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# Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications

*Edited by Bekir Salih and Ömür Çelikbıçak*

# GAS CHROMATOGRAPHY IN PLANT SCIENCE, WINE TECHNOLOGY, TOXICOLOGY AND SOME SPECIFIC APPLICATIONS

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# Meet the editors

Prof. Dr. Bekir Salih was born in Trabzon, Turkey in 1958. He received his B.S., M.S. and Ph.D. degrees from Hacettepe University, Turkey and was a postdoctoral fellow at Catholic University of Nijmegen in the Netherlands, Swiss Federal Institute of Technology ETH Zurich, Switzerland and Eötvös Loránd University, Hungary. After completion of his research mainly in the field of mass spectrometry, he continued his academic career as a faculty at the Faculty of Science in the Hacettepe University and he became Professor of Chemistry in 2001. He published more than 130 articles in his research fields including Mass Spectrometry, chromatography, polymers and their different applications.

Dr. Ömür Çelikbıçak was born in 1978 in Konya, Turkey. He received his Diploma (M.Sc.) and his Doctorate (Ph.D.) in Chemistry, in 2005 and 2011, respectively, both from the Hacettepe University, Turkey. He started his academic career as a research assistant at Hacettepe University, Chemistry Department under supervision of Prof. Dr. Bekir Salih in 2002. From 2008-2009 he was a research scholar in the mass spectrometry research laboratories at the Chemistry Department of The University of Akron, OH. Since 2011 he has been working at Hacettepe University as a post doctoral researcher. His research interests focus on the development of novel mass spectrometry methods and their chromatography coupled applications for the study of structures, interactions, fragmentations and detection of the various molecules in the gas phase.

# Contents

# Preface

Chromatography is the main separation technique used for the analysis of the real samples which have more and more different compounds having similar or completely different chemical and physical properties and also mainly affected each other during the analysis the target compound in the real sample. The development of the chromatography showed enormous race and many sophisticated chromatographic methods were discovered for different analytes related to their volatility, molecular weight, hydrophobicity, specificity to the phases or functional groups of the phases used in the chromatographic techniques. In the past, mainly preparative chromatographic techniques were used but nowadays miniaturized and modern systems which required very low volume of samples such as nano liters and low concentration of the analytes from part per trillion (ppt) to part per million (ppm) are prominently important. To parallel the development, new detection systems were also discovered in order to measure the analytes in the very low volume and with the most desired detection limits.

At the academic side, many researchers know how they find their way to touch suitable method and useful chromatographic techniques to analyze their samples with high accuracy and precision by the proper chromatographic technique and its required tools such as column, phases and detectors. Nevertheless, the people who are working at the industry ask very sophisticated question about the real sample "which compounds and in which level of their concentrations are in the samples?". To answer this question, there is no way to go out not to touch the chromatographic techniques.

The useful chromatographic technique is the Gas Chromatography for the volatile compounds, both organic and organometallic which having volatility up to 300 $^{o}$C. Using capillary column and some convenient detectors around hundreds of compounds having different detector responses could be analyzed quantitatively and qualitatively in the same run.

Today, hyphenated methods including Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Atomic Spectrometry have been widely used for the detection and quantification of the organic molecules and also organometallic species. Especially Gas Chromatography-Mass spectrometry technique is the best for the identification of the finger prints of the organic molecules and their quantification at

trace levels. This hyphenated technique does not need any reference material to identify the organic and organometallic species using chromatographic separation and mass spectrometric detection. Therefore, GC-MS system is a kind of most special and useful system among the other Gas Chromatographic Systems.

In the recent years, 2D Gas Chromatography (GCXGC) was discovered and has been widely used efficiently in today's Gas Chromatographic studies.

After all these developments in Gas Chromatography and its hyphenated methods, these techniques have been finding many applications in various sample analysis in different real samples such as environmental, biological, foods, drugs, narcotics, plants, soils, sediments and the other samples.

Using the information in the chapters, it may be possible to develop Gas Chromatographic procedures for different compounds in Plant Science, Wine Technology and Toxicology by the readers of the book.

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# Part 1

## Plant Science

1

# Determination of the Chemical Composition of Volatile Oils of Plants Using Superheated Water Extraction with Comprehensive Gas Chromatography-Time-of-Flight Mass Spectrometry

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## 1. Introduction

Plants or plant extracts can be used in traditional medical remedies. Plants have evolved the ability to synthesize chemical compounds that help them defend against attack from a wide variety of predators such as insects, fungi and herbivorous mammals. By chance, some of these compounds, whilst being toxic to plant predators, turn out to have beneficial effects when used to treat human diseases. Herbal medicines do not differ greatly from conventional drugs in terms of how they work. Many of the herbs and spices used by humans to season food yield useful medicinal compounds. They are of great economic importance which is not only related to their use as a spice or herbal tea. In fact, many of the medicinal plants are used traditionally in many other ways as their essential oils have antimicrobial, antifungal and antioxidant activity. Volatile oils of plants, also known as essential oils, often contain a mixture of flavour, fragrant and medicinal compounds. They contain complex mixtures including monoterpenes, sesquiterpenes and their oxygenated derivatives such as aliphatic aldehydes, alcohols and esters (Simandi et al., 1998; Vokou et al, 1998; Ozel et al., 2003; Ozel et al., 2006; Ozel & Kutlular, 2011).

Analysis of plants normally involves a sample preparation stage such as extraction or distillation followed by analysis with gas chromatography or liquid chromatography. The common methods used currently for the isolation of essential oils from natural products are steam distillation and solvent extraction (Ozel & Kaymaz, 2004). Losses of some volatile compounds, low extraction efficiency, degradation of unsaturated compounds through thermal or hydrolytic effects, and toxic solvent residue in the extract may be encountered with these extraction methods. Recently, more efficient extraction methods, such as supercritical fluid extraction (SFE) (Simandi et al., 1998) and accelerated solvent extraction (ASE) (Schafer, 1998) have been used for the isolation of organic compounds from various plants. Subcritical or superheated water extraction (SWE) is non-toxic, readily available, cheap, safe, non-flammable and is a recyclable option.

SWE was found to give recoveries comparable to those of steam distillation and Soxhlet extraction of essential oils from two *Origanum onites* samples (Kutlular & Ozel, 2009). The kinetics of SWE under optimum working conditions mean that the extraction is mostly completed in 15 minutes (Ozel et al., 2003). Soxhlet extraction is time-consuming and labour-intensive. Steam distillation is cheap but has no selectivity and is also time-consuming. SFE is a complicated system and often problems occur during extraction. ASE is quick but uses toxic organic solvents. In contrast, SWE is cheap, relatively fast and environmentally sound as solvents do not have to be used. It is also selective in that the operator is able to extract various polar and non-polar organic compounds by choice by varying the temperature as long as the water is kept in a liquid state using minor adjustments in pressure.

Plant volatile oils normally contain a complex mixture of organic compounds. They are largely composed of a range of saturated or partly unsaturated cyclic and linear molecules of relatively low molecular mass and within this range a variety of hydrocarbons and oxygenated compounds occur. Conventional one dimensional gas chromatography generally does not provide sufficient separation for complex mixtures. Since essential oils contain numerous components, it is possible that some components can obscure the analytes of interest. Two-dimensional gas chromatography is known as comprehensive gas chromatography (GCxGC). GCxGC is a fully multi-dimensional technique achieving a much increased peak capacity in limited analysis time. GCxGC has been shown to be an extremely powerful technique for the analysis of essential oils. High acquisition rates of time of flight-mass spectrometry (TOF-MS) offer a superior separation power. The coupling of GCxGC to TOF-MS is very effective (Marriott et al., 2000; Ozel et al., 2004)

## 2. Extraction of plant volatiles using superheated water

SWE is a technique based on the use of water as an extractant, at temperatures between 100 and 374 °C and at a pressure high enough to maintain the liquid state. Lab-scale SWE can be performed using 1-5 g of air-dried solid samples, a 5-20 mL stainless steel extraction cell, 0.5-5.0 mL $min^{-1}$ flow rate, temperatures of 100-175 °C, a pressure of 15-60 bar and 15-60 min of extraction time. A typical SWE system is shown in Figure 1. Previous workers (Ayala & Luque de Castro, 2001; Ozel et al., 2003; Ozel & Kaymaz, 2004) reported that SWE of essential oils is a powerful alternative, because it enables a rapid extraction and the use of low working temperatures. This avoids the loss and degradation of volatile and thermo labile compounds. Additional positive aspects of the use of SWE are its simplicity, low cost, and favourable environmental impact. Soxhlet extraction is time consuming (6-24 h) and uses a large amount of environmentally-unfriendly organic solvents (Ozel & Kaymaz, 2004). Steam distillation is also time-consuming, taking 6-24 hours.

In lab-scale SWE, separation of the compounds from the aqueous extract obtained is the critical stage. A liquid-liquid extraction technique often causes emulsion and breaking this can be very difficult. It has been found that solid phase extraction is a better technique for the removal of compounds from the aqueous environment of SWE when compared with liquid-liquid extraction (Rovio et al., 1999; Ozel et al., 2003). Headspace solid phase microextraction with GC-MS may be another alternative (Deng et al., 2005).

The optimal subcritical water extraction conditions can be decided upon by using various pressures, times, water flow rates and temperatures. Perez-Serrafilla et al. (2008) performed

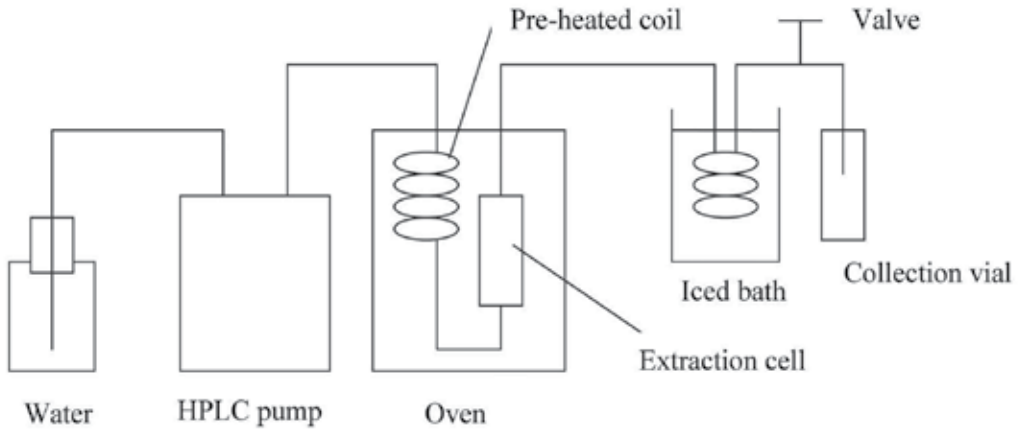


Fig. 1. Schematic representation of basic SWE apparatus

selective extraction of fatty acids and phenols using static and dynamic SWE. Selective extraction is also possible for target compounds using different conditions. The yields of essential oils of *Thymbra spicata* for a 30 minute extraction at a flow rate of 2 ml.min$^{-1}$ and 60 bar, and at four different temperatures (100, 125, 150 and 175°C) were carried out (Ozel et al., 2003). The yield increased with temperatures up to 150°C. A further increase to 175°C resulted in a small decrease in the yield.

The pressures of 20, 60 and 90 bar (Ozel et al., 2003) and 20, 50 and 80 bar (Deng et al., 2005) have been observed to cause no significant difference in the amounts of extracted oils from *Thymbra spicata* and *Fructus amomi*. Enough pressure should be preserved to keep the water in a liquid state. Optimization of SWE conditions has been studied (Ayala & Luque de Castro, 2001; Ozel et al., 2003; Ozel & Kaymaz, 2004; Deng et al., 2005). Ayala & Luque de Castro (2001) discovered that a temperature of 125 ˚C, 2MPa pressure and a 1 mL.min$^{-1}$ flow rate is optimum for SWE of essential oils. Conditions for SWE of *Origanum onites*, *Thymbra spicata* and *Fructus amomi* were optimal at a temperature of 150 ˚C, a pressure of 20-60 bar, a flow rate of 2 mL.min$^{-1}$ and 30 minutes of extraction time (Ozel & Kaymaz, 2004; Ozel et al., 2003; Deng et al., 2005). In SWE of marjoram by Jimenez-Carmona et al. (1999), of clove by Rovio et al. (1999) and of fennel by Gamiz-Gracia and Luque de Castro (2000), the yield reached its maximum at 150°C over a temperature range of 50-175°C.

Superheated water for the extraction of plant volatiles is a powerful technique because it enables a rapid extraction. The solubility of organic compounds in superheated water is high for two reasons. Firstly, solubility increases with increasing temperatures and secondly, water becomes less polar as the temperature rises. Other advantages of the use of SWE are its simplicity, low cost, speed and the fact it is considered more environmentally friendly. With solvent extraction, steam distillation and water extraction, the loss of some volatile compounds, long extraction times and the toxic solvent residue may be considered a disadvantage. The temperature is a very important parameter in superheated water extraction. In the extraction of eugenol and eugenyl acetate from cloves using superheated water, the workers found that the extraction kinetics were very fast at high temperatures (250°C and 300°C), giving a 100% recovery after 15 min, compared to extraction at 125°C,

where the same recovery took 80 min to achieve (Rovio et al., 1999). Conversely, at high temperatures essential oils may be destroyed (Ozel et al., 2003). Kinetic studies were carried out under optimum conditions for SWE (Ozel et al., 2003). Figure 2 shows the kinetics of the 5 main compounds in the SWE extract of *Thymbra spicata* (Ozel et al., 2003). Although the extraction was completed in 20 minutes, the time for the SWE was selected to be 30 minutes to make sure all the essential oils had been extracted (Ayala & Luque de Castro, 2001; Ozel et al., 2003; Ozel & Kaymaz, 2004).

