oil was the effective lipid for lipase production (3.5 U/l). When glucose in the medium was replaced with olive oil, the lipase yield was increased by 48.9% between 12 and 18 h.

2.4 *Mucor hiemalis*

The influence on lipase induction in *Mucor hiemalis* of different types of triacylglycerols containing mainly oleic acid (olive oil), erucic acid (mustard oil), or saturated fatty acids of 8 to 16 carbons (coconut oil) was studied (Akhtar et al., 1980). The fungus produced a significant amount of lipase in the presence of glucose, but the lipase activity increased markedly when olive oil was added to the medium at the beginning of the fermentation. Among the various sources of triacylglycerols used as the carbon source, olive oil was found to be most effective in inducing the lipase. The lipase of *M. hiemalis* can be considered to be both constitutive and inducible.

2.5 *Penicillium restrictum*

While supplementation with olive oil gave the best lipase results, the highest values of glucoamylase and protease activities (de Azeredo et al., 1999) were achieved with starch enrichment. This indicates that the type of carbon source used as supplementation plays a determinant role in the kind of major enzymes that will be produced by *P. restrictum*. Enriching the babassu cake with different carbon sources favours the synthesis of different enzymes: olive oil supplementation results in high lipase activities, while starch supplementation results in high glucoamylase activities. Therefore, according to the application desired, the basal medium may be differentially enriched to give high yields of the desired enzyme.

2.6 *Rhizopus homothallicus*

Different mixtures of triacylglycerols (Rodriguez et al., 2006): olive, sunflower, corn, peanut, walnut and grape seed oils, were used as energy and carbon sources in addition to lactose, and with urea as nitrogen source. It should be emphasized that the presence of the carbohydrate account for the early growth of the strain *Rhizopus homothallicus* and later growth occurs due to the added oil (Pokorny et al., 1994). This fungal strain was able to produce similar high lipase activities with all studied oils. In the fermentation system, lipase synthesis was not prevented at 4% of triglycerides. To complete these studies, the above medium using olive oil and urea was chosen to evaluate the effect of different carbohydrates on lipase production: glucose, fructose, glycerol, xylose, sucrose and lactose. There were little or no differences with these substrates. This fact suggests that carbohydrate type does not influence lipase production, probably because the carbohydrate concentration is low (5 g/ l) compared to the oil amount (40 g/l) added to culture media and because they are probably utilized before the oil and consequently, before lipase production (Cordova et al., 1998).

2.7 *Rhizopus oryzae*

In the study (Hama et al., 2006) utilizing *Rhizopus oryzae* cells as whole-cel biocatalysts, various substrate-relate compounds such as olive oil, oleic acid, oleyl alkohol, methyl carpate and Tween 80 were tested. It was found that the addition of olive oil on lipase production and localization in suspension cells were therefore investigated (Ban et al., 2001). Olive oil increased intracellular lipase production. However that extracellular hydrolysis activity was much higher in the absence of olive oil. Because the *Rhizopus oryzae* cells used in the study were able to produce lipase constitutively regardless of whether substrate-related compounds were present, it seems likely that these compounds are effective in retaining lipase within the cells.

Nitrogen and carbon sources influencing the growth and production of lipase by *Rhizopus oryzae* were studied by Fadiloglu & Erkmen (1999). High yields of enzyme activity were obtained when protease peptone was the nitrogen source in media with olive oil and without olive oil. Carbon sources increased lipase activity in the media without olive oil, but decreased it slightly in the presence of oil. Lipase activity was significantly higher in the media with olive ail than that without olive ail. Biomass concentration was also higher in the presence of oil (Fadiloglu & Erkmen 1999). Rapid induction of enzymes able to break down foodstuffs appearing in the environment of the micro-organism is clearly of great ecological advantage. This induction process effects a change in the phenotype allowing further production of energy required for metabolism and/or growth (Wiseman, 1975).

2.8 *Geotrichum candidum*

The fungus *Geotrichum candidum* 4013 produces two types of lipases (extracellular and cell-bound; Stránsky et al., 2007). Both enzymes were induced by addition of olive oil. The differences in the abilities of these two enzymes to hydrolyze *p*-nitrophenyl esters were observed. Yan and Yan (2008) tested a combination of different experimental designs to optimize the production conditions of cell-bound lipase from *Geotrichum* sp. A single factorial design showed that the most suitable carbon source was a mixture of olive oil and citric acid and the most suitable nitrogen source was a mixture of corn steep liquor and

NH_4NO_3. Burkert et al. (2004) studied the effects of carbon source (soybean oil, olive oil, and glucose) and nitrogen source concentrations (corn steep liquor and NH_4NO_3) on lipase production by *Geotrichum* sp. using the methodology of response surface reaching a lipase activity of 20 U mL^{-1}.

3. Conclusion

Inducible enzyme systems in micro-organisms display many features of microbiological and biochemical interest. There is no doubt that in the microbial systems investigated, the major induced enzyme (usually a hydrolase) was fonned *de novo.* Enzyme formation occurs from amino acids, rather than from inactive peptide or protein precursor existing prior to the addition of the inductor to the culture (Wiseman, 1975).

Most published experimental data have shown that lipid carbon sources (especially natural oils) stimulate lipase production. Among vegetable oils, olive oil has been referred as one of the best inductors of lipase production. The review showed that olive oil plays significant role in lipase production. It could be concluded that the higher content of unsaturated free fatty acids contained in oil, the higher intracellular and extracellular lipase activity could be obtained with the oil as inductor for cells cultivation.

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26

Olive Oil-Based Delivery of Photosensitizers for Bacterial Eradication

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1. Introduction

Olive oil is a natural product of *Olea europaea*. It contains triacylglycerols of unsaturated and saturated fatty acids as well as free acids and numerous other biologically active components. Modern pharmaceutical industries are turning to natural herbal sources in order to find effective, low allergenic and non-irritating components that can be used in drug delivery systems or as recipients for both hydrophobic and hydrophilic active agents. Combining hydrophobic compounds with olive oil components is not problematic at all. However, this is quite different for hydrophilic compounds. One possible way for overcoming this problem is by mechanochemical treatment. This method has become widespread for preparing powdered solid materials in a large variety of compositions and involves the use of a conventional high-energy ball mill to initiate chemical reactions and structural changes of materials in solid-phase processes. Mechanochemical activation appears to be an environmentally friendly method, since it does not require organic solvents (Grigorieva et al., 2004; Margetić, 2005; Lugovskoy et al., 2008; Lugovskoy et al., 2009). It was shown that the mechanochemical method enabled some olive oil components to covalently attach to talc or to titanium dioxide - the solid ingredients of creams, ointments and powders (Nisnevitch et al., 2011). The remaining components were deeply absorbed by solid phases. New solid-phase composite materials which combined useful properties of various components with a different nature were thus created. Talc combined with olive oil exhibited good antioxidant properties scavenging ca. 40% of free radicals. Olive oil phenols with one or two hydroxyl groups, such as hydroxytyrosol, caffeic acid, photocatechuic acid, syringic acid, derivatives of elenolic acid, derivatives of oleuropein, tyrosol and some others are among the olive oil components responsible for its *in vitro* antioxidative activity (Papadopoulos & Boskou 1991; Briante et al., 2001; Lesage-Meessen et al., 2001; Tovar et al., 2001; Vissers et al., 2004). These compounds retain their antioxidant properties when combined with talc by a mechanochemical method. Furthermore, the possibility of combining water-soluble ascorbic acid (vitamin C) with olive oil on a talc or titanium dioxide support using mechanochemical activation has been reported (Nisnevitch et al., 2011). These triple mixtures (support-olive oil-ascorbic acid) scavenged free radicals instantly and totally due to the presence of ascorbic acid, which is a well-known effective

antioxidant (Cathcart, 1985). The scavenging ability in the triple mixtures after mechanochemical treatment was as good as that of the double mixtures of ascorbic acid with the supports. Mechanochemical inclusion of ascorbic acid into composites of olive oil with talc or olive oil with titanium dioxide successfully combined hydrophobic and hydrophilic components and provided high antioxidant properties to the entire system despite the covalent bonding between the components (Nisnevitch et al, 2011).

New olive oil-based composite materials exhibit pronounced bactericidal properties. The antimicrobial activity of the mechanochemically treated triple mixtures which were pressed into pellets was examined against the Gram-positive *S. aureus* and the Gram-negative *E. coli* bacteria. Samples containing ascorbic acid on a titanium dioxide support were more effective against both bacteria than a talc support, probably because of weaker bonding of ascorbic acid to titanium dioxide than to talc, which contributed to better diffusion of the ascorbic acid out of the pellets. Gram-positive *S. aureus* was more sensitive to all the ascorbic acid-containing samples than the Gram-negative *E. coli*, but *E. coli* responded to addition of olive oil into both talc-ascorbic acid and titanium dioxide-olive oil mixtures. In the latter case, the inhibitory activity of the triple composites was higher than that of double ascorbic acid-support composites. The antimicrobial activity of all the ascorbic acid-containing samples depended on the ascorbic acid content in the pellets. Olive oil, olive fruit and olive leaf extracts are known to exhibit a broad antimicrobial, antimycoplasmal and antifungal spectrum due to the presence of long chain α,β–unsaturated aldehydes, phenolic glycoside oleuropein and several other phenol compounds (Fleming et al. ,1973; Kubo et al., 1995; Bisignano et al., 2001; Furneri et al., 2002; Medina et al., 2007; Covas et al., 2009; Kampa et al., 2009). Mechanochemical combination of natural antimicrobial agents from olive oil with ascorbic acid, which is a strong bacterial suppressor, enabled the production of highly active solid-phase antibacterial composites.

Hydrophilic and hydrophobic components can also be combined by encapsulating hydrophilic constituents in lipid vesicles called liposomes. Such lipid-based formulations are actually possible carriers for both hydrophobic and hydrophilic active components and can be applied as drug delivery systems. Liposome formulations possess enhanced abilities to penetrate the skin, thus improving the delivery process. Lipid-based drug administration can increase treatment efficiency in cases of skin infections and inflammations caused by bacterial invasion.

2. Olive oil-containing liposomes

Liposomes (nano or micro-scale vesicles) can be obtained using phospholipids' property of self-assembly in the presence of an aqueous phase. Phospholipids spontaneously form a closed spherical phospholipid bilayer such that phosphate groups are in contact with the aqueous phase on the internal and external surfaces, and lipid chains are hidden within the membrane. Such a phospholipid assembly results in large multilamellar liposomes, which are constructed from alternating concentric lipid and aqueous layers. Treatment of multilamellar liposomes by ultrasound, membrane extrusion or other methods leads to the formation of unilamellar liposomes which consist of a single lipid bilayer (Chrai et al., 2001). Liposomes are convenient carriers of both hydrophilic and hydrophobic molecules, where the former can be incorporated into aqueous layers of multilamellar liposomes or

encapsulated in the inner space of unilamellar ones, and hydrophobic compounds can be incorporated into the lipid bilayers (Chrai et al., 2002).

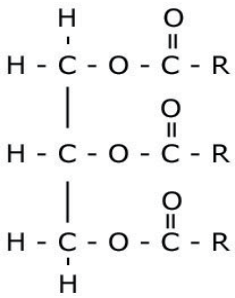


Fig. 1. Structure of a triacylglycerol. R – various residues of fatty acids.