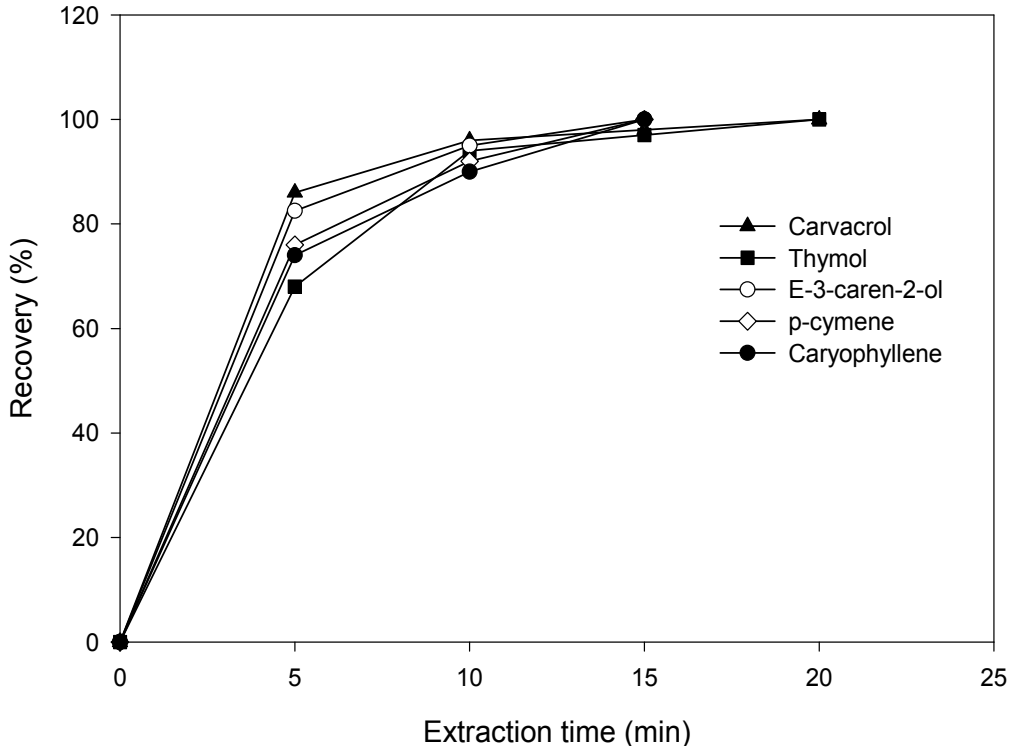


Fig. 2. Effect of extraction time on the extraction efficiency of some compounds of essential oils of *Thymbra spicata* using subcritical water extraction at a temperature of 150°C and a flow rate of 2 ml $min^{-1}$ (Ozel et al., 2003).

There is a growing interest in natural foods, because of the increased demand for non-synthetic natural antioxidants. Some species of s*alvia* are used as medicinal and aromatic plants. Salvia is used in food, cosmetics, perfumes and pharmaceutical products (Schwarz & Ternes, 1992). Different functional groups and/or chemicals such as flavonoids, vitamins, antioxidants and antimicrobials, can be extracted selectively using SWE (Rodriguez-Meizoso et al., 2006; Hartonen et al., 2007; Mendiola et al., 2007). Extraction of essential oils of *Salvia fruticosa* using superheated water has been studied using samples collected from Denizli, Fethiye, Korkuteli and Gundogmus (Turkey). The amounts and compositions of recovered essential oils can be influenced by different collection locations. In Table 1, it can be seen that there is a slight difference in the amounts and compositions of essential oils according to location, especially in the case of the *Salvia fruticosa* sample from Korkuteli (Ozel & Kutlular, 2011).

| Compound | RI | % | | | |
|---|---|---|---|---|---|
| | | Denizli | Fethiye | Korkuteli | Gundogmus |
| Essential oil content | - | 1.86 | 0.97 | 1.78 | 1.53 |
| α-Pinene | 939 | 2.18 | 1.16 | 8.65 | 4.21 |
| Camphene | 953 | 2.57 | 3.61 | 0.66 | 2.35 |
| Sabinene | 972 | -[e] | - | 0.29 | - |
| β-pinene | 976 | 1.11 | 2.18 | 13.39 | 1.08 |
| Octenol | 982 | 0.07 | 0.18 | - | 0.22 |
| Myrcene | 992 | 0.27 | 0.12 | - | 0.27 |
| α-Phellandrene | 1006 | - | - | 0.29 | - |
| 3-Carene | 1009 | - | - | 1.33 | - |
| 1,8-Cineole | 1030 | 37.25 | 33.85 | 16.64 | 42.72 |
| Limonene | 1033 | - | - | 0.25 | - |
| γ-Terpinene | 1074 | - | - | 0.59 | - |
| Terpinolene | 1088 | - | - | 0.62 | - |
| Linalool | 1098 | - | 0.58 | 5.85 | - |
| α-Thujone | 1101 | 4.76 | 7.06 | 2.76 | 3.10 |
| β-Thujone | 1113 | 9.02 | 3.87 | - | 1.52 |
| Fenchyl alcohol | 1130 | - | - | 0.46 | - |
| Camphor | 1139 | 17.91 | 22.12 | 3.96 | 28.27 |
| Borneol | 1162 | 4.79 | 6.45 | 0.84 | 1.99 |
| Pinocarveol | 1169 | 0.06 | - | 1.90 | - |
| Terpinen-4-ol | 1179 | 1.62 | - | 1.63 | - |
| α-Terpineol | 1194 | 0.77 | 3.27 | 9.16 | 4.03 |
| Dihydrocarvone | 1195 | - | 0.32 | - | - |
| Bornyl acetate | 1238 | 0.12 | - | - | 0.73 |
| Cinnamaldehyde | 1240 | 0.05 | 0.09 | 0.26 | - |
| Carvone | 1254 | 0.16 | - | 0.17 | - |
| Thymol | 1290 | 2.97 | 2.96 | 6.24 | 0.47 |
| Eugenol | 1364 | 0.13 | 3.97 | 3.21 | 0.12 |
| Caryophyllene | 1426 | 9.69 | 3.13 | 16.81 | 3.78 |
| Butylated Hydroxytoluene | 1516 | 0.10 | - | 0.11 | - |
| Eicosane | 2000 | 0.08 | - | - | - |
| Unknown | | 4.32 | 5.08 | 3.93 | 5.14 |
| Total compounds | | 21 | 17 | 24 | 15 |

RI, Retention index

Table 1. Percentage compositions of essential oils of *Salvia fruticosa* isolated using the SWE technique (Ozel & Kutlular, 2011).

Sample collection times also change the volatile profile of plant materials (Kutlular & Ozel, 2009). *Origanum onites* is a perennial species with woody stems. It is encountered in the Southern Greek mainland, the islands and the western and southern coastal areas of Turkey (Vokou et al., 1998). The essential oils of *Origanum* species have been proven to have

| Compound | RI | % | | |
|---|---|---|---|---|
| | | 15 June, 2006 | 25 June, 2006 | 05 July, 2006 |
| 3-Thujene | 938 | 0.01 | 0.03 | 0.02 |
| α-Pinene | 939 | 0.02 | 0.04 | 0.06 |
| Camphene | 953 | 0.01 | 0.01 | 0.02 |
| Benzaldehyde | 960 | 0.02 | 0.02 | 0.02 |
| β-pinene | 981 | 0.05 | 0.13 | 0.93 |
| Myrcene | 992 | - | 0.08 | 0.06 |
| 3-Octanol | 1004 | 0.02 | - | - |
| α-Phellandrene | 1006 | 0.01 | 0.02 | - |
| p-Cymene | 1027 | 0.88 | 0.92 | 0.61 |
| Eucalyptol | 1030 | 0.03 | 0.20 | 0.05 |
| Limonene | 1033 | 0.02 | 0.05 | 0.03 |
| Ocimene | 1052 | - | 0.01 | 0.02 |
| Acetophenone | 1068 | - | - | 0.02 |
| γ-Terpinene | 1074 | - | - | 0.01 |
| Terpinolene | 1088 | 0.23 | 0.52 | 0.38 |
| Undecane | 1100 | 0.02 | 0.01 | 0.03 |
| Linalool | 1100 | 0.14 | 4.44 | 5.14 |
| 2-Decen-1-ol | 1110 | 0.17 | 0.32 | 0.19 |
| α-Campholenal | 1125 | 0.01 | 0.01 | 0.02 |
| Camphor | 1139 | 0.01 | 0.04 | 0.01 |
| cis-Verbenol | 1140 | - | - | 0.01 |
| Borneol | 1162 | 0.34 | 0.41 | 0.79 |
| Pinocarveol | 1169 | 0.01 | 0.03 | 0.03 |
| Terpinen-4-ol | 1179 | 0.47 | 0.75 | 0.86 |
| cis-Linalool oxide | 1186 | 0.01 | 0.09 | 0.20 |
| α-Terpineol | 1195 | 0.19 | 0.39 | 0.56 |
| Carveol | 1197 | 0.03 | 0.03 | 0.14 |
| Dihydrocarvone | 1202 | 0.04 | 0.08 | 0.01 |
| Nerol | 1233 | 0.01 | 0.04 | 0.12 |
| Carvone | 1254 | 0.10 | 0.15 | 0.12 |
| Thymol | 1290 | 0.18 | 0.41 | 1.45 |
| Carvacrol | 1295 | 92.66 | 86.71 | 84.33 |
| Eugenol | 1364 | - | 0.19 | - |
| Eicosane | 2000 | 0.02 | - | 0.01 |
| Unknown | | 4.29 | 3.89 | 3.73 |

RI, Retention indice

Table 2. Percentage compositions of volatile components of *Origanum onites* leaves collected on various dates, isolated using the SWE technique (Kutlular & Ozel, 2009).

antibacterial, antifungal and antioxidant activities (Kutlular & Ozel, 2009). *Origanum* is accepted as a volatile yielding plant, its essential oils consist mainly of carvacrol and thymol type compounds which are well known phenolic antioxidants. Extraction of *Origanum onites* leaves collected on three different dates (15 June; 25 June; 5 July, 2006) using superheated water was performed (Kutlular & Ozel, 2009). The results are shown in Table 2. Carvacrol was found as the main compound. The total amount of essential oil increased with later collection dates in contrast to the amount of carvacrol which actually decreased with the date. Since carvacrol is one of the most valuable compounds, a compromise must be struck between the desired to obtain the greatest quantity of oil possible whilst at the same time obtaining the highest possible percentage of carvacrol. As a result, the date of sample collection is a very important parameter for the quantity and value of the essential oil of plant materials.

## 3. Analysis of plant volatiles using gas chromatography

Gas chromatography can be applied to the analysis of volatile and semi-volatile organic compounds, containing boiling points from near zero to over 400°C (Bartle & Myers, 2002). Plant volatiles such as essential oils, are often highly complex mixtures. Good separation methods are necessary to analyse these molecules. Hyphenation of different techniques is currently the preferred method for analysing these volatile essential oils (Welthagen et al., 2007). In general, the coupling of a GC separation step with a mass spectrometric detector is the most common set-up. For complex mixtures like essential oil, however, selectivity and separation capability of the GC-MS is not sufficient. Multidimensional GC separation with selective MS detection (such as TOF-MS) can improve separation and identification of complex plant volatiles (Welthagen et al., 2007). Small, highly polar molecules (such as amino acids and sugars) cannot be analysed using GC technology. A possible solution is the use of chemical derivatization methods where the polar groups of the target molecules are converted into less polar molecules.

In GC-MS, ions are produced by electron or chemical ionisation. But, the ions are now sorted according to molecular weight (or mass to charge, *m/z* ratio) by one of a range of analysers: magnetic sector quadropole, ion trap or time of flight (Bartle & Myers, 2002). Mass spectrum is related to the molecular weight and fragmentation of the analyte, allows identification through comparison with a library. The advantages of TOF-MS lie in the possibilities for accurate mass measurement and rapid rates of accumulation of spectra (up to 500 Hz), which allow GC peaks as narrow as 12ms to be identified (Bartle & Myers, 2002).

Injection of the sample into the column head is usually carried out using a syringe and a hypodermic needle. In order to be able to detect lower concentrations, a large volume injection technique has been developed allowing a sample size of 20-100 μL, rather than the formerly standard 1-2 μL (Bartle & Myers, 2002). Low levels of volatile compounds in plant materials may be analyzed by headspace, dynamic stripping or purge-and-trap sampling (Bartle & Myers, 2002). Direct thermal desorption (DTD) at one or more temperatures followed by pyrolysis can yield important information. Selection of temperatures is a very important parameter. The lowest temperature possible should be selected in order that the native volatile compounds can be determined without generating new compounds (Koning et al., 2008). This is illustrated by the study of essential oils of *Pistacia vera* (Ozel et al., 2004). A number of species can be seen to appear only at the higher temperatures of 200 and

250 °C, and these are known not to be components of essential oil of *Pistacia vera*, but are the browning reaction products (e.g. furfural, 2-furanmethanol, acetylfuran, 2-furanone, 2,4-dimethyl-furan, furfuryl acetate and furfuryl alcohol). Most of these components are low in concentration at 200 °C, however show a significant increase at 250 °C. The browning reaction products may be produced either by caramelization or by Maillard reactions during the higher temperatures of 200 and 250 °C.