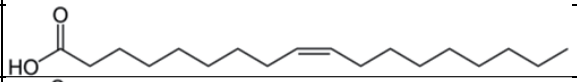
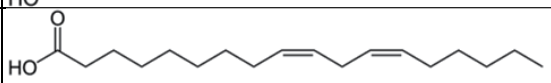
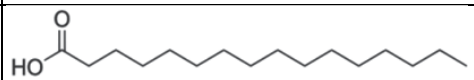
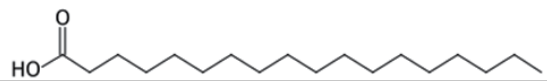
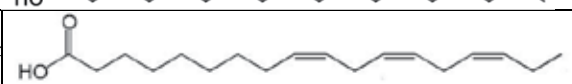
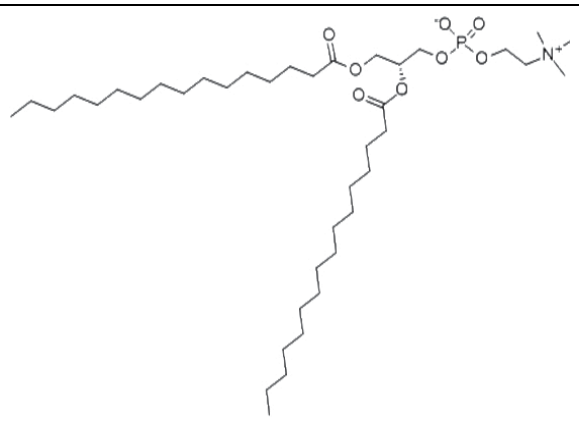
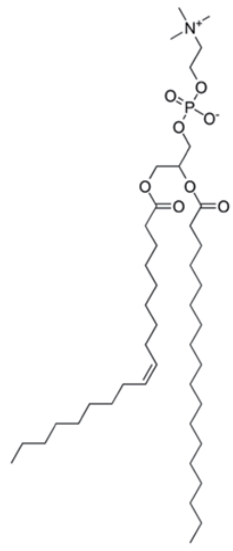
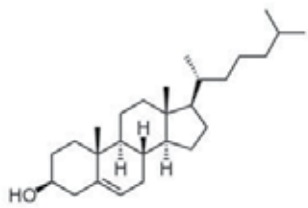
Fatty acid	Structure	% in Virgin Olive Oil (Hatzakis et al., 2008)		% in EPC (Ternes, 2002; Sigma aldrich. com)	% in DPPC (northern lipids.com)
		Triacylglycerols	Phospho-lipids		
Oleic acid		72.0-81.6	72.5-82.9	26-31	-
Linoleic acid		4.6-11.0	2.7-12.0	13-19	-
Palmitic acid		12.3-19.7	11.2-19.4	27-33	100
Stearic acid				13-15	-
α-Linolenic acid		0.08-0.53	0.11-0.47	0-0.2	-

Table 1. Main virgin olive oil, egg phosphatidylcholine and dipalmitoyl phosphatidylcholine fatty acids. (Nichols & Sanderson, 2002; oliveoilsource.com)

Liposomes can be exploited as carriers for controlled drug delivery and targeting to cells. Liposome formulations of drugs have several advantages over the use of drugs in their free form: liposomes guarantee delivery of a highly concentrated drug, liposomes protect the drugs from degradation during the delivery process, liposomes are applicable for polar as well as for nonpolar drugs, and ingredients of the liposomes themselves are nontoxic and biodegradable (Chrai et al., 2002). Liposome components participate in drug delivery, but not in drug function, such that liposomes actually play the role of excipients (Chen, 2008). Additional ingredients can be incorporated into the phospholipid bilayer in order to impart needed properties to liposomes, as indicated by the following examples: negatively charged phosphatidylinositol or positively charged stearylamine can be incorporated into the phospholipid bilayer in order to obtain charged liposomes (Robinson et al., 2001); addition of cholesterol provides rigidity to the liposome structure (New, 1994). The latter example is

explained by an increase in the gel-to-liquid crystalline phase transition temperature (T_c) of the lipid liposome layer upon the addition of cholesterol (Beaulac et al., 1998).

Component	Structure
Dipalmitoyl phosphatidylcholine (DPPC)	
Egg phosphatidylcholine (EPC)*	
Cholesterol	

*Alternative fatty acids residues are listed in the Table 1.

Table 2. Compounds used as a basis for liposome preparations.

The major ingredients of olive oil are triacylglycerols (Fig. 1) of unsaturated and saturated fatty acids (Table 1), mainly of oleic acid, but it also contains mixed triacylglycerols of palmitic-oleic-oleic, linoleic-oleic-oleic, palmitic-oleic-linoleic, stearic-oleic-oleic, linolenic-oleic-oleic and other acids (Nichols & Sanderson, 2002; oliveoilsource.com).

Olive oil also contains a small amount of free fatty acids and several minor constituents necessary for health – tyrosol, hydroxytyrosol and their derivatives such as oleuropein, oleuropein aglycone, dialdehydic form of oleuropein aglycone, decarboxymethyl form of oleuropein aglycone and ligstroside aglycone; phenolic acids, for example, 4-hydroxybenzoic acid, protocatechuic acid, syringic acid and 4-hydroxy-phenylacetic acid; flavonoids and lignads, for instance, apigenin, luteolin, pinoresinol and acetopinoresinol; squalene, α-tocopherol, vitamins E and K, pigments chlorophyll, pheophytin, carotenoids and other compounds (Boskou et al., 2006a,b; Boskou 2009a,b). In addition, olive oil includes phospholipids at a concentration range of 11-157 mg/kg in virgin olive oil (Hatzakis et al., 2008) and 21-124 mg/kg in cloudy (veiled) virgin olive oil (Koidis & Boscou, 2006).

Component	DPPC liposomes, % (w/w)	EPC liposomes % (w/w)
Dipalmitoyl phosphatidylcholine	62	-
Egg phosphatidylcholine	-	64
Olive oil	30	28
Cholesterol	8	8

Table 3. Weight compositions of olive oil based liposomes.

The liposomes used in this work were composed of dipalmitoyl phosphatidylcholine (DPPC, Northern Lipids Inc., Canada) or egg yolk phosphatidylcholine (EPC, Sigma, USA) also named L-α-lecithin. These phospholipids are constructed based on the phosphatidylcholine. However, DPPC has a homogeneous composition and contains only saturated palmitic acid residues, contrary to the heterogeneous composition of EPC, which includes several saturated and unsaturated fatty acid residues (Table 1). EPC liposomes are composed of two different fatty acid residues, where one residue is usually saturated and the other is unsaturated, as demonstrated in Table 2 (Kent & Carman, 1999). The most common fatty acids incorporated into the EPC structure are presented in Table 1. Phosphatidylcholine, the major membrane phospholipid in eukaryotic cells, is the source of the bioactive lipids lysophosphatidylcholine, phosphatidic acid, diacylglycerol, lysophosphatidylcholine, platelet activating factor and arachidonic acid. It also plays a role as a reservoir for several lipid messengers (Kent & Carman, 1999).

We incorporated virgin olive oil (Yad Mordechai, Israel) into the lipid bilayer in order to enhance the biocompatibility of liposomes and enrich them with natural salubrious components. For this purpose, organic solutions of DPPC or EPC together with olive oil were prepared, and the organic solvent was evaporated in a round-bottom flask to dryness in a vacuum rotary evaporator to obtain a thin lipid film which was vigorously agitated with buffer solutions with or without water-soluble active agents.

Fig. 2. A schematic representation of unilamellar olive oil including liposome. Phospholipid molecules are orange coloured, olive oil components are green, cholesterol molecules are grey coloured. Internal liposome volume containing water-soluble active components is pink coloured.

As can be seen from Table 1, both used by us phospholipids are built from the same fatty acids as olive oil triacylglycerols and olive oil phospholipids, although in different proportions. This fact points to high compatibility between the used phospholipids and olive oil. Various combinations of phospholipids and olive oil were attempted, and it was found that a homogeneous lipid film could not be obtained with any combination of olive oil and EPC and at a high olive oil content added to DPPC. A small amount of cholesterol (Table 2) was added to the lipid mixture solution in order to increase the lipid film's rigidity. Even and homogeneous films were attained after this addition, which resulted in stable liposomes. The multilamellar liposomes were transformed into unilamellar liposomes by sonication, as described previously (Nisnevitch et al., 2010; Nakonechny et al., 2010). Final compositions found to be appropriate for liposome preparation are presented in Table 3.

A schematic representation of the olive oil-based unilamellar liposomes is presented in Fig. 2. Triacylglycerol olive oil components are organically incorporated into the phospholipid-based structure, hydrophobic olive oil constituents such as polyphenols or vitamins are incorporated into the liposome bilayer and the aqueous solution is located in the inner liposome space. The prepared liposomes were used for encapsulation of active bactericidal factors as described in part 4.

The prepared olive oil-based liposomes were characterized by average size, evaluated by measuring the turbidity spectra. This method is based on the determination of an equation of the turbidity spectra curves, estimation of the power "n" in the equation (1):

$$\log \frac{I_o}{I} = K\lambda^{-n} \quad (1)$$

where $\log \frac{I_o}{I}$ – a measured turbidity value, I_o – initial light intensity, I – light intensity and λ – wavelength, and the liposome average size evaluation with a calibration curve representing "n"-values' dependence on vesicle sizes (Trofimov & Nisnevich, 1990; Nisnevitch et al, 2010). Higher "n"-values correspond to smaller vesicle sizes. Turbidity spectra of DPPC and EPC liposomes with and without addition of cholesterol and olive oil were measured (Fig. 3), and corresponding type (1) equations were found in each case. As can be seen from Table 4, "n" values in these equations and correspondingly, vesicle sizes, are different for DPPC and EPC liposomes, and vary when cholesterol and olive oil are incorporated into the phospholipid layers.

As can be seen from Table 4, the DPPC-based liposomes are smaller than the EPC ones obtained using the same treatment conditions. This phenomenon can be explained by two factors – by the lipid structure and by the lipid phase state of the liposomes. The homogeneous composition of DPPC, which contains only saturated palmitic acid residues, enables dense lipid packing in the liposome bilayers, in contradistinction to the heterogeneous composition of EPC, which includes several saturated and unsaturated fatty acid residues (Table 1). Such a denser package leads to the formation of unilamellar DPPC liposomes with a smaller diameter. At the temperatures of our experiments (from room temperature to 37°C), DPPC exists in a gel phase state (T_c of DPPC is 41°C (avantilipids.com)), whereas EPC is found in a liquid crystal state (T_c of EPC is –10°C (Kahl et al., 1989)). Acyl chains of phospholipids are more disordered and bulky in a fluid state, thus causing an increase in surface area per phospholipid molecule which results in bigger liposomes in the case of EPC liposomes (New, 1994).

Liposome composition	DPPC-based liposomes		EPC-based liposomes	
	"n"-value in equation (1)	Vesicle size, nm	"n"-value in equation (1)	Vesicle size, nm
Phospholipid alone	2.44	200	1.57	> 400
Phospholipid and cholesterol	2.50	190	2.00	> 400
Phospholipid, cholesterol and olive oil	2.03	> 400	1.36	> 400

Table 4. Turbidity spectra parameters and vesicle size for liposomes of various compositions.

Addition of cholesterol to phospholipids resulted in an increase in the "n"-value, which means that the vesicle size decreased upon the addition of cholesterol. The liposome size increased again after olive oil was incorporated into the membrane structure (Table 4). These facts can be explained by taking the correlation between liposome rigidity and size into account. Addition of cholesterol caused the liposome vesicles to become more rigid and respectively smaller, and further addition of olive oil led to disturbance of the lipid layer and to an increase in size (Table 4).