The one dimensional GC technique cannot always provide sufficient separation of all components of plant volatiles. In order to enhance peak capacity, multidimensional gas chromatography can be used. Marriott & Shellie (2002) define multidimensional analysis in chromatography as 'any technique that combines two or more distinct separation/analysis steps'. The first dimensional separation is based on separation by boiling point in a non-polar column. The second dimensional separation is based on separation by polarity using a polar column. The inclusion of this makes this overall a two dimensional chromatogram.

Today, there are two kinds of two dimensional GC: heart-cutting and comprehensive two-dimensional gas chromatography (GCxGC). In the heart-cutting technique, one or more unresolved fractions from the first column are transferred to a second column having a different polarity where the separation of the compounds will be achieved. In general, the first column is non-polar with a length of 30 to 60 m, whilst the second is 30 m long with a higher polarity. The heart-cut can be directly transferred to the second column or it can be trapped on a cryogenic device and transferred later. With comprehensive GC (GCxGC), the entire sample, not only fractions, are separated on two different columns. The columns are shorter, typically 15 to 60 m (100% polydimethylsiloxane or 5% diphenyl 95% polydimethylsiloxane stationary phase) for the first and only 1 to 2 m (50% diphenyl 50% polydimethylsiloxane or polyethylene glycol) for the second column. The short length of the second column enables very fast separations whilst collecting the fractions from the first column. The most important component of the system is the 'modulator', which will accumulate the fractions coming from the first column on a short segment of column, and then release it quickly into the second one. There are different kinds of modulators but in general the mechanisms involve alternate cryofocussing and thermal desorption of the trapped analytes. GCxGC has advantages over the heart-cutting technique (Marriott et al., 2000; Marriott & Shellie, 2002).

GC–on-a-chip is receiving more attention now especially from field workers. There is interest in the development of a microfabricated gas chromatography system suitable for the separation of volatile organic compounds and compatible with use as a portable measurement device. A planar 2-dimensional GC chip with fully circular channel profiles has been microfabricated from glass using acid-etching techniques. Coupling of the directly heated column to a low cost, low-power photoionization detector showed reasonable separation of gasoline vapour. Comprehensive separation has given some promising results from the separation of ppm gas mixtures of a set of volatile organic compounds when coupled with commercial GC detectors (Halliday et al., 2010)

GCxGC typically generates peak capacity of the order of several thousand, making it highly appropriate technology for the separation and analysis of complex samples such as essential oil. Plant essential oil is used in numerous traditional medicines. Essential oil is usually rich in monoterpenes, sesquiterpenes and oxygenated derivatives. One dimensional GC lacks the

resolving power to provide adequate separation of the complex mixture of essential oil. GCxGC has an estimated separation increase of about ten times over one dimensional GC. Another advantage of GCxGC, regarding the second dimension retention times of the compounds, is demonstrated by the lines on the chromatogram indicating groups such as hydrocarbons, alcohols, aldehydes etc. (Schnelle-Kreis et al., 2005). Due to the very fast separations, GCxGC also needs very fast detection systems such as TOF-MS.

A typical DTD with GCxGC-TOF-MS separation / total ion chromatogram (TIC) of *Rosa damascena* is shown in Figure 3. The chromatographic peak data consists of first dimension retention times, second dimension retention times and peak area (TIC). 54 compounds can be identified from this chromatogram. The first dimension separation axis extends to 2900 s and the second dimension axis ends at 6 s. The TIC is created as a reconstructed chromatogram from the peaks detected by an automatic peak detection algorithm. The peak finding routine, based on deconvolution techniques, detected several hundred peaks and a mass spectrometry library search resulted in the majority of them being assigned a library match. (Ozel et al., 2006)

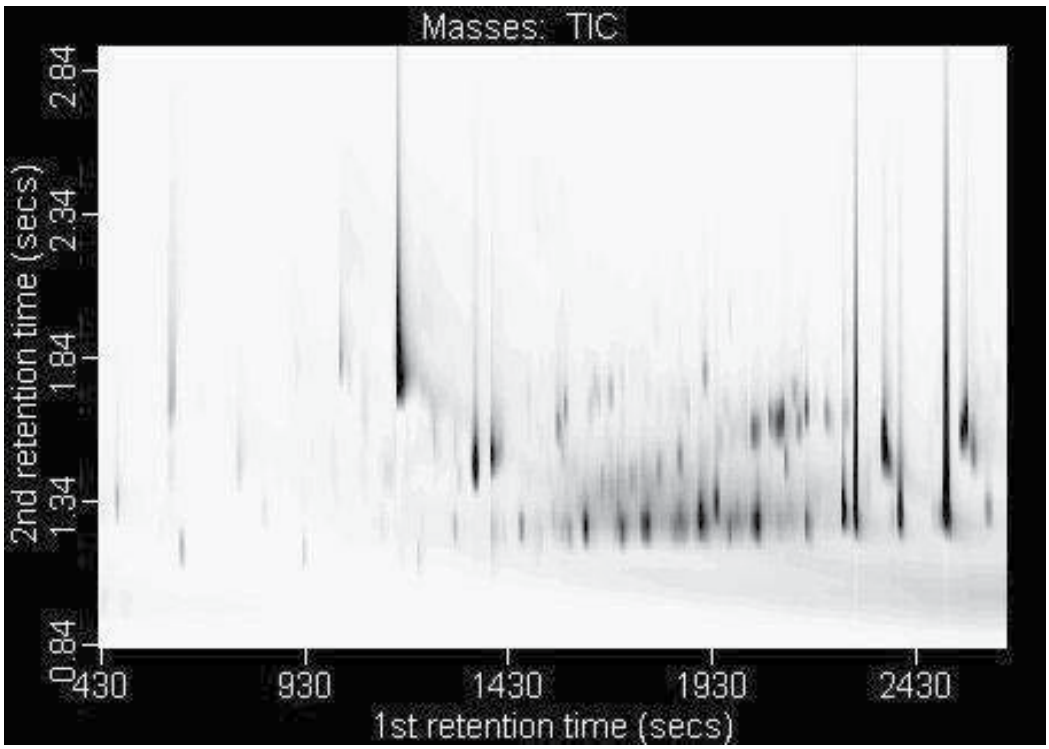


Fig. 3. GCxGC-TOF-MS total ion current (TIC) plot of *Rosa damascena* Mill. volatile18 components at 150°C using the DTD technique (Ozel et al., 2006).

Ozel et al., (2006) studied the volatile profile of *Rosa damascena* using various techniques, namely DTD, SWE and water distillation. DTD volatiles showed a greater total number of different components than either of the other two methods. The numbers of volatile components identified with a percentage higher than 0.05%, were 54, 37, and 34 for the

DTD, SWE and WD techniques respectively. DTD, unlike the other two methods, does not require any sample preparation beforehand, thus making it faster. In the above study, DTD was also shown to be quantitative. Commercial producers of essential oils could therefore use the DTD technique to select which batches of leaves or flowers would produce the highest quality oil, as they would be able to see quickly if their desired components and in what amounts were present. It was concluded that DTD is a promising method for qualitative and quantitative analysis of volatiles which can yield a highly accurate and comprehensive chromatographic profile with a low contamination risk, without the need for costly and time consuming sample preparation techniques.

## 4. Conclusion

The main aim of this chapter is to highlight the extraction of various plants using highly effective, quick, environmentally-friendly superheated water followed by analysis using the high-resolution separation technique of GCxGC-TOF-MS. It has been discussed in many earlier studies that SWE is a very promising alternative to traditional extraction techniques (Simandi et al., 1998; Vokou et al, 1998; Ozel et al., 2003; Ozel et al., 2006; Ozel & Kutlular, 2011). Water / steam distillation and solvent extraction are widely used techniques in the essential oil industry. Solvent extraction is time-consuming and toxic. Steam distillation is cheap although time consuming and with no selectivity. In contrast, SWE is cheap, selective, fast and environmentally friendly. Lab-scale SWE can extract only a small quantity of essential oil during the plant extraction process. In addition, it is necessary to use a solvent during liquid-liquid extraction or SPE before GC analysis. However, realistically, in a big SWE extraction unit solvent will not be needed as the oil will float on the water making collection easy. With increasing interest in avoiding organic solvents in the extraction of volatile organic compounds from plant samples, SWE has been shown to be a feasible alternative for use in industrial essential oil production.

Using the DTD technique, the volatile components of plant materials can be analysed with no extraction process or sample preparation. On an industrial scale, water distillation and occasionally SWE would still be the main methods used in actual extraction of essential oil but DTD could be used to check which components are being missed out in this process very quickly and easily without the need for time consuming, expensive sample preparation. It could also be used to check samples of batches of plant materials from different regions, times of collection or different parts of the plant for their desirable or undesirable components without first having to go through the expensive, time consuming extraction techniques. DTD can also be used quantitatively to determine the amounts in which essential oils are present in samples. Thus DTD could help a producer decide upon which batch to use.

TOF-MS can operate at acquisition frequencies of more than 100 spectra per second. This provides the capability to deconvolute mass chromatograms for compounds with retention times that differ by more than about 120 ms. Combining DTD with GCxGC-TOF-MS enables a rapid study of the characteristic classes of compounds emitted when solid plant materials are treated at different temperatures during preparation. The major advantages of the GCxGC technique are improved peak resolution and enhanced sensitivity over one dimensional GC. The coupling of TOF-MS with GCxGC greatly enhances the information that can be drawn from analysis of complex plant volatile mixtures.

## 5. Acknowledgements

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# 2

# Application of Gas Chromatography in the Analysis of Flavour Compounds in Field Peas

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## 1. Introduction

Flavour compounds influence the taste and quality of foods both of which are very important criteria in food selection and consumer acceptance. Pulse legumes such as field peas are increasingly used in foods such as soup mixes, purees, bakery and other processed products (Heng et al., 2004). In some parts of the world, particularly in Western countries, the presence of off-flavours in peas can be an obstacle to their consumption.

Different chemical compounds such as alcohols, aldehydes, ketones and various heterocyclic compounds play a major role in the flavour of peas. As flavour compounds have different characteristics, changes in their concentrations and profiles can affect the taste and flavour of the finished food product.

Flavour can be analyzed either using sensory methods or with analytical instruments such as gas-chromatography (GC). Separating and analyzing a mixture of volatile compounds in foods without decomposition is an important feature of this latter technique. As most flavour compounds in foods are volatile, simplified GC methods may offer an appropriate technique for the separation and characterisation of volatiles in different food matrices.

In GC, the mobile phase or carrier phase is an inert gas such as helium and the stationary phase is a very thin layer of liquid or polymer on an inert solid support inside a column. The volatile analytes interact with the walls of the column, and are eluted based on the temperature of the column at specific retention times (Grob & Barry, 2004). The eluted compounds are identified with detectors. Flame ionization and mass spectrometry are the most commonly used detectors for flavour analysis (Vas & Vékey, 2004).

Flavour compounds in foods may, however, be at concentrations too low to be accurately detected by GC; concentration of volatiles may, therefore, be required prior to GC operation (Werkhoff et al., 1998; Deibler et al., 1999; Prosen & Zupančič-Kralj, 1999; Zambonin, 2003). Different methods such as purge and trap, static headspace, liquid-liquid, solid phase

extraction, and solid phase microextraction are used for extraction and concentration of volatile compounds. Among various separation and concentration techniques, head space solid phase microextraction (HS-SPME) using a fused-silica fibre combined with gas chromatography-mass spectrometry (GC-MS) has gained increasing attention for the extraction and analysis of volatile, semi-volatile, polar and non-polar compounds in foods such as vegetables, legumes, beverages and dairy products. In comparison with conventional extraction techniques, HS-SPME is a solvent-free, less expensive, fast, and simple technique and involves the adsorption of volatile compounds onto an adsorbent fibre. In fibre-SPME, adsorption is based on the equilibrium partitioning of the analytes between the solid-phase of the SPME fibre, liquid or solid sample matrix. Upon heating, adsorbed analytes are desorbed onto a GC column and analyzed by gas chromatography (Pawliszyn, 1995; Penũalver et al., 1999; King et al., 2003; Vas & Vékey, 2004; Anli et al., 2007).

The flavour profile of legumes, such as peas, is anticipated to become an important quality trait for both traditional and novel food applications. More specifically, knowledge of the flavour profile of peas and the impact of different parameters will be important in selecting the right cultivar as well as storage, handling and processing conditions for different food applications. Unfortunately, data on the impact of different parameters on the flavour profile of peas has been lacking. The main objective of this research, therefore, was to use an optimised HS-SPME-GC-MS technique (Azarnia et al., 2010) to evaluate differences in the flavour profiles of 11 pea cultivars grown in Saskatchewan which is the largest field pea producing province in Canada (AAFC, 2006). Previous work done in our laboratory focused on differences in the flavour properties of different raw pea flours. As pea is cooked before consumption, this work was, therefore, conducted on whole cooked peas.

## 2. Materials and methods

### 2.1 Materials

Chemicals were purchased from Sigma–Aldrich (Oakville, ON, Canada). Selection of pure volatile standards was carried out as previously reported by Azarnia et al., 2010. Carboxen–polydimethylsiloxane, SPME-fibre (CAR/PDMS, 85 μm, Supelco, Oakville, ON, Canada) was used for the GC analysis. Yellow- (CDC Golden, Eclipse, Cutlass, CDC Centennial), green- (Cooper, CDC Striker, CDC 1434-20), marrowfat- (Rambo, MFR042) and dun- (CDC Dundurn, Kaspa) type were evaluated in this study. These field pea cultivars were grown under uniform conditions using recommended agronomic practices for field pea on land managed by the Crop Development Centre, University of Saskatchewan, Canada. These cultivars were selected based on our preliminary results which showed higher differences in the total area of volatile compounds compared to other cultivars. Furthermore, CDC Golden, Eclipse, Cutlass, Cooper and CDC Striker are widely grown in Western Canada. These cultivars were grown in two different locations (i.e. Meath Park, MPK and Wilkie, WIL, near Saskatoon, Saskatchewan, Canada) in crop years of 2008 and 2009.

### 2.2 Methods

#### 2.2.1 Standard preparation

The preparation of standard solutions as well as the evaluation of the reproducibility of the method during each GC run was carried out as described in Azarnia et al., 2010.

### 2.2.2 Solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS) analyses

Volatile compounds in pea cultivars were determined using HS-SPME-GC-MS as described by Azarnia et al., 2010. Briefly, 3 g of each sample were extracted at 50 °C for 30 min using CAR/PDMS fibre. A MPS2 multipurpose sampler (Gerstel Inc., Baltimore, MD) was used for HS-SPME. Analyses were carried out with a Varian CP-3800 gas chromatograph (Palo Alto, CA). Adsorbed volatile compounds were desorbed at 300 °C for 3 min into a split/splitless injector (Glass insert SPME, 0.8 ID; Varian, Mississauga, ON, Canada). Pure helium gas (1 mL/min) was used for the elution of compounds on a VF-5MS capillary column (30 m x 0.25 mm x 0.25 µm, Varian Inc., Mississuaga, ON, Canada). The initial temperature of the GC oven was 35 °C which was held for 3 min, and then increased to 80 °C at a rate of 6 °C per min, and finally to 280 °C at a rate of 20 °C per min, and held for 2 min. The total time of analysis was 22.5 min. A Saturn 2000 MS detector (Varian Inc., Palo Alto, CA) was used for detection of compounds, and the mass range was 30–400 m/z. The total ion current was obtained using an electron impact ionization source at 70 eV at a scan rate of 1 s/scan. Calibration and tuning of the equipment were carried out as recommended by the manufacturer. Identification of volatile compounds were carried out either using National Institute of Standards and Technology (NIST) database (V. 05) through mass spectra library search or by comparing mass spectra and retention times of the compounds with those of the pure commercial volatile standards. After determination of the area count of each volatile compound from the average of two replicate assessments, a semi-quantitative comparison was carried out by calculation of the relative peak area, RPA, of each volatile compound. Results were expressed as percentage of total volatile compounds.

### 2.2.3 Preparation of cooked-whole seeds

Seeds were soaked in water (ratio of 1:2, seeds:water) and kept at room temperature (~ 22°C) for 24 h. After draining, the seeds were cooked in boiling water (ratio of 1:2; seeds:water) for 20 min. 3 g of the cooked-whole seeds were weighed into 10 mL headspace amber vials (Supelco, Oakville, ON, Canada) and then mashed twice inside the vial by using a spatula.

### 2.2.4 Statistical analysis

Each experiment was carried out in two replicates. Peak area count of each volatile compound was obtained for each replicate. Analysis of variance (ANOVA) using a general linear model (GLM) procedure of the Statistical Analysis System (SAS, 2004, Cary, USA) was performed to evaluate differences between parameters. The parameters evaluated were type, cultivar, location, crop year, and interactions between them. Means comparison between parameters was carried out by Duncan's multiple range test using SAS software.

## 3. Results and discussion

### 3.1 Effect of type, cultivar, and location on Total Volatile Compounds (TVC) and chemical families

The impact of type, cultivar, and location on TVC and different chemical families (i.e. alcohols, aldehydes, ketones, esters, sulfur compounds, hydrocarbons) in field pea cultivars

was evaluated and results are, respectively, presented in Figures 1-7. The data were subjected to ANOVA and Duncan's multiple range test and were separately reported for each crop year (Tables 1-4). Furthermore, the effect of crop year on the flavour profile of pea cultivars was studied and statistical results are presented in Table 5.

### 3.1.1 Effect of type, cultivar and location on TVC

Changes in the value of TVC in different field pea cultivars grown in the year of 2008 and 2009 are shown in Fig. 1. ANOVA results showed that TVC in peas grown in different crop years was significantly ($P < 0.01$) affected by the pea type and cultivar (Tables 1 & 3). Based on Duncan's test (Table 1), in the year of 2008, peas grown in MPK had higher TVC compared to those grown in WIL. Rambo from marrowfat type had the highest mean value of TVC, whereas CDC Striker from green-type had the lowest value of TVC. The highest mean value of TVC was observed in the field peas from marrowfat-type, whereas peas from green-type had the lowest value of TVC (Table 1). In the year of 2009, no significant ($P > 0.05$) differences were found between the cultivars grown in different locations (Table 3). Rambo and Kaspa, respectively, had the highest and the lowest mean value of TVC. Amongst the different pea types, marrowfat-type had the highest value of TVC, whereas dun-type had the lowest value of TVC (Table 3).

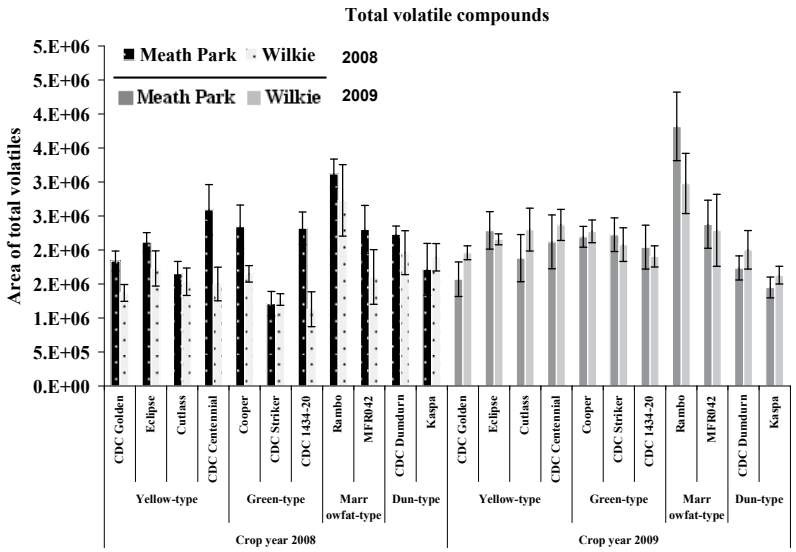


Fig. 1. Changes in total volatile compounds content in cooked field peas as affected by type, cultivar, location and crop year. Results are from a two replicate analysis and expressed as mean ± standard deviation.

### 3.1.2 Effect of type, cultivar and location on different chemical families

#### 3.1.2.1 Alcohols

Changes in the alcoholic compounds in pea cultivars are shown in Fig. 2. In the year of 2008, the mean value of alcohols were significantly ($P < 0.01$) affected by the type, cultivar and

location (Table 2). Pea cultivars grown in WIL location had higher mean value of alcohols than those grown in MPK location. 3-Methyl-1-butanol and 1-hexanol had, respectively, the highest and the lowest mean values (Table 2). In the year of 2009, the mean value of alcohols was significantly affected by the type and cultivar, whereas no significant differences were found between the cultivars grown in different locations (Table 3). 1-Propanol and 2-ethyl-1-hexanol had the highest mean values and 1-hexanol had the lowest mean value (Table 4).

Alcohols in peas are mostly formed from enzymatic oxidation of lipids. Physical damage, storage and processing of seeds could lead to the formation of alcohols (Eriksson, 1967; de Lumen et al., 1978; Oomah & Liang, 2007). Volatile alcoholic compounds have distinct characteristics and they could therefore affect the taste and flavour of peas. For example, 1-propanol has an alcoholic odour and a fruity flavour; 2-methyl-1-propanol has a wine odour, 3-methyl-1-butanol has a fruity, banana, sweet odour with a bittersweet taste; 1-hexanol has an herbaceous, mild, sweet, green fruity odour and an aromatic flavour; 1-heptanol has an aromatic and fatty odour and a spicy taste, whereas 1-octanol has a fresh, orange-rose odour and an oily, sweet taste (Burdock, 2002).

| [1]ANOVA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Main effects** | | | | **Interactions** | | | | | | | |
| [2]cv (+++) | [3]l (+++) | [4]t (+++) | [5]r (++) | cv*l (+++) | l*t (NS) | cv*r (NS) | t*r (NS) | l*r (NS) | | | |
| **Duncan grouping** | | | | | | | | | | | |
| **Cultivar** | Rambo (a) | CDC Dundurn (b) | CDC Centen-nial (bc) | Cooper (bcd) | MFR042 (bcd) | Eclipse (bcd) | Kaspa (cde) | CDC 1434-20 (de) | CDC Golden (e) | Cutlass (e) | CDC Striker (f) |
| **Location** | Meath Park (a) | Wilki (b) | | | | | | | | | |
| **Type** | Marro wfat (a) | Dun (b) | Yellow (bc) | Green (c) | | | | | | | |

[1]ANOVA performed using general linear model. +++=$P$<0.01, NS= Not significant ($P$>0.05).
[2]cv=Cultivar, [3]l=Location, [4]t=Type, [5]r=Replicate. Items with different letters within a row are significantly different at $P$<0.05 (a>b>c>d>e>f).

Table 1. ANOVA results and Duncan's multiple range test for total volatile compounds in field pea cultivars grown in 2008

#### 3.1.2.2 Aldehydes

Relative peak area of aldehydes in pea cultivars grown in different locations and crop years is presented in Fig. 3. The mean value of aldehydes was significantly ($P < 0.01$) affected by the type of cultivar. However, no significant ($P > 0.05$) differences in aldehydes were observed between cultivars grown in different locations (Tables 2 & 4). 3-Methyl butanal was the most abundant aldehyde in all the pea cultivars studied (Tables 2 & 4).

Enzymatic or autoxidative decomposition of unsaturated fatty acids, mainly linoleic and linolenic acids could lead to the formation of aldehydes in peas (Hornostaj & Robinson, 2000; Barra et al., 2007). Differences observed in the concentration of these carbonyl compounds could be due to differences in linoleate compositions in pea cultivars (Oomah &

| Chemical family | [1]ANOVA | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Main effects | | | | Interactions | | | | |
| | [2]cv | [3]l | [4]t | [5]r | cv*l | l*t | cv*r | t*r | l*r |
| Alcohols | +++ | +++ | +++ | NS | +++ | NS | ++ | NS | NS |
| Aldehydes | +++ | NS | +++ | NS | +++ | NS | NS | NS | NS |
| Ketones | +++ | +++ | ++ | NS | +++ | NS | ++ | NS | NS |
| Esters | +++ | NS | +++ | NS | +++ | +++ | +++ | NS | NS |
| Sulfur compounds | +++ | ++ | +++ | NS | +++ | NS | ++ | NS | NS |
| Hydro-carbons | +++ | +++ | +++ | NS | +++ | +++ | NS | NS | NS |
| Pyrazines | +++ | +++ | +++ | NS | +++ | NS | NS | NS | NS |

**Duncan grouping for each chemical family in peas belonging to different pea- types and grown in different location**

| | Pea-type | | | | Location | |
|---|---|---|---|---|---|---|
| Alcohols | Marrowfat (a) | Dun (b) | Yellow (bc) | Green (c) | Wilkie (a) | Meath Park (b) |
| Aldehydes | Green (a) | Dun (b) | Yellow (b) | Marrowfat (b) | Meath Park (a) | Wilkie (a) |
| Ketones | Dun (a) | Green (ab) | Yellow (ab) | Marrowfat (b) | Meath Park (a) | Wilkie (b) |
| Esters | Green (a) | Yellow (b) | Dun (b) | Marrowfat (c) | Meath Park (a) | Wilkie (a) |
| Sulfur compounds | Dun (a) | Yellow (b) | Green (b) | Marrowfat (c) | Wilkie (a) | Meath Park (b) |
| Hydro-carbons | Green (a) | Dun (b) | Yellow (b) | Marrowfat (b) | Meath Park (a) | Wilkie (b) |
| Pyrazines | Dun (a) | Yellow (b) | Green (b) | Marrowfat (c) | Wilkie (a) | Meath Park (b) |

**Duncan grouping for individual flavor compounds in peas belonging to each chemical family**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Alcohols | 3-Methyl-1-butanol (a) | 2-Ethyl-1-hexanol (b) | 2-Methyl-1-propanol (c) | 1-Propanol (c) | 1-Octanol (dc) | 1-Heptanol (d) | 1-Hexanol (e) |
| Aldehydes | 3-Methyl-butanal, (a) | Hexanal (b) | 2-Methyl-butanal, (c) | | | | |
| Ketones | 2-Butanone (a) | 2-Pentanone (b) | | | | | |
| Esters | Ethyl acetate (a) | Hexanoic acid, methyl ester (b) | | | | | |

Table 2. (Continued)

| | | | | |
|---|---|---|---|---|
| Sulfur compounds | Dimethyl sulfide (a) | Methanethiol (b) | Dimethyl disulfide (c) | 2-Acethylthiazole (d) |
| Hydro-carbons | Trichloro-methane (a) | Furan,2-ethyl (b) | Toluene (c) | |
| Pyrazines | 2,3-Diethyl-5-methyl pyrazine | | | |

[1]ANOVA performed using general linear model. +++=P<0.01, ++=P<0.05, NS= Not significant (P>0.05).
[2] cv=Cultivar, [3]l=Location, [4]t=Type, [5]r=Replicate. Items with different letters within a row are significantly different at P<0.05 (a>b>c>d>e).