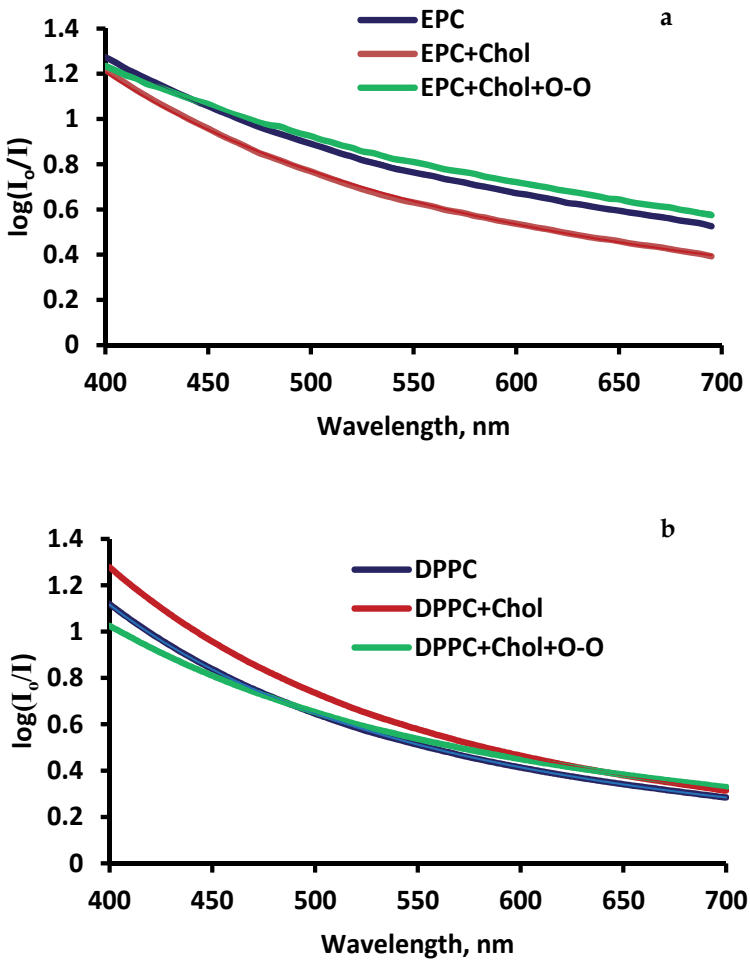


Fig. 3. Turbidity spectra of liposomes with and without additions of cholesterol (Chol) and olive oil (O-O). a – EPC-based liposomes, b – DPPC- based liposomes.

3. Photosensitizers encapsulated in olive oil-containing liposomes

Bacterial resistance to antibiotics has become a serious problem worldwide, causing an urgent need to develop new approaches and ways to overcome the evolution and spread of drug-resistant strains (Patterson, 2006; Maragakis et al., 2008; Moellering et al., 2007). One alternative to treatment of infections by antibiotics is photodynamic antimicrobial chemotherapy (PACT), which is based on the use of non-toxic compounds – photosensitizers, which can be activated by visible light. Excited photosensitizer molecules return to a ground level by transfering their energy to dissolved molecular oxygen with production of reactive oxygen species, which leads to direct damage of cellular components (Macdonald & Dougherty, 2001; Wainwright, 1998). This process is explained in Fig. 4.

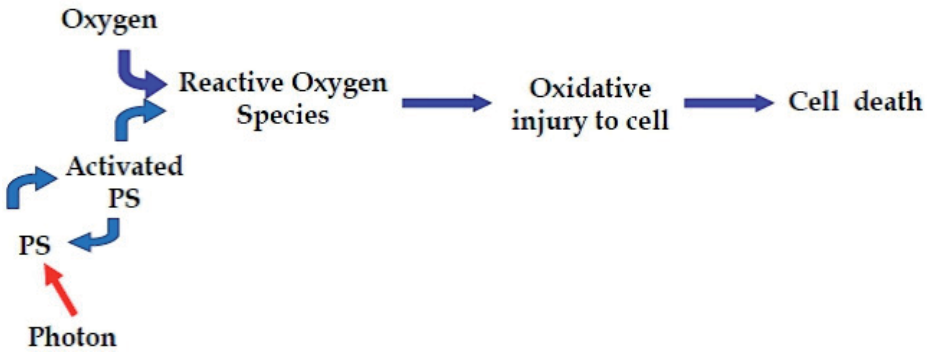


Fig. 4. A scheme of photosensitizer (PS) activation upon illumination which visible light and its cytotoxic action.

Photosensitizes refer to several chemical groups - porphyrins, phenothiazinium, phthalocyanines, xanthenes, chlorin derivatives and others. However, a feature common to all of these groups is the presence of conjugated double bonds, which allow effective absorbance of light energy. The history, mechanism of action and biomedical applications of PACT have been reviewed extensively (Nitzan & Pechatnikov, 2011; Malik et al., 2010; Reddy et al., 2009; Randie et al., 2011; Daia et al., 2009). Two photosensitizers, Rose Bengal and Methylene Blue, were used in this work. Rose Bengal relates to a xanthene (halogenated xanthenes) group of photosensitizers, and is negatively charged under physiological conditions. Methylene Blue represents a phenothiaziniums group and exists in cationic form. The structures of these compounds are shown in Fig.5.

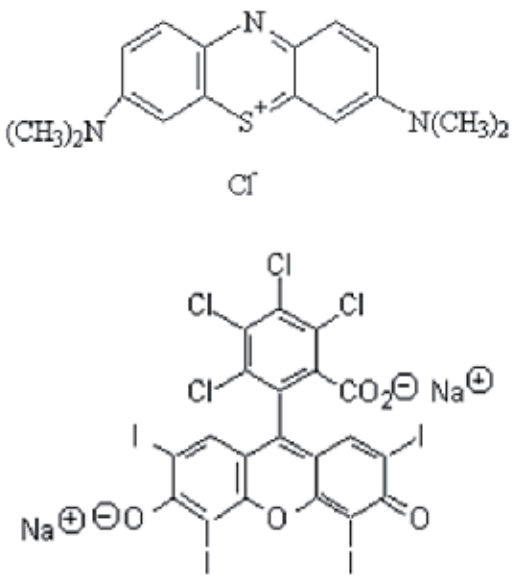


Fig. 5. Structures of photosensitizers Methylene Blue (upper) and Bengal Rose (lower).

Both photosensitizes absorb visible light, and their absorption spectra are presented in Fig. 6.

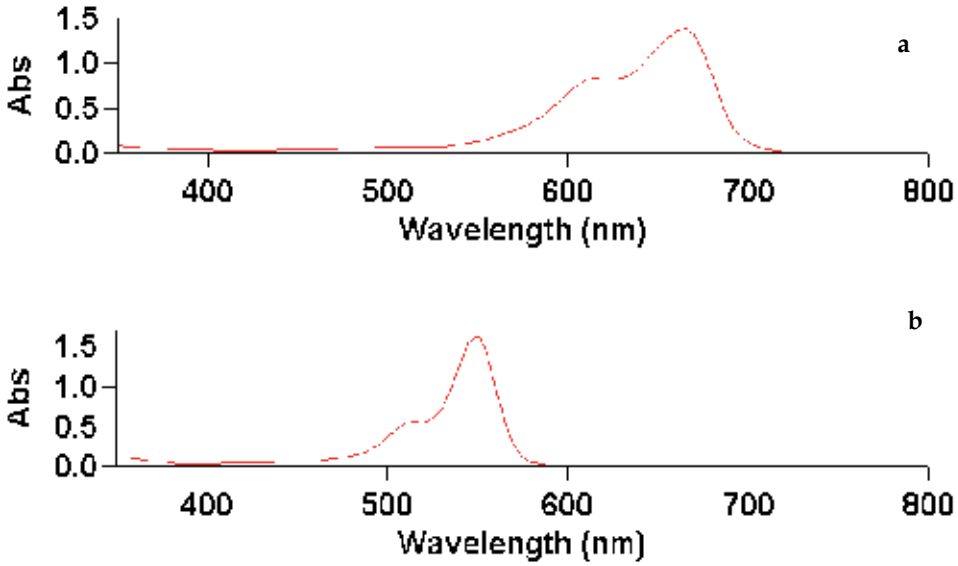


Fig. 6. Absorption spectra of (a) Methyle Blue and (b) Bengal Rose.

The described photosensitizers were encapsulated into DPPC and EPC liposomes with and without addition of olive oil as previously described by us (Nisnevitch et al., 2010). Liposomes with encapsulated photosensitizers were separated from free photosensitizers by centrifugation, and absorption of free photosensitizers was measured at the appropriate wavelengths (665 nm for Methylene Blue and 550 nm for Rose Bengal, Fig. 6).

$$\frac{A_o \cdot V_o - A \cdot V}{A_o \cdot V_o} \cdot 100\% \tag{2}$$

where - A_0 - absorbance of the initial photosensitizer in the volume V_o and A- absorbance of the free photosensitizer in the volume V. The encapsulation rate reached 50±5% in all cases.

The extent of the photosensitizers encapsulation in liposomes was estimated by formula (2) as the ratio of the encapsulated photosensitizer amount, taken as the difference between initial and free photosensitizer amount, and the initial amount.

4. Bactericidal properties of photosensitizers encapsulated in olive oil-based liposomes

Application of liposomal forms of various drugs is widely used in cases of cancer and bacterial infection treatment. Treatment of tumours by liposomal forms of doxorubicin led to a manifold accumulation of the drug in the malignant cells (Drummond et al., 1999). Entrapment of photosensitizers into liposomes was also successfully applied for eradication of cancer cells (Derycke & de Witte, 2004). Liposome-encapsulated tobramycin, unlike its free form, was demonstrated to be highly effective against chronic pulmonary *P. aeruginosa* infection in rats (Beaulac et al., 1996). Drug administration using liposomes provided a delivery of active components in a more concentrated form and contributed to their

enhanced cytotoxicity. A mechanism of drug delivery by liposomes was examined for Gram-negative and Gram-positive bacteria. Gram-negative and Gram-positive bacteria differ in their cell wall structure. Gram-negative cells possess an outer membrane which contains phospholipids, lipoproteins, lipopolysaccharides and proteins, peptidoglycan and cytoplasmic membrane. Gram-positive bacteria do not have an outer membrane, and their cell wall consists of peptidoglycan and an inner cytoplasmic membrane (Baron, 1996).

In Gram-negative bacteria, fusion between drug-containing liposomes and the bacterial outer membranes occurs, which results in the delivery of the liposomal contents into the cytoplasm. This mechanism was verified by scanning electron microscopy (Mugabe et al., 2006; Sachetelli et al., 2000), and it is schematically shown on the Fig. 7a.