Table 2. ANOVA results and Duncan's multiple range test for chemical families in cooked pea cultivars grown in the year of 2008

Liang, 2007). Hexanal and pentanal are commonly identified in fruits and vegetables (Oomah & Liang, 2007). Propanal and hexanal, have been reported to be responsible for off-flavour in stored unblanched frozen peas (Barra et al., 2007). Timely harvesting of peas may prevent the formation of undesirable flavours derived from enzymatic reactions (Hornostaj & Robinson, 2000). Aldehyde compounds are known to contribute to the flavour and aroma of various plants and plant foods (Hornostaj & Robinson, 2000). Hexanal, as an example has a fatty, green, grassy, fruity odour and taste; 3-methyl butanal has a choking, acrid, fruity, fatty, almond odour; 2-methyl butanal has a choking odour and a coffee or chocolate flavour and taste, whereas benzaldehyde has a bitter almond taste (Burdock, 2002).

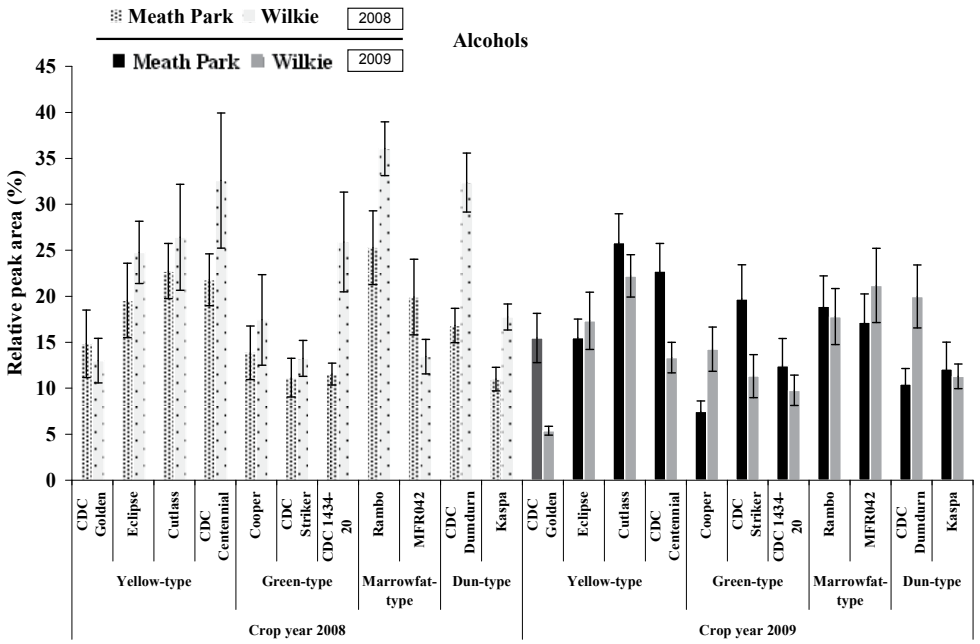


Fig. 2. Changes in total alcohol content in cooked field peas as affected by type, cultivar, location and crop year. Results are from a two replicate analysis and expressed as mean ± standard deviation. Relative peak area (%) = Peak area of total alcohols/ Total peak area of volatile compounds x 100.

| [1]ANOVA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Main effects** | | | | **Interactions** | | | | | | | |
| [2]cv (+++) | [3]l (NS) | [4]t (+++) | [5]r (++) | cv*l (+++) | l*t (+++) | cv*r (+++) | t*r (NS) | l*r (++) | | | |
| **Duncan grouping** | | | | | | | | | | | |
| **Cultivar** | Rambo (a) | MFR042 (b) | CDC Centen-nial (bc) | Cooper (bc) | Eclipse (bc) | CDC Striker (bcd) | Cutlass (bcd) | CDC 1434-20 (cde) | CDC Dun-durn (de) | CDC Golden (ef) | Kaspa (f) |
| **Location** | Meath Park (a) | Wilki (a) | | | | | | | | | |
| **Type** | Marrow fat (a) | Green (b) | Yellow (b) | Dun (c) | | | | | | | |

[1]ANOVA performed using general linear model. . +++=P<0.01, NS= Not significant (P>0.05).
[2]cv=Cultivar, [3]l=Location, [4]t=Type, [5]r=Replicate. Items with different letters within a row are significantly different at *P*<0.05 (a>b>c>d>e>f).

Table 3. ANOVA results and Duncan's multiple range test for total volatile compounds in field pea cultivars grown in the year of 2009

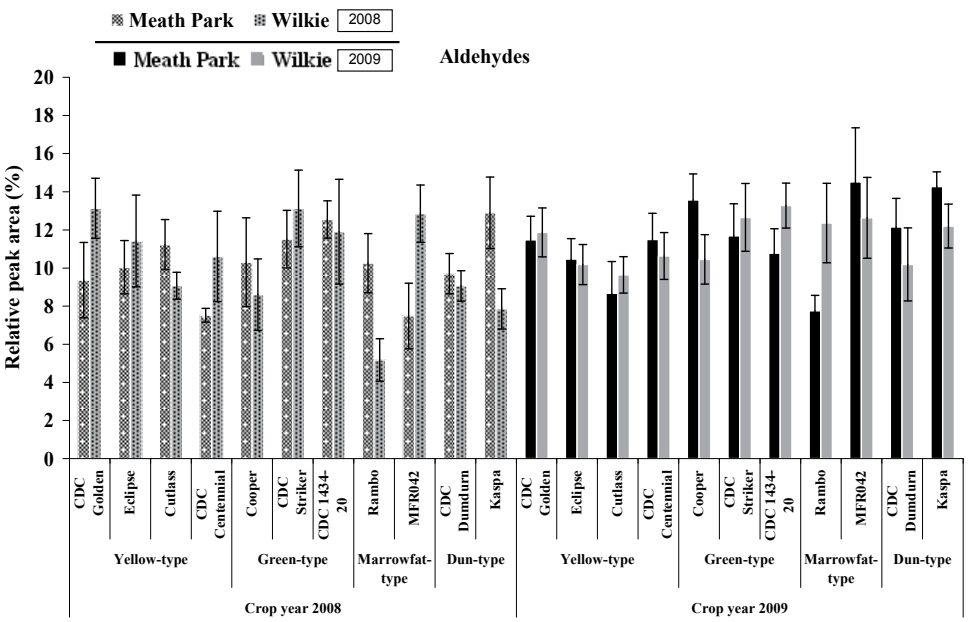


Fig. 3. Changes in total aldehyde content in cooked field peas as affected by type, cultivar, location and crop year. Results are from a two replicate analysis and expressed as mean ± standard deviation. Relative peak area (%) = Peak area of total aldehydes/ Total peak area of volatile compounds x 100.

### 3.1.2.3 Ketones

Fig. 4 shows relative peak areas of ketones in the different pea cultivars studied. A significant difference ($P < 0.01$) in the mean value of ketones was observed between pea cultivars from different locations (Tables 2 & 4). Pea cultivar grown in MPK had higher mean value of ketones compared to those from WIL (Table 2). In the 2009 crop year, pea cultivar grown in WIL had higher mean value of ketones than those from MPK (Table 4). 2-Butanone had higher mean value compared to 2-pentanone in all the pea cultivars studied (Tables 2 & 4).

Ketones are products derived from lipid oxidation. They have different characteristics which could affect the flavour of peas. 2-Pentanone, and 2-butanone have been described as having a wine or acetone odour, and a sweet apricot odour, respectively (Burdock, 2002).

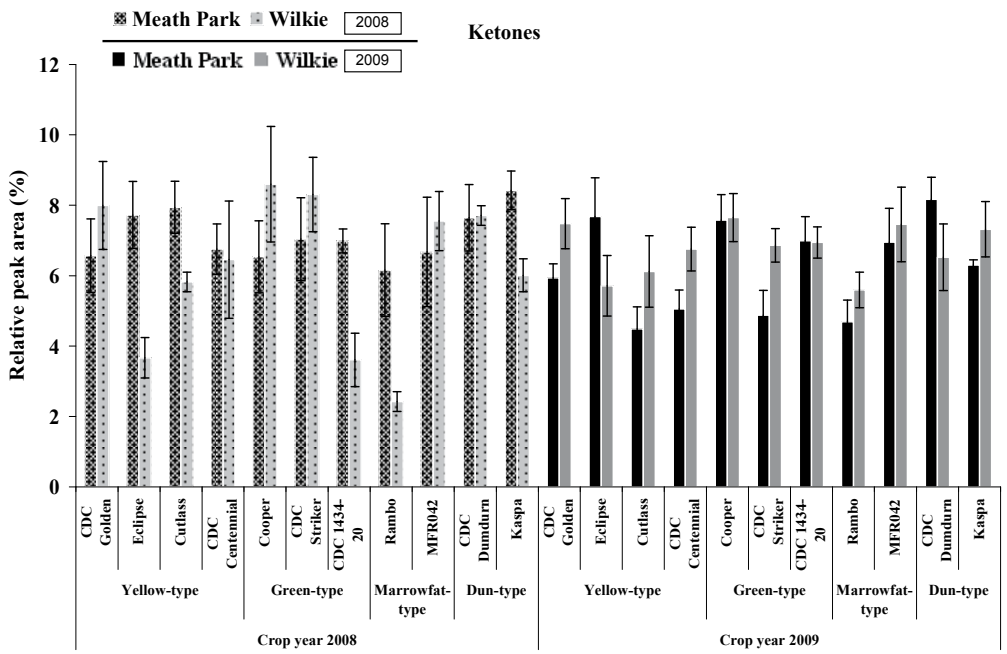


Fig. 4. Changes in total ketone content in cooked field peas as affected by type, cultivar, location and crop year. Results are from a two replicate analysis and expressed as mean ± standard deviation. Relative peak area (%) = Peak area of total ketones/ Total peak area of volatile compounds x 100.

### 3.1.2.4 Esters

The relative peak area of esters found in the pea cultivars is shown in Fig. 5. No differences ($P > 0.05$) were found between the cultivars grown in different locations (Tables 2 & 4). Ethyl acetate was the most abundant ester in all the pea cultivars studied (Tables 2 & 4). This compound has an ether and brandy odour and a fruity, sweet taste and has also been reported in soybeans and beans (Burdock, 2002; del Rosario et al., 1984). Hexanoic acid, methyl ester also identified in the peas reportedly has an ether and pineapple odour (Burdock, 2002).

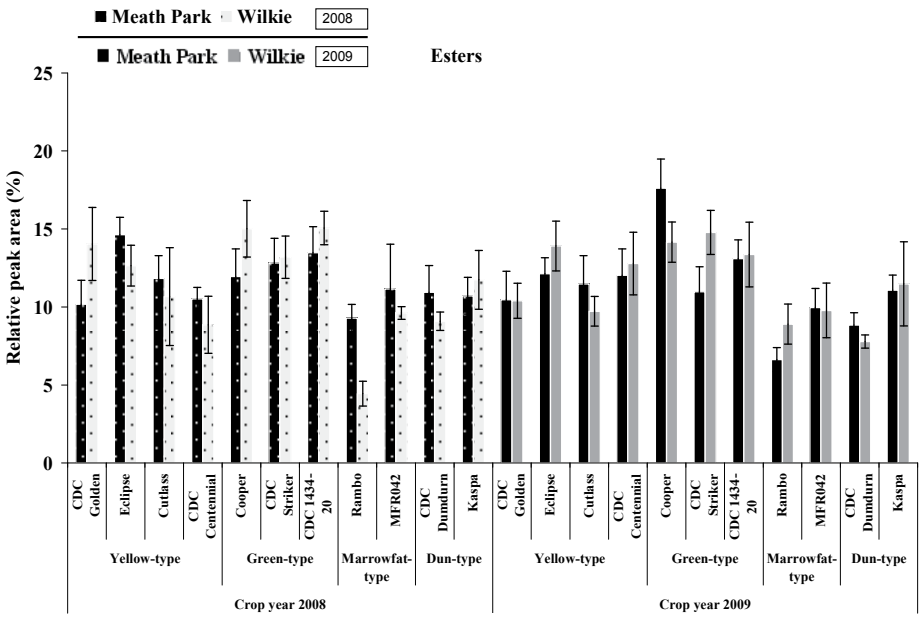


Fig. 5. Changes in total ester content in cooked field peas as affected by type, cultivar, location and crop year. Results are from a two replicate analysis and expressed as mean ± standard deviation. Relative peak area (%) = Peak area of total esters/ Total peak area of volatile compounds x 100.

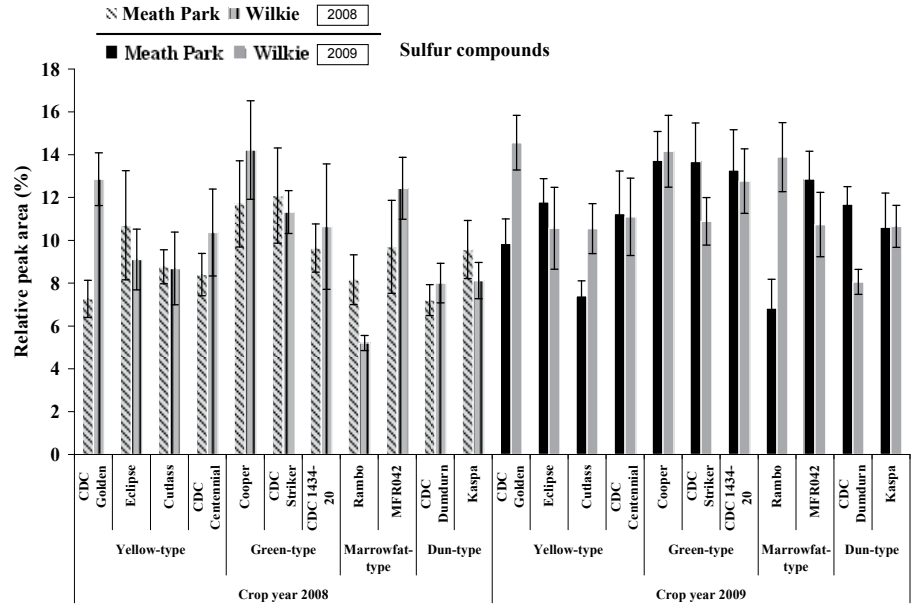


Fig. 6. Changes in total sulfur compounds content in cooked field peas as affected by type, cultivar, location and crop year. Results are from a two replicate analysis and expressed as mean ± standard deviation. Relative peak area (%) = Peak area of total sulfur compounds/ Total peak area of volatile compounds x 100.

### 3.1.2.5 Sulfur compounds

Differences in sulphur compounds found in the pea cultivars are presented in Fig. 6. Significant differences ($P < 0.01$) were found between the pea cultivars. In both years, pea cultivars grown in WIL had higher mean value of sulfur containing volatile compounds than those grown in MPK (Tables 2 & 4). Dimethyl sulfide was the most abundant sulfur compound in the peas studied (Tables 2 & 4).

Volatile sulphur compounds are natural compounds in foods and could be formed during heat processing and storage (Maga et al., 1973). Formation of these compounds has been reported in blanched peas (Jakobsen et al., 1998). Sulphur compounds contribute to the overall flavour and aroma of foods (Jakobsen et al., 1998). For example, dimethyl disulfide, one of the major sulphur containing compounds identified, has a diffuse, intense onion odour. Dimethyl sulfide, on the other hand, has an intense, cabbage odour (Burdock, 2002).

### 3.1.2.6 Hydrocarbons

The relative peak area of hydrocarbons found in the pea cultivars is presented in Fig. 7. In the 2008 and 2009 crops, significant ($P < 0.01$) differences in the mean value of hydrocarbons were observed between the peas grown in different locations. In both years, peas grown in MPK had higher hydrocarbons compared to the ones from WIL (Tables 2 & 4). The most abundant hydrocarbon was trichloromethane, followed by furan,2-methyl and toluene (Tables 2 & 4).

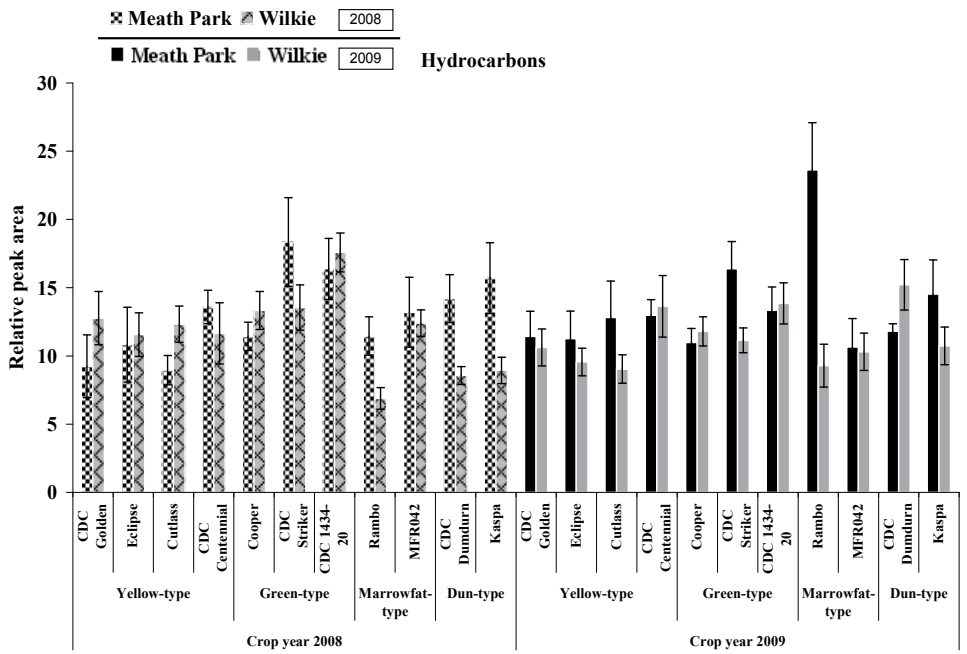


Fig. 7. Changes in total hydrocarbons content in different cooked field peas as affected by type, cultivar, location and crop year. Results are from a two replicate analysis and expressed as mean ± standard deviation. Relative peak area (%) = Peak area of total hydrocarbons/ Total peak area of volatile compounds x 100.

| Chemical family | [1]ANOVA | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Main effects | | | | Interactions | | | | |
| | [2]cv | [3]l | [4]t | [5]r | cv*l | l*t | cv*r | t*r | l*r |
| Alcohols | +++ | NS | +++ | NS | +++ | NS | NS | NS | NS |
| Aldehydes | +++ | NS | +++ | NS | +++ | ++ | NS | NS | NS |
| Ketones | +++ | ++ | +++ | NS | +++ | NS | NS | NS | NS |
| Esters | +++ | NS | +++ | NS | +++ | NS | NS | NS | NS |
| Sulfur compounds | +++ | +++ | +++ | ++ | +++ | +++ | +++ | NS | NS |
| Hydrocarbons | +++ | +++ | ++ | +++ | +++ | NS | NS | NS | NS |
| Pyrazines | +++ | +++ | +++ | ++ | +++ | NS | ++ | NS | NS |

**Duncan grouping for each chemical family in peas belonging to different pea- types and grown in different location**

| | Pea-type | | | | Location | |
|---|---|---|---|---|---|---|
| Alcohols | Marrowfat (a) | Yellow (ab) | Dun (bc) | Green (c) | Wilkie (a) | Meath Park (a) |
| Aldehydes | Dun (a) | Green (a) | Marrowfat (b) | Yellow (c) | Wilkie (a) | Meath Park (a) |
| Ketones | Dun (a) | Green (ab) | Yellow (bc) | Marrowfat (c) | Wilkie (a) | Meath Park (b) |
| Esters | Green (a) | Yellow (b) | Dun (c) | Marrowfat (c) | Wilkie (a) | Meath Park (a) |
| Sulfur compounds | Green (a) | Marrowfat (b) | Dun (b) | Yellow (b) | Wilkie (a) | Meath Park (b) |
| Hydrocarbons | Green (a) | Dun (ab) | Marrowfat (ab) | Yellow (b) | Meath Park (a) | Wilkie (b) |
| Pyrazines | Dun (a) | Yellow (ab) | Green (b) | Marrowfat (c) | Meath Park (a) | Wilkie (b) |

**Duncan grouping for individual flavor compounds in peas belonging to each chemical family**

| | | | | | |
|---|---|---|---|---|---|
| Alcohols | 1-Propanol (a) | 2-Ethyl-1-hexanol (a) | 1-Octanol (b) | 3-Methyl-1-butanol (c) | 1-Hexanol (d) |
| Aldehydes | 3-Methyl-butanal (a) | Hexanal (b) | Benzal-dehyde (c) | 2-Methyl-butanal (d) | |

Table 4. (Continued)

| | | | | | |
|---|---|---|---|---|---|
| Ketones | 2-Butanone (a) | 2-Pentanone (b) | | | |
| Esters | Ethyl acetate (a) | 3-Methyl-1-butanol- acetate (b) | | | |
| Sulfur compounds | Dimethyl sulfide (a) | Methan-ethiol (b) | 2-Acethyl-thiazole (c) | Dimethyl trisulfide (d) | Dimethyl disulfide (e) |
| Hydro-carbons | Trichloro-methane (a) | Furan,2-ethyl (b) | Toluene (c) | Undecane (c) | |
| Pyrazines | 2,3-Diethyl-5-methyl pyrazine | | | | |

[1]ANOVA performed using general linear model. +++=$P$<0.01, ++=$P$<0.05, NS= Not significant ($P$>0.05). [2]cv=Cultivar, [3]l=Location, [4]t=Type, [5]r=Replicate. Items with different letters within a row are significantly different at $P$<0.05 (a>b>c>d>e).

Table 4. ANOVA results and Duncan's multiple range test for chemical families in cooked pea cultivars grown in the year of 2009

In general, hydrocarbons are derived from oxidation of unsaturated fatty acids in foods (Märk et al., 2006; Oomah & Liang, 2007). Trichloromethane (chloroform), produced on exposure to chlorinated organic compounds, is a natural compound in plants (Lovegren et al., 1979). Volatile alkanes reportedly contribute to the desirable odour or flavour characteristics of green beans and peas (Perkins, 1988).

#### 3.1.2.7 Pyrazines

2,3-Diethyl-5-methyl pyrazine was the only pyrazine identified in the pea cultivars studied. Significant ($P < 0.01$) differences were observed between pea cultivars grown in different locations (Tables 2 & 4). CDC Golden and Rambo had, respectively, the highest and the lowest mean value of this compound (Tables 2 & 4). In 2008, peas grown in WIL had higher values of this compound compared to those grown in MPK (Table 2). In the 2009 crop, peas from MPK had higher values of this compound than those from WIL (Table 4).

Pyrazines have low vapour pressure and an intense smell and contribute to desirable flavours and aroma of fresh vegetables (Müller & Rappert, 2010). 2,3-Diethyl-5-methyl pyrazine has a nutty, meaty, roasted hazelnut odour (Burdock, 2002).

### 3.2 Effect of the crop year on the flavour profile of field pea cultivars

ANOVA analysis was carried out on the data pooled from the two crop years to evaluate the impact of this parameter on the flavour profile of pea. Results showed that TVC in pea was significantly ($P < 0.01$) affected by crop year (Table 5). Cultivars grown in the year 2009 had higher TVC than those from the 2008 year (Table 5). There were significant differences in alcohols, aldehydes, sulfur compounds and pyrazine between the cultivars grown in different years. No significant differences in ketones, hydrocarbons and esters were found between the crops grown in different years (Table 5). In general, higher values of alcohols, sulfur compounds and pyrazine were observed in the peas from 2008, whereas crops from 2009 had higher values of aldehydes (Table 5).

| [1]ANOVA | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Main effects** | | | | | | **Interactions** | | | | |
| | [2]cv | [3]l | [4]t | [5]cy | [6]r | cv*l | cv*cy | cy*l | t*l | t*cy |
| Total volatiles | +++ | +++ | +++ | +++ | NS | +++ | +++ | +++ | +++ | +++ |
| Alcohols | +++ | +++ | +++ | +++ | NS | +++ | +++ | +++ | ++ | NS |
| Aldehydes | +++ | NS | +++ | +++ | NS | +++ | +++ | NS | +++ | NS |
| Ketones | +++ | +++ | +++ | NS | NS | +++ | ++ | +++ | NS | NS |
| Esters | +++ | NS | +++ | NS | NS | NS | NS | NS | NS | NS |
| Sulfur compounds | +++ | NS | +++ | ++ | NS | +++ | +++ | +++ | NS | NS |
| Hydrocarbons | +++ | +++ | +++ | NS | NS | +++ | +++ | NS | +++ | +++ |
| Pyrazines | +++ | NS | +++ | ++ | NS | +++ | +++ | +++ | NS | NS |

| **Duncan grouping** | | |
|---|---|---|
| Compound | Crop year | |
| Alcohols | 2008 (a) | 2009 (b) |
| Aldehydes | 2009 (a) | 2008 (b) |
| Ketones | 2008 (a) | 2009 (a) |
| Esters | 2009 (a) | 2008 (a) |
| Sulfur compounds | 2008 (a) | 2009 (b) |
| Hydrocarbons | 2008 (a) | 2009 (a) |
| Pyrazines | 2008 (a) | 2009 (b) |
| Total volatiles | 2009 (a) | 2008 (b) |

[1]ANOVA performed using general linear model. +++=$P$<0.01, ++= $P$<0.05, NS= Not significant ($P$>0.05). [2]cv=Cultivar, [3]l=Location, [4]t=Type, [5]cy=Crop year, [6]r=Replicate. Compounds belonging to each chemical family with different letters within a row are significantly different at $P$<0.05 (a>b).