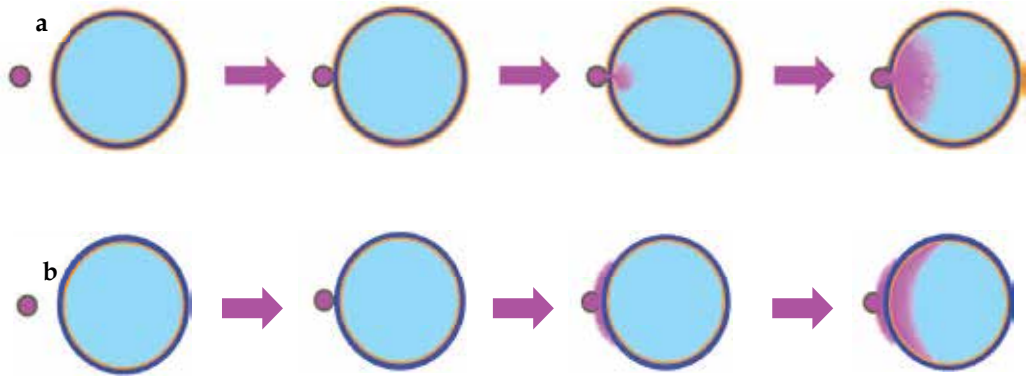


Fig. 7. A schematic representation of liposome-encapsulated drug delivery to (a) Gram-negative and (b) Gram-positive bacteria cells.

In Gram-positive bacteria, liposomes are assumed to release their content after interaction with the external peptidoglycan barrier, enabling passive diffusion through the cell wall (Furneri et al., 2000). This drug delivery mechanism is demonstrated in Fig. 7b. Application of liposomal forms of drugs leads to prolongation of their action in infected tissues and provides sustained release of active components (Storm & Crommelin, 1998).

Gram-positive and Gram-negative bacteria respond differently to PACT, with the former being more susceptible to the treatment. Gram-negative bacteria do not bind anionic photosensitizers (Minnock et al., 2000), unless additional manipulations facilitating membrane transport are used (Nitzan et al., 1992), due to the more complex molecular and physico-chemical structure of their cell wall. PACT is considered to have good perspectives in the control of oral and otherwise localized infections (Meisel & Kocher, 2005; O'Riordan et al., 2005). Local application of liposome-entrapped drugs can prolong their action in infected tissues and provide sustained release of active components (Storm & Crommelin, 1998). It should be mentioned that bacterial resistance to phosphosensitizers has not been reported to date.

Liposome formulations of photosensitizers showed high efficiency in eradication of both Gram-negative and Gram-positive bacteria. Liposome or micelle-entrapped hematoporphyrin and chlorin *e6* were found to be effective against several Gram-positive bacteria, including methicillin-resistant *S. aureus* (Tsai et al., 2009).

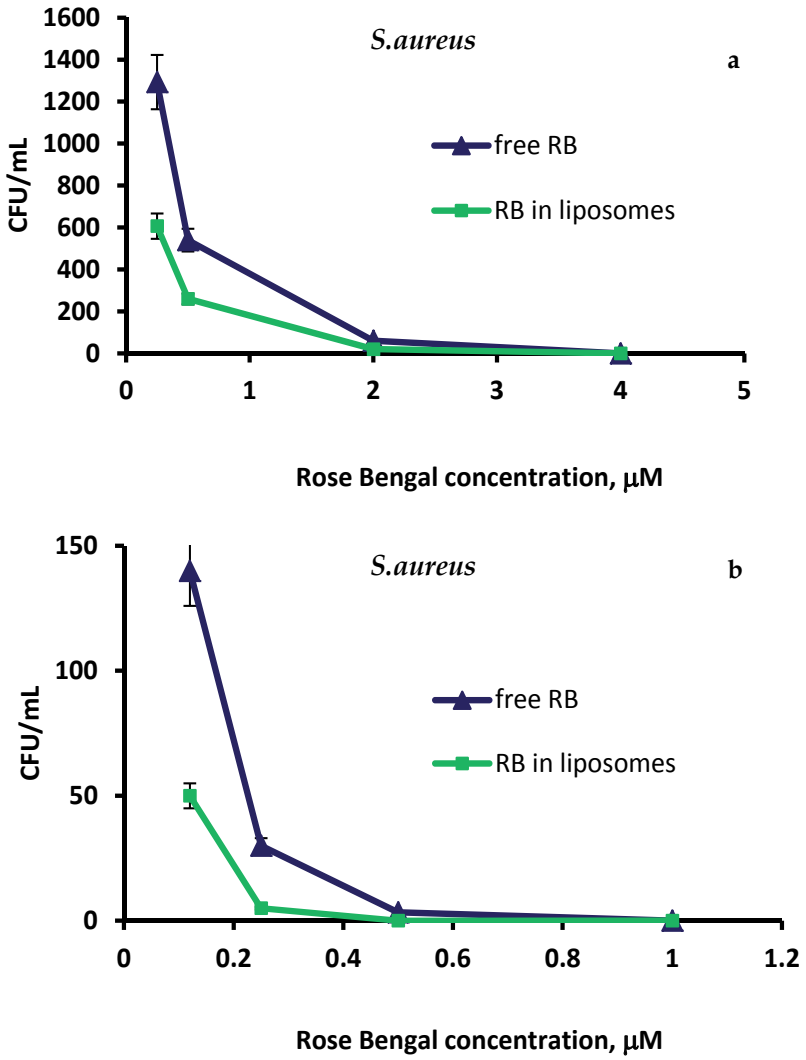


Fig. 8. Eradication of *S. aureus* by various concentrations of Rose Bengal (RB) in a free form and encapsulated into EPC–olive oil liposomes under white light illumination at initial bacteria concentration of (a) $3 \cdot 10^9$ cells/mL and (b) $3 \cdot 10^7$ cells/mL.

Encapsulation of photosensitizers into liposomes does not always result in enhancement compared to the free-form cytotoxic activity. The activity of m-tetrahydroxyphenylchlorin in liposomal form was comparable to the free form activity of PACT inactivation of a methicillin-resistant *S. aureus* strain (Bombelli et al., 2008). When tested against methicillin-resistant *S. aureus,* chlorophyll *a* was reported to be more efficient in free form than in a liposomal formulation, whereas hematoporphyrin as well as a positively charged PS 5-[4-(1-dodecanoylpyridinium)]-10,15,20-triphenyl-porphyrin were less effective in free form than upon encapsulation in liposomes. These results were explained by differences in photosensitizer chemistry which may influence their association with liposomal components, lipid fluidity and localization in liposome vesicles (Ferro et al., 2006; 2007).

We have previously shown that Methylene Blue encapsulated in liposomes composed of DPPC or EPC effectively deactivated several Gram-positive and Gram-negative bacteria, including *S. lutea*, *E. coli*, *S. flexneri*, *S. aureus* and MRSA, and that liposomal Rose Bengal also eradicated *P. aeruginosa* (Nisnevitch et al., 2010; Nakonechny et al., 2010; 2011).

Olive oil-containing liposomes loaded with photosensitizers were tested for their antimicrobial activity under white light illumination against two Gram-positive bacteria of the genus *Staphylococcus* – *S. aureus* and *S. epidermidis*. Although *S. epidermidis* is part of the normal skin flora, it can provoke skin diseases such as folliculitis, and may cause infections of wounded skin, in particular around surgical implants. *S. aureus* is defined as a human opportunistic pathogen and is a causative agent in up to 75% of primary pyodermas, including carbuncle, ecthyma, folliculitis, furunculosis, impetigo and others (Maisch et al., 2004).

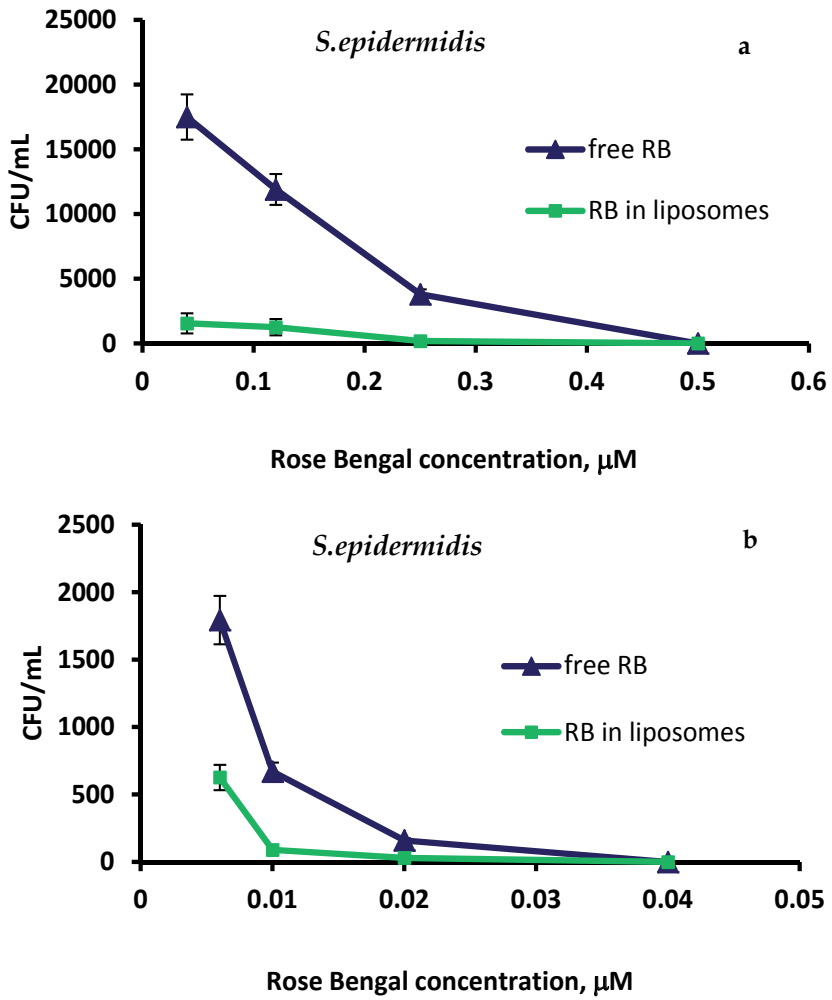


Fig. 9. Eradication of *S. epidermidis* by various concentrations of Rose Bengal (RB) in a free form and encapsulated into EPC–olive oil liposomes under white light illumination at initial bacteria concentration of (a) $3 \cdot 10^8$ cells/mL and (b) $3 \cdot 10^6$ cells/mL.

The water-soluble photosensitizers Rose Bengal and Methylene Blue were encapsulated in the above-described unilamellar liposomes at various concentrations and were examined under white light illumination against various cell concentrations by a viable count method as described previously (Nakonechny et al., 2010) and the number of bacterial colony forming units (CFU) was determined. This number characterized the concentration of bacterial cells which survived after a treatment.

The antimicrobial effect of liposomes incorporated with olive oil and loaded with Rose Bengal was strongly dependent on its concentration (Fig. 8 and 9). As can be seen from Fig. 8a, treatment of *S. aureus* with EPC-based liposomes caused a million-fold suppression of the bacterial cells at 0.25 μM of Rose Bengal and total eradication at a concentration of 2 μM when tested at an initial cell concentration of $3 \cdot 10^9$ cells/mL. Total eradication of *S. aureus* at an initial concentration of $3 \cdot 10^7$ cells/mL occurred already at a liposome-encapsulated Rose Bengal concentration of 0.5 μM (Fig 8b).

A principal similar trend was observed for *S. epidermidis*. It was necessary to apply liposome-encapsulated Rose Bengal at a concentration of 0.25 μM for total eradication of bacteria at an initial concentration of $3 \cdot 10^8$ cells/mL (Fig. 9a), and it was enough to apply 0.02 μM encapsulated photosensitizer for killing bacteria at $3 \cdot 10^6$ cells/mL (Fig. 9b). *S. epidermidis* exhibited a higher sensitivity than *S. aureus* for the liposome formulation of Rose Bengal compared with its free form. For *S. aureus*, liposomal Rose Bengal was only twice as effective as its free form – at each Rose Bengal concentration its liposomal form caused two-fold higher suppression of the bacteria. In contradistinction, *S. epidermidis* was suppressed three to twelve times more effectively by Rose Bengal encapsulated in liposomes than by the free photosensitizer.

Bacterial eradicating ability of the encapsulated as well as of the free Rose Bengal was demonstrated to depend on the initial concentration of the bacteria. When tested at the same Rose Bengal concentration, a suppression of both bacteria varied from partial to total. As can be seen from Fig. 10a, a 0.25 μM concentration of Rose Bengal encapsulated in EPC-olive oil liposomes caused a decrease of up to $6 \cdot 10^2$ cells/mL in the *S. aureus* concentration when taken at an initial concentration of $3 \cdot 10^9$ cells/mL (corresponding to 6.7 $\log_{10}$ CFU/mL) and up to zero cell concentration when taken at $3 \cdot 10^7$ or $3 \cdot 10^6$ cells/mL. In the case of *S. epidermidis,* 0.01 μM encapsulated Rose Bengal induced bacterial reduction of up to $1.5 \cdot 10^4$ cells/mL from the initial concentration of 10^8 cells/mL, and to the zero concentration at an initial concentration of $3 \cdot 10^6$ cells/mL (Fig. 10b).

DPPC-based liposomes were also examined, in addition to EPC-based olive oil-containing liposomes. The results showed high antimicrobial efficiency of the olive oil-containing liposomes in both bases, which was not less than that of the liposomes without olive oil supplements. Fig. 11 relates to the antimicrobial activity of Rose Bengal, applied against *S. epidermidis*, in free form or encapsulated in olive oil-containing ECP- and DPPC-liposomes, as well as to EPC-liposomes without olive oil. The data presented in Fig. 11 indicate that at each initial concentration, all liposomal forms of Rose Bengal eradicated bacteria more effectively than its free form (P-value 0.015), but there was no statistically significant difference in the photosensitizer activity when encapsulated in various types of liposomes (P-value 0.86).

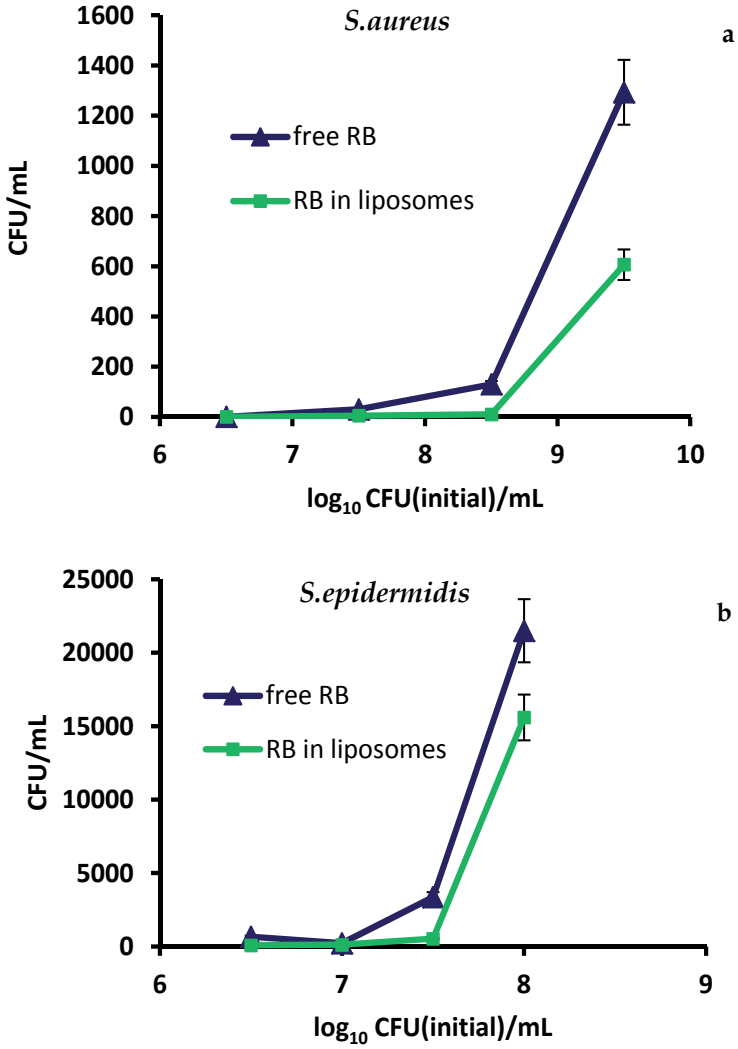


Fig. 10. Eradication of (a) *S. aureus* by 0.25 μM and (b) *S. epidermidis* by 0.01 μM Rose Bengal (RB) in a free form and encapsulated into EPC–olive oil liposomes under white light illumination at various initial bacteria concentrations presented in a logarithmic form.

Olive oil-containing liposomes with encapsulated Methylene Blue were tested against *S. epidermidis*. Bacterial sensitivity to this photosensitizer was much lower than to Rose Bengal in both free and liposomal forms. Thus, at the same initial bacterial concentration of $3 \cdot 10^6$ cells/mL, total eradication of *S. epidermidis* by liposomal Rose Bengal was achieved at 0.02 μM (Fig. 9b), and by liposomal Methylene Blue only at a concentration of 62.5 μM (Fig. 12). As to the general effect of free and liposomal Methylene Blue, it can be said that this photosensitizer exhibits the same trends as Rose Bengal. A liposome-encapsulated form was twice to three times more effective than the free form at all Methylene Blue concentrations (Fig. 12).

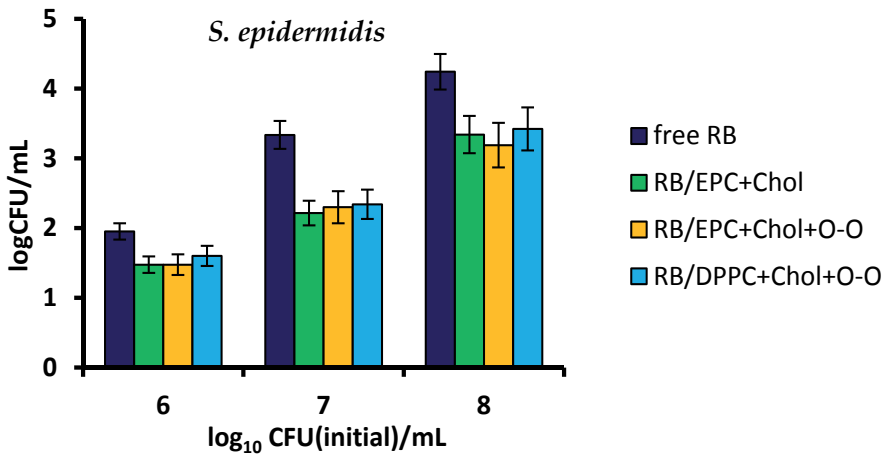


Fig. 11. Eradication of *S. epidermidis* under white light illumination by 0.01μM Rose Bengal (RB) in a free form and when encapsulated into liposomes with or without olive oil (O-O) and cholesterol (Chol) at various initial bacteria concentrations presented in a logarithmic form.

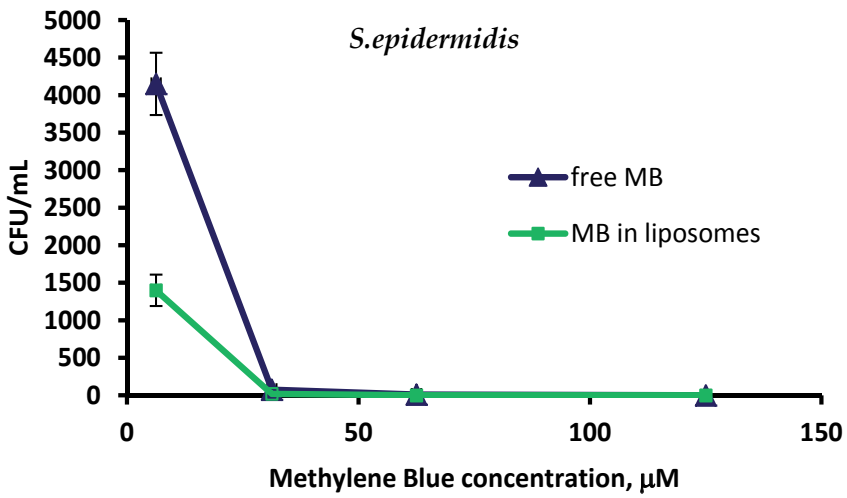


Fig. 12. Eradication of *S. epidermidis* by various concentrations of Methylene Blue in a free form and encapsulated into EPC–olive oil liposomes under white light illumination at initial bacteria concentration of $3 \cdot 10^6$ cells/mL.

It is important to mention that in no case did olive oil incorporation into the membrane of liposomes with encapsulated photosensitizers cause any decrease in their antimicrobial activity.

5. Perspectives for application of olive oil-containing liposomes

Several types of drug delivery systems containing lipids for oral, intravenous or dermal administration are described in the literature (Wasan, 2007). One of them is an oil-in-water

emulsion, composed of isotropic mixtures of oil triacylglycerols, surfactant and one or more hydrophilic solvents. The typical particle size of such systems is between 100 and 300 nm (Constantinides, 1995). Another system, called a lipidic self-microemulsifying drug delivery system, represents transparent microemulsions with a particle size of 50-100 nm (Constantinides, 1995; Holm et al., 2003). The described emulsions and microemulsions were based on structural triacylglycerols or sunflower oil. Such systems were proven to appropriately deliver lipophilic drugs such as cyclosporine A, saquinavir, ritonavir and halofantrine (Charman et al., 1992; Holm et al., 2002). A soybean lecithin-based nanoemulsion enriched with triacylglycerols was used for efficient delivery of Amphotericin B (Filippin et al., 2008). An additional example represents solid lipid nanoparticles which were shown to not only deliver glucocorticoids, but also to enhance drug penetration into the skin (Schlupp et al., 2011). Colloid dispersions of solid triacylglycerol 140 nm-sized nanoparticles stabilized with poly(vinyl alcohol) were applied for delivery of the drugs diazepam and ubidecarenone (Rosenblat & Bunje, 2009). Soybean and olive oils were suggested as drug delivery vehicles for the steroids progesterone, estradiol and testosterone (Land et al., 2005). All of the above-mentioned examples illustrate successful use of lipid-based systems for delivery of hydrophobic drugs. However, they are all unsuitable for carrying hydrophilic components.

Liposomes are devoid of this serious disadvantage and are applicable for delivery of both hydrophobic and hydrophilic agents. In case of dermal application, lipid-based drug formulations exhibit enhanced abilities to penetrate into skin, improving the delivery process of active agents, thus enabling an increase in treatment efficiency in cases of skin infections and inflammations caused by bacterial invasion. Liposomes were shown to carry the encapsulated hydrophilic agents into the human stratum corneum and possibly into the deeper layers of the skin (Verma et al., 2003). Packaging of drugs into liposomes enables a more concentrated delivery, enhanced cytotoxicity, improved pharmacokinetic qualities, sustained release and prolonged action of active components.