Table 5. ANOVA and Duncan's multiple range test results for total volatile compounds and chemical families in peas grown in two different crop years

## 4. Conclusion

Our results showed that the flavour profile of peas was affected by market class, cultivar location, and crop year. The highest total volatile compound (TVC) was observed in cultivars from marrowfat-market class. Crops grown in Meath Park location had the highest TVC. Furthermore, different volatile compounds were identified in pea cultivars. In both crop years, cultivars from the green-market class had the highest mean values of esters and hydrocarbons, whereas the highest value of alcohols was observed for the marrowfat-market class, and the dun-market class had the highest mean values of ketones and pyrazine. 3-Methyl-butanol, 1-propanol, 2-ethyl-hexanol, 3-methyl-butanal, trichloromethane, 2-butanone, dimethyl sulfide, ethyl acetate and 2,3-diethyl-5-methyl pyrazine were the most abundant volatile compounds observed in the pea cultivars.

## 5. Acknowledgment

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3

# Chemotaxonomic Study Based on the Variation of Quinone Compounds in the Heartwood of Javanese Teak Using GC-MS

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## 1. Introduction

Teak that often appears in Java is one of the species most associated with the Javanese since ancient times. Teak is generally classified as a fancy wood and has also been used as a general purpose of timber. This tree grows throughout the island of Java beginning in easternmost Java (Banyuwangi, East Java Province) and ending in Sukabumi, West Java Province to the west.

Past research has confirmed that many quinones are present in teak heartwood (Sandermann and Simatupang 1966, Windeisen et al. 2003, Lukmandaru and Takahashi 2009). It is also proved that the quinone and its derivatives exhibit antitermitic activities (Sandermann and Simatupang 1966; Rudman and Gay, 1961). It is reported that the composition of these quinones vary with tree age (Lukmandaru and Takahashi 2009; Lukmandaru 2009), site and tree type (Sandermann and Simatupang 1966; Windeisen et al. 2003). This study investigated the variations in quinone constituents of teak (tectoquinone, deoxylapachol, isodeoxylapachol, lapachol, and tectol) and chemotaxonomic study based on the quinones and other related components.

## 2. Materials and method

### 2.1 Sample material

The tree samples were collected from three provinces, West Java (Purwakarta region, 32 trees), Jogjakarta (Gunungkidul region, 13 trees) and Central Java (Randublatung region, 42 trees). Those three regions (Fig. 1) have been known to produce teak trees annually in considerable amount. The condition of the sites and tree characteristics are described in Table 1. The wood from the base part from two opposite radii of the outer heartwood were converted into wood powder (40-60 mesh) and were then combined to form a single sample for further analysis.


Fig. 1. Map of the island of Java, Indonesia. Teak tree samples were taken from three regions: Purwakarta, Randublatung and Gunungkidul.

| Factor | Purwakarta | Randublatung | Gunungkidul |
|---|---|---|---|
| Origin/type | Plantation forest | Plantation forest | Community forest |
| Province | West Java | Central Java | Jogjakarta |
| Altitude (m) | 110 | 140 | 270 |
| Soil type | Latosol, loamy sand | Humous margalitic, loamy sand | Black calcareous, loam |
| Annual rainfall range (mm) | 1200 - 1800 | 1300 - 2000 | 1400 - 1800 |
| Temperature range ($^0$C) | 22 - 28 | 20 – 34 | 22 – 36 |
| Relative humidity range (%) | 76 - 80 | 70 - 74 | 68 - 72 |
| Number of samples | 32 | 42 | 13 |
| Diameter breast height range (cm) | 39 - 137 | 23 - 83 | 25 - 38 |

Table 1. Description of the sampling and sites.

### 2.2 Analysis of extractive components

Wood powder (about 2 g) were extracted using a soxhlet apparatus with ethanol-benzene (1:2,v/v) for 8 hours. After evaporation of the solvent, the extracts (concentration of 100 mg/mL) were analyzed using GC (Hitachi Model G-3 500), NB-1 bonded capillary 30m. Operation temperature was 120−300$^o$C with a heating rate of 4$^o$C /min and held at 300$^o$C for 15 min. Injector and detector temperatures were set at 250$^o$C. Helium was used as the carrier gas, the split ratio was 80:1, and the injected volume was 1.0 µL.

The identification of components was based on a comparison between authentic components, references (Windeisen et al. 2003; Lemos et al. 1999; Perry et al. 1991), and GC–MS analysis results. The following authentic components were used: tectoquinone (25753-31 Kanto Chemical), lapachol (142905 Sigma-Aldrich), 2-hydroxymethyl anthraquinone (17241-59-7 Acros Organics), squalene (37309-30 Kanto Chemical), and palmitic acid (32016-30 Kanto Chemical). Mass spectrometry measurements were obtained from GC-MS analysis on a Shimadzu QP-5000 with operation conditions being similar to GC analysis. The MS operating parameters were temperature ionization voltage of 70 eV, transfer line temperature at 250$^o$C, and scan range of 50–500 atomic mass unit.

### 2.3 Statistical analysis

SPSS 10.0 version with Windows was used for principal component analysis and discriminant analysis.

## 3. Results and discussion

Fig. 2 shows the gas chromatogram of the ethanol-benzene extracts in the outer heartwood. Nine constituents, namely, deoxylapachol, palmitic acid, lapachol, isodeoxylapachol, tectoquinone, unknown compound 1 (UN1), unknown compound 2 (UN2), squalene, and tectol were detected. The peaks 1 and 4 were provisionally assigned as deoxylapachol and its isomer (Perry et al., 1991;Windeisen et al., 2003) due to the almost identical mass spectra of the two compounds (Lukmandaru and Takahashi, 2009). The molecular masses of unidentified compound 1 (UN1) and 2 (UN2) were found to be m/e (base peak) = 244 and 242, respectively. On the basis of their chemical structures, the quinones were from naphtaquinone (lapachol, desoxylapachol and its isomer), anthraquinone (tectoquinone), as well as naphtaquinone dimer (tectol). Table 2 summarizes the composition of components determined by capillary gas chromatography and relative contents of the constituents for 87 typical individuals of teak from 3 habitats. The contents of these nine constituents showed wide variations among individuals and habitats. Triterpene squalene was generally the most abundant component in ethanol-benzene soluble extracts (varied from 6 - 65 %) while the quinone fraction ranged from 15 to 64%.

| Components | Purwakarta (*n*=32) | | | Randublatung (*n*=42) | | | Gunungkidul (*n*=13) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Min. | Max | Average (St. dev.) | Min. | Max. | Average (St. dev.) | Min. | Max. | Average (St. dev.) |
| Deoxylapachol | 0 | 26.79 | 6.65 (7.95) | 0 | 17.09 | 3.44 (3.97) | 0 | 23.05 | 2.90 (6.60) |
| Palmitic acid | 0.49 | 3.34 | 1.34 (0.60) | 0 | 10.45 | 2.03 (1.67) | 0 | 13.98 | 3.35 (3.41) |
| Lapachol | 0 | 12.92 | 4.25 (4.06) | 0 | 17.42 | 3.37 (4.10) | 0 | 14.03 | 4.39 (3.87) |
| Isodeoxylapachol | 0 | 13.44 | 5.17 (3.08) | 2.15 | 13.78 | 5.71 (2.37) | 0 | 25.94 | 6.05 (6.66) |
| Tectoquinone | 4.07 | 51.71 | 14.61 (10.46) | 2.16 | 21.18 | 8.05 (5.28) | 3.17 | 23.92 | 11.31 (6.24) |
| UN1 | 0 | 4.00 | 0.48 (0.88) | 0 | 11.24 | 1.75 (2.63) | 0 | 7.28 | 1.81 (1.96) |
| UN2 | 0 | 9.80 | 1.90 (2.20) | 0 | 3.82 | 1.10 (0.80) | 0 | 11.32 | 2.16 (3.02) |
| Squalene | 7.59 | 62.60 | 41.89 (12.03) | 12.45 | 65.23 | 42.75 (14.66) | 6.86 | 54.02 | 26.29 (15.10) |
| Tectol | 2.25 | 28.91 | 10.71 (5.92) | 3.34 | 17.99 | 9.71 (2.86) | 0 | 20.34 | 6.75 (6.22) |

Note : UN : unknown compound,

Table 2. The relative contents of ethanol-benzene soluble components in the outer heartwood of Teak in three habitats.

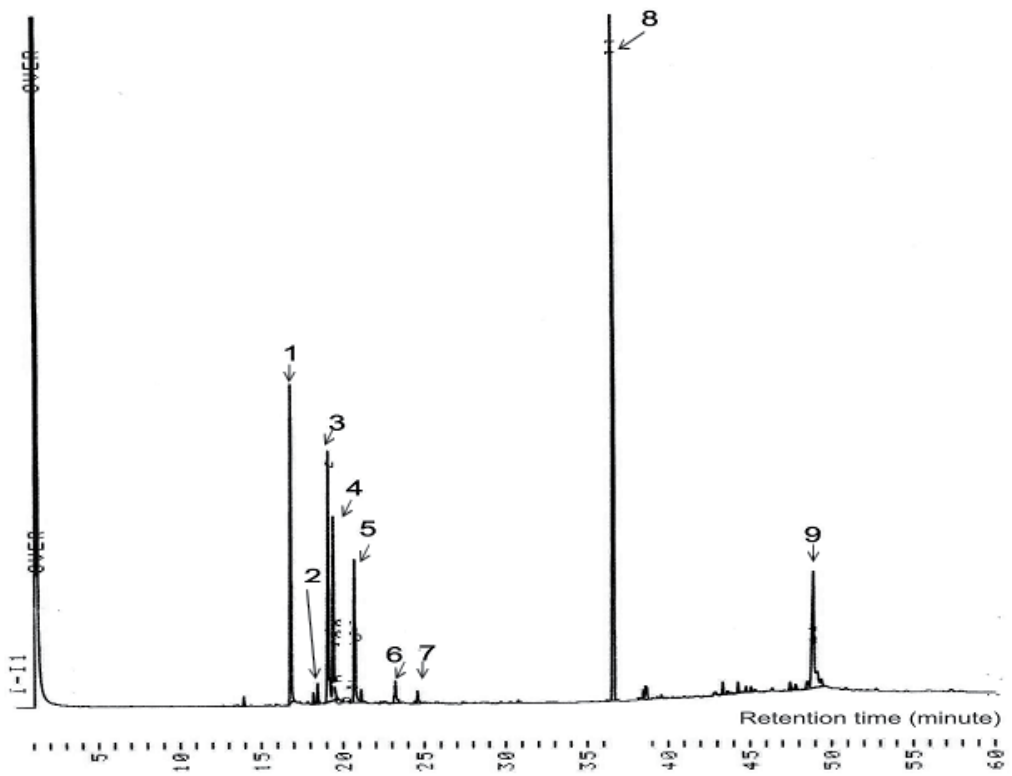


Fig. 2. Gas chromatogram of the ethanol-benzene extract from the teak outer heartwood. Nine major compounds are detected : peak 1and 4 = desoxylapachol and its isomer; peak 2 = palmitic acid; peak 3 = lapachol; peak 5 = tectoquinone; peak 6 = unidentified compound 1; peak 7 = unidentified compound 2; peak 8 = squalene; and peak 9 = tectol.