In this chapter we considered only one type of antimicrobial agents delivered by olive oil-containing liposomes, but the list of active drugs can be continued and expanded. Incorporation of olive oil into the lipid bilayer increases the biocompatibility of liposomes and enriches them with a broad spectrum of natural bioactive compounds. Integration of olive oil into the liposome lipid bilayer enriches the liposome features by new properties. Such enriched liposomes can not only fulfill a passive role in drug delivery, but can also supply active components for post-treatment recovery of skin. It has been proven that daily treatment with olive oil lowered the risk of dermatitis (Kiechl-Kohlendorfer et al., 2008). Olive oil vitamins and antioxidants could help overcome skin damage caused by skin infection and by the active treatment itself. Olive oil-containing liposomes can thus be converted from passive excipients into active supporting means of drug delivery systems. Totally natural and biocompatible olive oil-containing liposomes carrying any of the antimicrobial agents can be administrated in ointments and creams for application on skin areas contaminated with bacteria.

6. Conclusions

Olive oil can be incorporated into the liposome phospholipid bilayer, composed of an egg phosphatidylcholine or a dipalmitoyl phosphatidylcholine bilayer. The photosensitizers Rose Bengal and Methylene Blue encapsulated in olive oil-containing liposomes showed

high efficiency in the eradication of Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* bacteria. The effectiveness of the antimicrobial agents was concentration-sensitive and depended on the initial concentration of the bacteria.

Application of olive oil-containing liposomes for drug delivery can change their perception as having a passive role of lipid-based excipients, converting them into a new generation of active and supporting drug carriers, supplying natural bioactive components for post-treatment recovery of skin.

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Part 5

Regional Studies

27

Olive Oil Sector in Albania and Its Perspective

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1. Introduction

Albania, situated on the eastern shore of the Adriatic Sea, may be divided into two major regions: a mountainous highland region (north, east, and south) constituting 70% of the land area, and a western coastal lowland region that contains nearly all of the country's agricultural lands and is the most densely populated part of Albania. Due to the mountains landscape and especially because of its many divisions, the climate varies from region to region. It is warmer in the western part of the country which is affected by the warm air masses from the sea (the Adriatic costal region has a typical Mediterranean climate). This climate makes Albania an important producer of olives and olive oil for the region.

The transition of Albanian economy from a centrally planned to a market economy is associated with the implementation of a considerable number of structural and institutional reforms necessary for a sustainable market economy. Trade liberalization policies were implemented associated with elimination of price controls as the economy was decentralized to balance the supply and demand of goods and services.

Despite the progress made, especially in terms of macroeconomic and financial stability, Albania continues to have one of the lowest levels of income per capita in. In addition, there is a big income gap between rural and urban areas, since the agricultural sector comprise about 58% of total labour force and count for 25% of Albania Gross Domestic Product (INSTAT, 2010). Albania's economic growth can be achieved primarily through strengthening the agricultural sector. The current macroeconomic situation along with the climatic, geographic, and cultural advantages as comparable to neighbouring countries provide the opportunity for a fast and sustainable growth of the agricultural sector. Even though the olive production does not take a large share in the total agricultural production, it is an industry with huge potentials that has been steadily growing during the years.

Like many of the other agricultural products, the major supply of oil (vegetable and olive) in Albania comes from imports. This is because of the inconsistent and unreliable supply of

local raw material needed for the oil processing industry. In addition, the distribution infrastructure linking to the markets is also poor. With current prices and expected yield, the farmers do not have the incentives to grow oil-bearing plants because of the low economic returns. Furthermore, many processing plants had been destroyed after the 1990s. However, if Albania reaches an average yield, similar to that of its neighbouring countries (Greece and Italy), there will be a great potential for Albania to develop an olive industry comparable to its neighbours with similar climatic and soil conditions. To make this a reality olive productivity has to increase along with a favourable marketing situation conducive to exports. The surface plant with olives is 42 thousand hectares, with a total number of olive trees of around 5 million. Because of the insufficient services olive tree have low growth rates with a very high yield fluctuation. The result is mall quantity and low quality olive oil. Almost 10.3 million US $ have been invested in the olive oil processing industry since 1992. The major part of the processing machinery in use is obsolete.

The olive and olive oil sector is an important segment of Albanian primary production and agro industry. Primary production of olives accounts for approximately 16% of total fruit output in value, including grapes. The number of planted trees is nearly 5 million and is rapidly increasing, as a response to sustained demand, good prices and government subsidies for expanding the production base.

Official data on olive oil production show an output ranging between 6,400 Mt (Million ton) in bad harvest years and 11,900 Mt in good harvest years. There is a structural production deficit of approximately 1,000 Mt per year, mostly covered by imports of bottled olive oil from Italy and other EU countries. Main production areas of olives for olive oil are in the center and south of the country. In these areas, 90% to 95% of cultivars are for olive oil production. (Leonetti et al, 2009)

Processing industry has a specializing and modernizing trend, producing mostly olive oil and table olives (15-20% of total olive production). Official data for 2009 show that there are 108 enterprises processing all edible oils including olive oil, and 16 enterprises processing table olives. The structural deficit of table olives is covered mainly by imports from Greece.

2. Olive cultivation in Albania

Albania is one of the few countries in Europe and the only country in the Central-East Europe that has the favourable climatic and geographical conditions for olive cultivation. The olive cultivation story in Albania is very old. The people of the rural areas are used with the cultivation of olives, and a good tradition has been heritage from one generation to the other.

The demand for olive oil and table olives in the domestic market is very high. On the other hand, with an adequate technological improvement in the olive processing industry, this product could be traded in the international market.

Olives are among the most important fruit tree crops grown in Albania, covering an estimated 8% of the arable land. As shown in Figure 1, the Albanian olive production zone covers the entire coast from Saranda (South) to Shkodra (North) and inland river valleys in the districts of Peqin/Elbasan, Berat/Skrapar, and Tepelene/Permet.

Olive tree in Albania is cultivated in the regions along the western costal lowland. Geographically 3.3% is cultivated in the plain zone and 96.7% in the hilly zone. In 77% of the

farms olives are cultivated in organized plantations whereas in the remaining 23% of the farms this culture is found in a not organized form. The olive concentration in plantations gives the possibility for more careful services and the use of adequate technologies. According to the data taken from INSTAT (Institute of Statistics, 2008), the dynamic of the surface and the number of the olives during the years is as follows.

Fig. 1. Map of Albania showing olive cultivation area (USAID, 2011)

According to Figure 2 the surface of olive plantation and the number of olive trees has increased by four times in the year 1990 compared with the year 1938. After the 1990s, as the result of the late processing of the Land Agrarian Reform in this sector, the olive production industry has suffered a lot of considerable damages. As many other sectors of the country's economy, this sector was characterized by a visible depreciation in the main indicators. Huge olive blocks like those in Fier, Mallakaster, Berat and Lushnje were burned and destroyed. The transformation of the State Farms into private economies in this sector of the economy has been very slow. Even today, there are regions where the reform changes have not yet been completed. Table 1 shows olive production and yield in the main regions of the country and Table 2 describes in numbers the overall country situation.

Although there has been a considerable investment in the new olive plantations, the production investments and the services for this culture have been minimal. Today the olive production has low and fluctuating yields. The extensive character of the olive cultivation and the insufficient treatments that are usually done to the olives are the cause of this phenomenon. The yield fluctuation in the olive production has been and still is a serious phenomenon for our country. According to statistical data, the ratio between an "empty" year (year with very low production) and the year with a good production is very high.

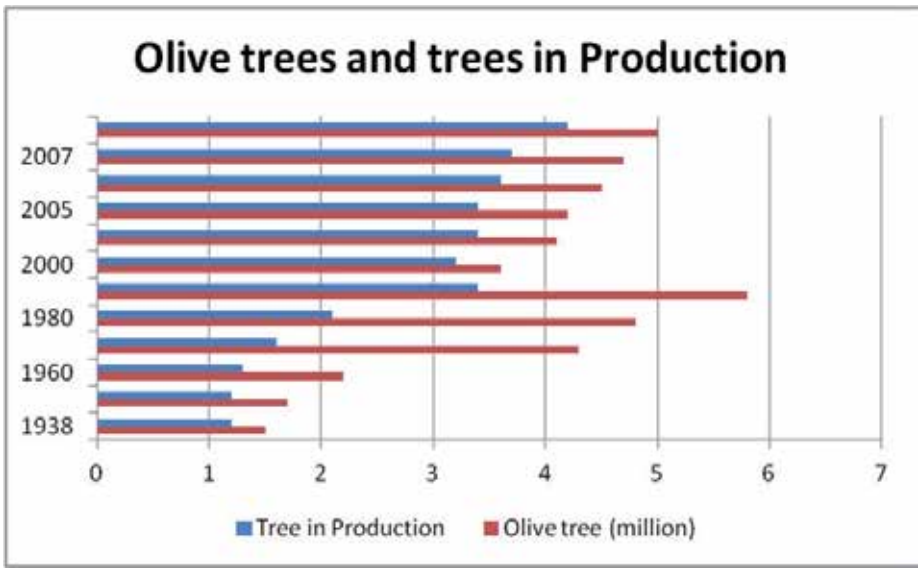

Fig. 2. Olive trees and trees in production for the period 1938-2008 (INSTAT, 2010)

Nr	Region	Number of olives (000 trees)		Yield (Kg/tree)	Production (Ton)
		Total	In production		
1	Berat	628	492	22,0	10841
2	Vlorë	532	495	13,0	6436
3	Elbasan	364	331	10,0	3315
4	Fier	347	311	12,7	3955
5	Tiranë	318	294	9,1	2664
6	Sarandë	312	310	6,6	2048
	TOTAL	**2501**	**2233**	**13,1**	**29259**
	REPUBLIC	**3564**	**3200**	**13,1**	**42012**

Table 1. Olive production data for the main regions 2009 (Ministry of Agriculture, Food and Consumer Protection, 2010)

Nr.	Region	Number of olives (000 trees)		Yield (Kg/tree)	Production (Ton)
		Total	In production		
1	Berat	628	492	22,0	10841
2	Delvinë	127	126	3,3	419
3	Durrës	57	52	19,3	1004
4	Elbasan	364	331	10,0	3315
5	Fier	347	311	12,7	3955
6	Gramsh	2	2	20,3	31
7	Gjirokastër	5	4	34,4	150
8	Kavajë	75	75	13,4	998
9	Kruje	104	87	4,5	393
10	Kuçovë	39	37	20,4	753
11	Laç	10	10	13,0	126
12	Lezhë	18	15	9,9	148
13	Lushnjë	227	209	19,5	4070
14	Mallakastër	197	161	20,9	3362
15	Peqin	65	64	6,5	412
16	Përmet	2	1	12,8	15
17	Sarandë	312	310	6,6	2048
18	Skrapar	1	1	11,6	14
19	Shkodër	93	81	6,0	485
20	Tepelenë	43	43	8,6	373
21	Tiranë	318	294	9,1	2664
22	Vlorë	532	495	13,0	6436
	TOTAL	**3564**	**3200**	**13,1**	**42012**

Table 2. Number of heads, yields and olive production according to the regions, 2009 (Ministry of Agriculture, Food and Consumer Protection, 2010)

3. Olive age and cultivars in Albania

According to the age of the olives there is a visible distinction that divides the olive plantations into two groups;

1. Centennial olive plantations are mainly found in the urban areas of Sarandë, Vlorë, Berat, and Elbasan. These are native varieties with high economic values that consist of the main part of olive production of the country.
2. Olive plantations planted after the 1960s, which are found by the sea and in the central part of the country.

Based on the statistical data the proportion of the olives according to their age result as follows: Olive plantations above 100 years old (30% of the total olive trees), Olive plantations from 30-40 years old (45%) and Olive plantations from 10-20 years old (25%).

One of the most important factors affecting productivity of the olive tree is its cultivar. Albania is rich with more than 28 varieties grown throughout the country. The nine most cultivated are listed in Table 3. With the exception of the Frantoio variety introduced from Italy, the other eight most commonly grown varieties are native to Albania. The two leaders are "Kalinjot", which covers about 40% of the total plantations for oil and table use; and "Kokermadh i Beratit", representing approximately 21% of table olives. The interaction of the Albanian varieties with the local environment (soil, climate, altitude) and cultural practices results in the special characteristics and tastes distinctive to the oils produced in various regions of throughout Albania.

Varieties	Number of Trees	Surface(Ha)	Maximum Oil Yield (% of weight)	Main Use
Kalinjot	2,335,000	17,700	27	Table & Oil
Kokërrmadh i Beratit	1,000,000	7,700	18	Table
Frantoio	470,000	2,600	19	Oil
Kokërrmadh Elbasani	450,000	4,000	20	Table & Oil
Mixan	430,000	3,770	25	Oil
Ulli i Bardhë Tiranes	200,000	1,500	28	Oil
Nisiot	120,000	900	12	Oil
Ulli i Hollë I Himares	70,000	800	15	Oil

Table 3. Olive cultivars in Albania (Ministry of Agriculture, Food and Consumer Protection, 2009)

4. Olive harvesting and collecting

Olive collection in Albania starts at the beginning of October and goes on until February. The harvesting is mostly done manually, and no modern equipment is used. During harvesting no selection between olives is done. Farmers use combined harvesting of olives that fall from the wind or as the effect of diseases and olives that are taken from the trees. This way of harvesting has a big influence on the manufactured oil quality.

The Albanian distribution system is traditional and extremely fragmented, without a real wholesaling sector. Especially for olive oil, distribution to retailers is mainly performed by the bottlers themselves. Wholesalers play a more important role in distributing table olives. More in general, food processing companies are distributing directly to retail outlets bypassing or relaying less on wholesalers. Two major changes occurred in the last three years, which will induce major changes in the distribution system. The establishment of a network of wholesale markets, facilitating wholesale trading and gradually introducing more transparency in price formation and on the other hand the development of organized distribution, with the entrance of two foreign-owned supermarket chains and the parallel growth of some domestic larger retailers into supermarket chains.

More organized logistics are necessary to cope with such evolution. Total mark up in the post-production section of the food chain is also likely to increase, as prices are already high. This is likely to put more pressure on producers to reduce sales prices. For olive oil and table olives, such evolution is likely to induce the following changes:

- Organized distribution needs regular supplies of relatively large quantities of products. The role of bottlers will further increase and medium producers will be forced to upgrade their distribution system or to reduce the share of olive oil sold with their own brand. This evolution is also representing a challenge for the small modern processors which will be forced to increase the resources devoted to marketing, as increasing number of wealthy customers will make their purchases in supermarkets.
- An increasing role will be played by wholesale markets in distribution of table olives, thus facilitating in the short term a further increase in the number of small wholesalers/processors. Generally, wholesalers and importers will become more important players in the table olive trading.

Most urban dwellers buy olive oil in mini-markets and traditional retail outlets whereas imported olive oil is almost exclusively sold through supermarkets. Organized distribution is catching an increasing share of customers. These outlets do not represent any more the higher end of retailing business. Supermarkets are adjusting their prices to those ones of traditional retailers, aiming at widening the range of customers beyond the middle income consumers' segment. Restaurants and other catering outlets are buying, with few exceptions, the cheapest qualities of olive oil. Limited purchasing of higher quality olive oil is made by high-end restaurants. Apart from self-consumption, olive oil in rural areas is mostly informally traded and purchased from local oil mills. A smaller share, estimated in 30% of the total or less, is sold usually by the liter (i.e. not bottled), in traditional retail outlets. Retail shops and green markets are the prevalent market channels in rural areas where there is no olive oil production.

Until the end of the 1970s the olive oil processing was done in traditional primitive ways by the peasants themselves. Gradually with the increases in yield, some plants were built. These were very old technology fashioned plants. Only at the beginning of the 1980s some presses were imported from Italy, and this was the start of innovations in the oil manufacturing plants. Actually almost half of olive oil existing processing plants use the "Pieralisi" type presses for the olive oil production (Figure 3). Second popular kind of press is Alfa Laval with 15% and the next significant types are Eno Rossi (11%) and Mix (5 %). The situation shows that the processing olive oil technology is dominated by three phase decanters.

5. Financing the olive oil sector

After the 1990s, a lot of investments were done in the olive oil processing industry. According to a study done by IFDC in 2002, the total amount of investments in this sector is 1442 million Lekë (or 10.686.230,92 euro). The regions with the highest amount of investment are Vlora with 25.0% of the total, Tirana with 17.6%, Saranda with 17.5%, and Fier with 13.8% of the total investments.

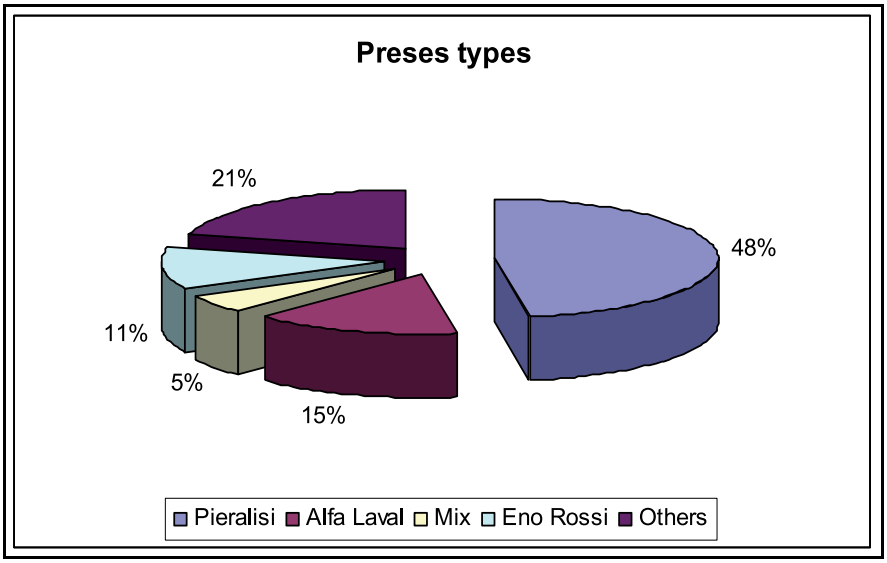

Fig. 3. Olive oil processing presses used in Albania (Ministry of Agriculture, Food and Consumer Protection, 2009)

There are three main investment sources in Albania, as far as the agricultural sector is concerned, own financial sources, bank credits and other funds. The investments are mainly done by the private financial sources of the entrepreneurs. This is followed by a smaller part of those that have taken some bank credits. Figure 4 below, shows schematically the share that each of these forms holds in the total investment structure.

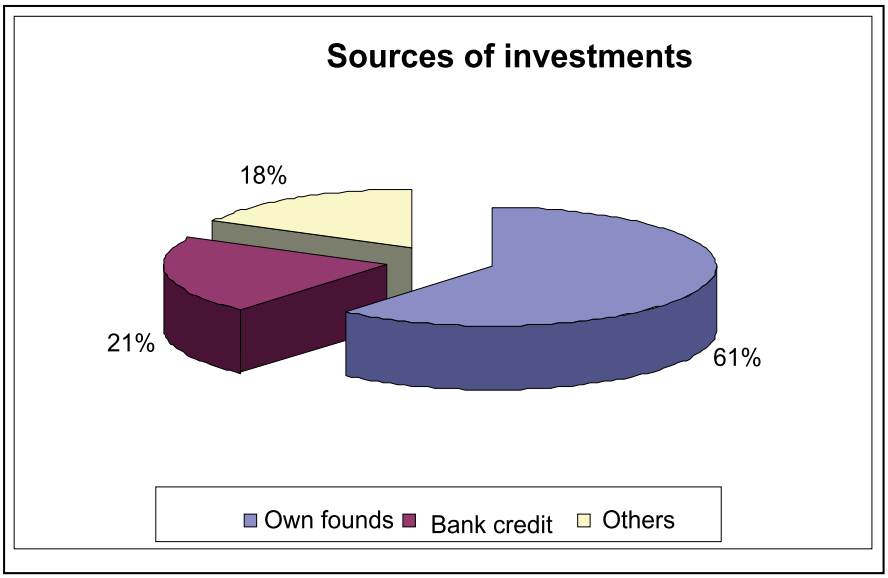

Fig. 4. Sources of financial invetments (Ministry of Agriculture, Food and Consumer Protection, 2009)

6. International trade

Albanian olive oil exports are very encouraging as the industry is maturing and achieving all attributes required for the olive oil quality. The figures however remain modest: 22 tons were exported in 2004; 16 tons in 2005; 54 tons in 2006; 15 tons in 2007 and 4 tons were exported in 2008. The first success was the export of "Shkalla enterprise" certified organic and extra-virgin olive oil to the niche market in Switzerland. This represents a small, but stable export and with potential to increase. This was the first sign of the "recovery" of Albanian olive oil export to the neighboring countries since 1996. The transaction was particularly important because, for the first time, the processing plant was certified. Furthermore, the payment was delivered by the letter of credit, in contrast to cash, that had been the practice until then.

Albanian imports on the other side are significant and range between 850 – 1100 Mt per year, of which almost 90% is supplied by Italy and Greece. Large part of the imported oil is in bulk to be than bottled in Albania. Albanian import of olive oil has increased since year 2000. In 2005 and 2006, due to major increase of EU olive oil prices and higher levels of domestic olive production, imports of olive oil dropped. In 2008, imports of olive oil were considerably higher than the same period of the previous years, due to the low olive oil production in 2007, caused by low olive production. This evolution of imports shows how the olive oil demand in Albania is price sensitive. The olive oil price increased by almost 40% from year 2004 to 2005 and was associated with almost 20% reduction in imports. Simultaneously, the continuous increase of domestic production of olives and olive oil has partially compensated the increasing demand, and contributed to lowering demand for imports. Imports usually increase in the last three months of each year, when consumption is higher and the olive oil of the new crop is not yet ready. Imports reach a minimum in summer. In general, the yearly peak of imports of olive oil follows by one or two months that one of table olives. In 2008 imports remained high also in January and February, due to the scarcity of domestic production.

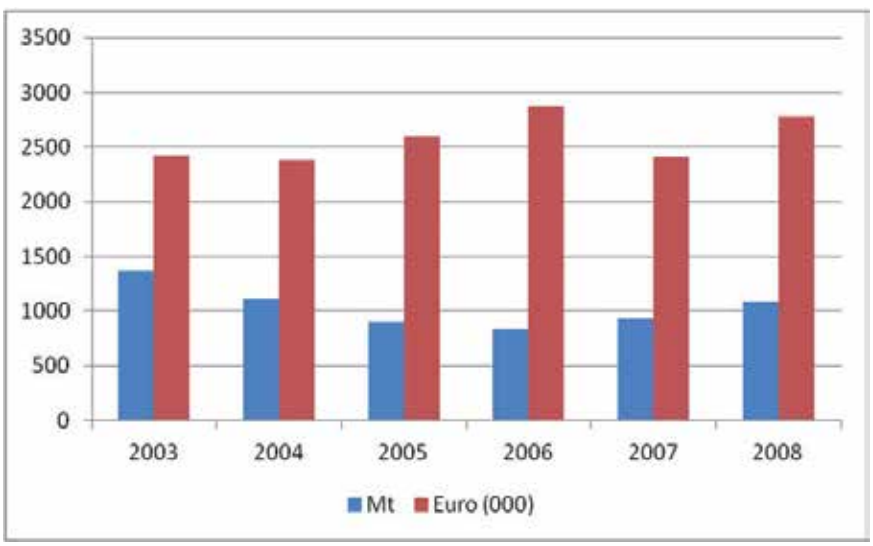

Fig. 5. Olive oil import trends from EU in terms of quantity and value (INSTAT, 2009)

At present, imports cover about 10% of market-based demand, but imports from the highly competitive Mediterranean producers could lead to an increase in market share of imported olive oils in the next years. Increasing domestic demand, years of relatively lower productivity and a general scarce competitiveness of the Albanian products are among the main factors that caused such a situation.

7. Economic issues behind olive oil processing industry

According to the results of the studies that have been undertaken for the olive oil industry indicated that olive oil production is privately profitable. The Private Cost Ratio has been measured and the coefficient was evaluated at 0,703, meaning that this production is profitable for the private enterprises. Still according to these results indicate that Albania has not a comparative advantage in olive oil producing industry in the production year 2008-2009. The calculations resulted in DRC = 2.2. This means, it is not socially desirable to produce and expand olive oil production in Albania, as the use of domestic factors is not efficient in economic terms. Comparing the two values of the above ratios, in current situation, olive oil production can be appreciated as competitive within the country with private prices, but it cannot be appreciated as competitive with social prices.

A sensitivity analysis was done to see how the DRC ratio reacts to different changes in different parameters of the model. Changes were done in the parameters like world market prices of olives and olive oil, exchange rate and labor force price. As a result of the sensitivity analysis it was seen that the olive oil production is very sensitive to changes in input (olives) and output (olive oil) prices of the world market and also to changes in the exchange rate. But it is not sensitive towards changes in the labor force prices. The major determinants of the Albanian olive oil comparative advantage are the favorable world price of olive oil, the exchange rate and the price of olives as input factors for the olive oil manufacturing. The explored values of private profitability and the DRC suggest that that olive oil production is privately and socially profitable, however two important conclusions are to be emphasized particularly: firstly, the private profitability is higher than the social profitability, and secondly, social profitability is largely depended on the situation at the international market.

Due to the changes in the sensitivity analysis it can be seen that a reduction on the olives as input in the olive oil industry, the domestic resource cost ratio enters in the interval values in which we can say that olive oil production is competitive in Albania. In this stage there is need of state policy intervention in order to help the olive oil producers for having lower prices of inputs. Policies like subsidies of the prices of inputs are suggested in this case.

In recent years, Albania has seen a rapid evolution in its citizens' consumption behaviors and life styles due to economic growth, improvement in the standard of living, fast urbanization and trade liberalization within the country. One consequence of this has been the gradual segmentation of the food and beverages market, similar to what has been seen in other transitioning countries (Berisha and Mara, 2005: WB, 2007). The transition from a centrally planned socialist economy to a market oriented economy has also given rise to an urban middle-income class of consumers. Another important study on olive oil consumer preferences conducted in Tirana/Albania 2010 has resulted with some other result on the olive oil (Chan Halbrendt et al, 2010). According to this study 6 consumers' segments and profiles were identified, based on set of preferences and willingness to pay. The fact that it is now

possible to clearly identify several segments of consumers marks a milestone in the process of evolution of agri-food marketing, with major consequences on development policies. Origin is a key choice factor for 82% of respondents, in three out of six consumers' segments.

The confidence on quality and safety of domestic product is low. This conclusion emerges from the analysis of several factors: i) imports are growing notwithstanding the consumers' preference for Albanian olive oil; ii) consumers have little confidence on reliability of domestic industrial producers and controls made by competent authorities, so they prefer to buy olive oil directly from trusted farmers, or from the oil mills or to buy imported products; iii) during the analysis there was a scarce correspondence between low income and preference for low prices, as high prices are considered one of the few reliable proxies for quality.

The majority of purchased olive oil is still traded as not bottled product, being sourced either directly from farmers and oil mills or as by quantity in traditional shops. 44% of the interviewed consumers in Tirana confirm that they buy directly from farmer and olive oil mill respectively. This percentage should be much higher in smaller cities or rural areas characterized by olive and olive oil production and consumption. When considering also self-consumption of farmers in production areas, it is possible to conclude that most probably, more than 70% of the olive oil consumed in the country is sold as a non-bottled product and is subject to little quality control.

Under the current extensive inefficient conditions in which the olive culture is cultivated in Albania, there is however a profitability for farmers to produce. This profitability and comparative advantage can be improved if the olive culture is cultivated more intensively. If the farmers are sure that the processing industry will act as a reliable market for their products, they will increase the production. On the other hand the increased olive cultivation will provide more raw materials for the processing industry, assuring its functioning with full capacity. The better utilisation of existing capacities in processing industry will allow favouring from the low of economies of scale and at the end effect result in lower production cost.

8. The future of olives and olive oil in Albania

In the last years olives and olive oil has become one of the priorities of the Albanian Government policies. Recently Albanian Government is undertaking an extremely ambitious policy for expanding the production base, targeting a fivefold increase of the total number of olive trees, i.e. up to 25 ml trees. For this purpose, most subsidies provided from 2007 to the agricultures sector from the State (scoring about 10 m Euro in 2008) are addressed to the olive cultivation and olive oil production. Focusing investments in increasing yields (production per tree), stabilizing output from one year to another and improving harvesting and pest management practices would be at present a more cost/effective option for ensuring a sustainable development of the sector. Priority actions include: i) improvement of value chain governance tools, including harmonization of laws to EC, ii) increased technical assistance to farmers to increase productivity and stabilize output; iii) support to value chain operators for facilitating access to services, iv) supporting establishment and strengthening of farmers' associations and cooperatives and; v) optimize

the use of effluents and by-product in olive oil industry, to mitigate environmental impact of olive oil production and increase profitability in olive oil processing.

The Government of Albania lunched since early 2009 the idea of supporting the plantation of 20 million olive trees, which would eventually transform Albania into a world level competitor. At present, domestic demand of olive oil scores around 12,200 Mt and that one of table olives 14,000 Mt per year (excluding self-consumption).Yearly yields and output are highly variable, as Albanian olive orchards receive poor or no services and are highly vulnerable to weather conditions. According to previous surveys and according to the evaluation of specialists, average yield of olive trees in Albania is circa 15 kg/tree.

Under these assumptions and estimations, there is a deficit of 1,500 tons of olive oil which is not very different from the recorded official imports of olive oil – circa 1,000 tons of olive oil (the current yields may be even a bit more than 15 kg/tree, i.e. if assumed 15.5 kg/tree, than we obtain a deficit corresponding exactly to the recorded imports). Improving average yield to 17 kg/tree (+13%) to the current 5,011 million of trees (thus excluding in these calculations the expected increased number of trees in the coming years) there a surplus of production will be already achieved. Considering that many trees will enter in full production in the next years, this objective seems easy to achieve. According to expert (agronomist) evaluations, under irrigation and proper treatment, it is possible to achieve average yield of 25 kg/tree (conservative assessment). At this level, suficit is of equivalent 7,550 tons.

As a conclusion, Albania can meet its demand for olives and olive oil, and even achieve surplus, by simply improving services to the current olives; moreover, even without further support for new plantings, the expansion of the production base will continue, even if at a slower pace: before the introduction of subsidies for new plantings, the average growth of the production base was of 166,000 new trees/year; in 2007-2008, after the introduction of subsidies, this amount increased to 257,000 per year.

In their study, Leonetti et al, 2009, introduced 5 different scenarios considering several investment and related implications.

- Scenario 1 – Average future plantings in accordance to the trend recorded before the introduction of subsidies.
- Scenario 2 - Average future plantings in accordance to the trend recorded after the introduction of subsidies (2007- 2008).
- Scenario 3 - 20 million trees are planted within 5 years, starting in year 2009, at a pace of 4 million trees per year.
- Scenario 4 - 20 million trees are planted within 10 years, starting in year 2009, 2 million trees per year
- Scenario 5 - 20 million trees are planted within 15 years, starting in year 2009, 1.33 million trees per year.

The number of trees according to each scenario is reflected respectively in Figure 6. For the production, based on expert assessment, they assume that old trees (planted till 2008) have a yield of 15 kg/tree, whereas the new ones, 25 kg/tree. In the second year, the new trees achieve 3 kg/tree, third year 8 kg/trees, fourth 20kg/tree, fifth 25 kg/tree.

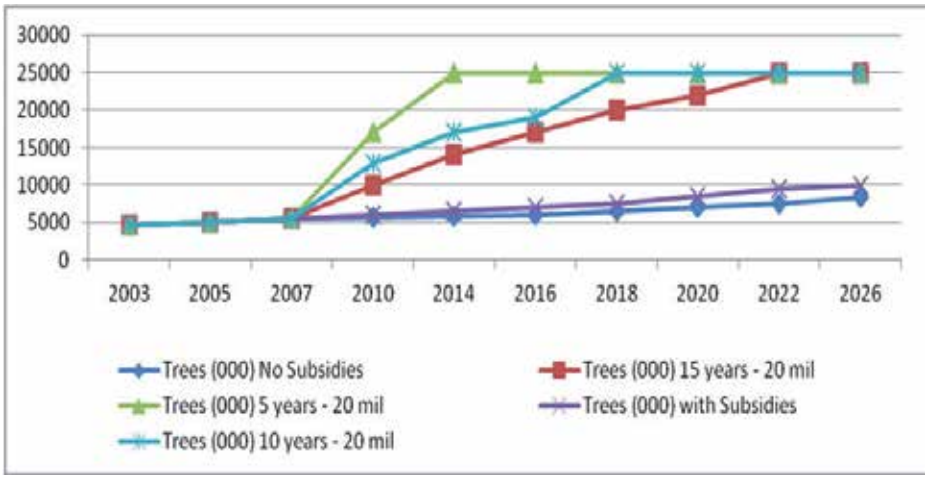

Fig. 6. Dynamics of Olive trees under different scenarios (000 trees), (Adapted from Leonetti et al, 2009)

9. Concluding remarks

Olive oil production can become a very important aspect in the Albanian agriculture economy. Due to the favourable climatic conditions the main input, olives, can be cultivated in a more intensive form, despite the fact, that the areas under olive cultivation in Albania compared with the areas in Greece or Italy are very insignificant.

After a relatively long hiatus related with the democratic political changes in of early '90-s, the Albanian olive and olive oil industry is showing signs of a healthy recovery. Since the country restored political and economic stability in 1999, the olive sector has attracted notable attention from the government and private investors, giving rise to a considerable growth of the sector. The planting of new trees has increased tremendously as a result of the government's supporting programs and private initiatives. New plantings have been established with modern practices and good management. The oil processing industry has also experience significant growth. Small processing plants have become more efficient in producing high quality oil for domestic and foreign markets. Still, the sector faces important challenges to overcome such as high cost of production, dominance of low quality olive oil production, shortage of raw materials, weak contracting relationship between the growers and the processors, and deficiencies in marketing.

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The health-promoting effects attributed to olive oil, and the development of the olive oil industry have intensified the quest for new information, stimulating wide areas of research. This book is a source of recently accumulated information. It covers a broad range of topics from chemistry, technology, and quality assessment, to bioavailability and function of important molecules, recovery of bioactive compounds, preparation of olive oil-based functional products, and identification of novel pharmacological targets for the prevention and treatment of certain diseases.

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