To evaluate the relationship between the detected nine constituents and 87 individuals, principal component analysis was conducted. The result of the principal component showed that the individuals can be classified into three main groups (Table 3). First principal component were represented by palmitic acid, isodeoxylapachol, and tectol as the principal components. Tectoquinone, UN2, and squalene were representative of the second principal component. As the isodeoxylapachol and tectoquinone contents showed relatively high values, the content of those compounds were examined for each individual. The isodeoxylapachol histogram is shown in Fig. 3. It showed a curve that the there was no individual with content from 10 to 12 % and from 14 to 24 % individuals. This curve can be divided into two types with high content (over 6%: 37 individuals with average content of 8.41 % and standard deviation of 3.46 %), and low content (0–6%: 50 individuals with average content of 3.46 % and standard deviation of 1.53 %) of isodeoxylapachol.

| Constituents | First principal component | Second principal component | Third principal component |
|---|---|---|---|
| Deoxylapachol | -0.07 | -0.30 | 0.61 |
| Palmitic acid | 0.75 | 0.10 | -0.10 |
| Lapachol | -0.14 | 0.06 | 0.63 |
| Isodeoxylapachol | **0.78** | -0.10 | 0.24 |
| Tectoquinone | 0.08 | **0.81** | -0.30 |
| UN1 | 0.17 | -0.01 | 0.72 |
| UN2 | -0.20 | 0.84 | 0.01 |
| Squalene | -0.54 | -0.64 | -0.40 |
| Tectol | 0.66 | 0.05 | -0.16 |
| Cumulative distribution | 0.24 | 0.45 | 0.62 |

Note : UN : unknown compound

Table 3. Factor loadings of nine constituents in principal component analysis

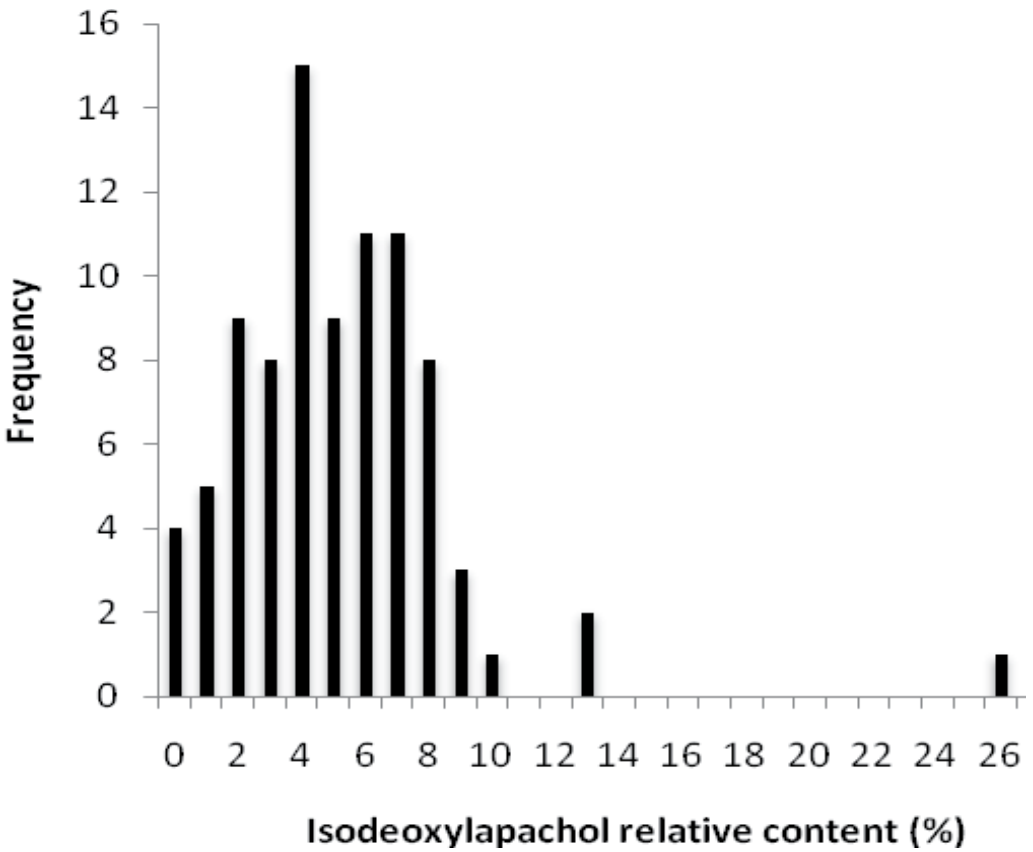


Fig. 3. Histogram of isodeoxylapachol relative content in the outer heartwood of Teak

For tectoquinone, there was no individual with content from 24 to 28% as well as from 44 to 48% (Fig. 4) but the individuals could be divided into two groups with low contents (0-16%: 72 individuals with an average content of 8.25 % and a standard deviation of 4.05 %), and high contents (over 16 %: 15 individuals with an average content of 24.45 % and a standard deviation of 9.71 %). On the basis of this result, we classified the 15 individuals with high tectoquinone contents as TypeT, the 37 individuals with high isodeoxylapachol contents as TypeI. Since two constituents were found in some individuals, we classified the individuals according to whether they contain only one of the constituents or two of them. There were six individuals only containing with high isodesoxylapachol and tectoquinone levels as well as the 42 individuals with low isodesoxylapachol and tectoquinone levels as TypeL.

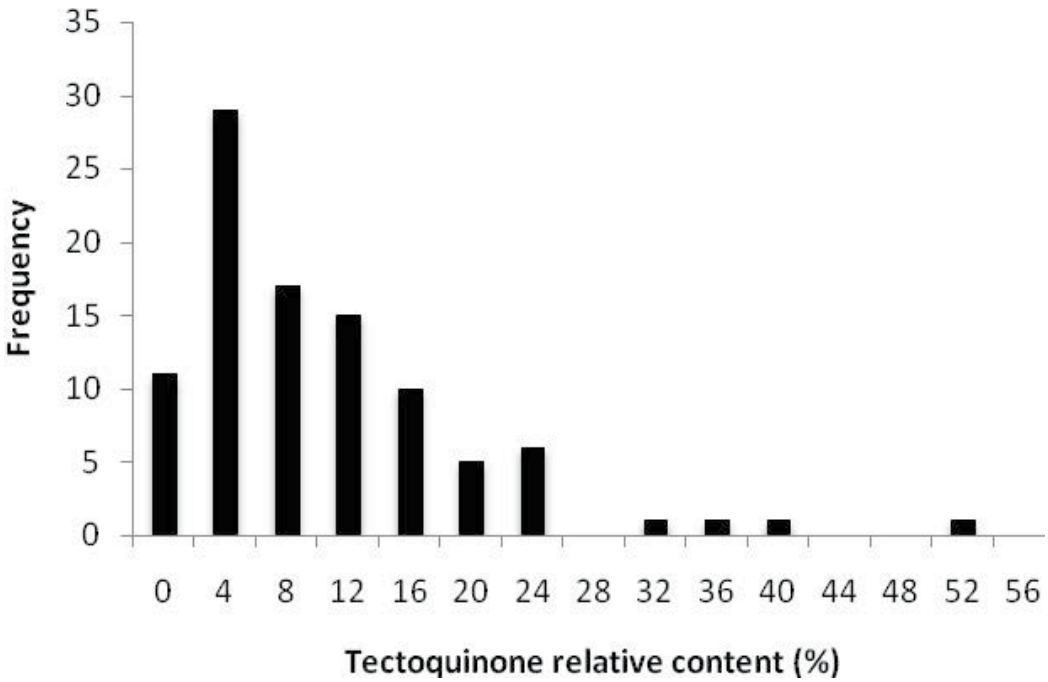


Fig. 4. Histogram of tectoquinone relative content in the outer heartwood of Teak

On the basis of geographical distribution (Table 4), TypeI accounted for 42.5 % (37/87) of the population and equally distributed in both Purwakarta and Randublatung regions. It is also noted that in seven out of 13 tree samples taken from community forest in Gunungkidul region were classified as TypeI. TypeT accounted for 17.24 % (15/87) of the population. This type was particularly minor in Randublatung region (9.52 %). TypeL which can produce small amounts of both isodesoxylapachol and tectoquinone, accounted for 48.2 % (42/87) of the population. This type was the most common in Randublatung region (57.1 %). It is noticed that from six individuals containing with high isodesoxylapachol and tectoquinone, only one individual was found in Purwakarta region.

| Region | Number of individuals | Classification by quinone components | | |
|---|---|---|---|---|
| | | T | I | L |
| Purwakarta | 32 | 8 | 13 | 13 |
| Gunungkidul | 13 | 3 | 7 | 5 |
| Randublatung | 42 | 4 | 17 | 24 |
| Total | 87 | 15 | 37 | 42 |

Note : UN : unknown compound; T : tectoquinone relative content over 16 %; I : isodeoxylapachol relative content over 6 %; L : tectoquinone relative content 0-16 % and isodeoxylapachol relative content 0-6 %

Table 4. Frequency of individuals classified based on contents of quinone components

As the final step, discriminant analysis was used. The discriminant ratio was 90.8% (79/87). Only 2 individuals of TypeL was discriminated as TypeI, whereas only 3 individuals of TypeL were discriminated incorrectly as TypeI. Teak individuals can be thus classified into three types (TypeI, TypeT, and TypeL) on the basis of the contents of quinone constituent (isodeoxylapachol and tectoquinone) and also their distribution corresponding to variations in the contents. For more comprehensive investigations, the subsequent works should be conducted with larger samples including plantation teak trees from East Java as well as from community forests that scattered in the island of Java.

## 4. Conclusions

This is the first report on the chemotaxonomical study of teak heartwood. The tree samples were collected from three regions, Purwakarta, Gunungkidul, and Randublatung, for a total of 87 individuals. Based on the principal component analysis, three types (T,I and L) can be obtained from the relative content of isodeoxylapachol and tectoquinone. TypeT, TypeI and TypeL refers the heartwood with high tectoquinone (over 16 %); high isodeoxylapachol (over 6 %); and low tectoquinone - isodeoxylapachol relative contents, consecutively. Based on geographical distribution, it is found that TypeI is the major type in Gunungkidul while TypeL is the most abundant in Randublatung.

## 5. Acknowledgement

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# 4

# Gas Chromatographic Analysis of Plant and Insect Surface Compounds: Cuticular Waxes and Terpenoids

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## 1. Introduction

The surfaces of all higher plants are covered by a layer of cuticular waxes. These are composed mainly of long-chain aliphatic components but also of cyclic compounds. The primary role of the waxes is to prevent uncontrolled water loss. The chemical composition of plant cuticular waxes can affect the resistance of plants to herbivores and herbivore behaviour. Cuticular waxes and their separate components enhance or deter insect oviposition, movement or feeding.

Most plants have trichomes on their aerial surfaces. The trichomes may be simple hairs or more specialized glandular trichomes, whose main function may be the production and accumulation of chemicals such as essential oils. The vast majority of these consists of monoterpenoids, sesquiterpenoids and diterpenoids with a high vapour pressure. They may be absorbed on the cuticular wax layer. The trichome secretions are closely related to plant-insect or plant-microbe interactions. Terpenoids can attract, repel or initiate defence reactions in insects. Apart from their ecological roles, plant terpenoids are widely used in the pharmaceutical and fragrance industries. The properties of essential oils are correlated with their qualitative and quantitative compositions.

The surfaces of insects are also covered by a layer of wax. Insect cuticular waxes are also involved in various types of chemical communication between individuals of a species and reduce the penetration of chemicals and toxins as well as infectious microorganisms. Analyses and identification of insect waxes is the first step towards developing methods of insect control.

Improvements in analytical techniques have led to the characterization of plant and insect surface compounds and provided new insights in chemical ecology. Moreover, an enormous number of plant terpenoid analyses are associated with their pharmaceutical and fragrance applications. Qualitative and quantitative analyses of cuticular waxes and terpenoids are usually achieved by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Peak identification is based primarily on retention times, retention indices and comparison of recorded spectra with an MS library. This review will describe gas

chromatographic applications in the analysis of various classes of cuticular waxes and terpenoids.

## 2. Cuticular waxes

Cuticular waxes are commonly present on the surfaces of higher plant and insect species, but they often differ from one another in composition. Generally speaking, cuticular waxes consist of complex mixtures of long-chain nonpolar compounds, with the molecular weights of individual components ranging from about 200 to 600 Da, but from time to time exceeding 1000 Da.

### 2.1 Chemical composition of plant cuticular waxes

Plant surface waxes are complex mixtures of relatively non-polar aliphatic and cyclic compounds (Baker, 1982; Bianchi, 1995; Jetter et al., 2006). The surface waxes of plants consist of various groups of long-chain lipids such as hydrocarbons, wax esters (esters of long-chain alcohols and fatty acids, alkyl esters), fatty acids, long-chain alcohols, aldehydes, ketones, β-diketones and hydroxy-β-diketones. Different classes of compounds may contain a series of homologues with different numbers of carbon atoms in the chain, but those most often encountered have from 20 to 36 carbon atoms, although wax esters with more than 60 carbons are also known. Some less common constituents include branched-chain hydrocarbons, alkenes, terpene hydrocarbons, diols, ω-hydroxyacids, branched-chain fatty acids, branched-chain esters, methyl esters, benzoic acid esters, acetates, polyesters of ω-hydroxyacids (estolides), and many others. Cyclic compounds are also common in surface waxes: terpenoids (triterpenols, triterpenoid acids, ketones and aldehydes) and sterols, and their esters; aromatic compounds have been found in only a very few plant species, however. The relative percentages of homologous compounds often form typical patterns that can be helpful in the interpretation of gas chromatograms. For example, the distribution patterns of n-alkanes are typically bell-shaped. The chemical composition of plant surface waxes depends mainly on the plant species and may be different on its various organs. Surface waxes cover not only the leaves but also the stem, fruits, flowers and seeds. Their composition may also depend on plant age and environmental conditions such as illumination, temperature or the presence of chemicals. The composition of surface waxes developing during the evolution of plants became increasingly complex and varied. The chemical compositions and biochemical aspects of plant surface waxes are described in more detail in several monographs (Hamilton, 1995a; Kolattukudy, 1976; Riederer & Müller, 2006).

### 2.2 Chemical composition of insect cuticular waxes

The cuticular waxes of insect species may contain the following chemical classes: hydrocarbons, fatty acids, alcohols, triacylglycerols and wax esters (Gołębiowski et al., 2011; Nelson & Blomquist, 1995). The waxes of some species also contain aldehydes, ketones, esters and sterols. The wax compositions of insects can vary depending on stage, sex, age, and their position in the colony hierarchy. Cuticular waxes can also vary within species as a response to living conditions such as temperature, dryness and available food. The major function of insect waxes is protection against desiccation, but they also prevent microbial infections, affect the adsorption of chemicals and play a role in chemical communication

between species. Hydrocarbons, e.g. n-alkanes, n-alkenes and methyl-branched hydrocarbons, often constitute a major fraction of insect cuticular waxes: they were found to be the major compounds in the cuticular waxes of adults, nymphs and eggs of *Zygogramma exclamationis* (Nelson and Charlet, 2003). 79.4% of cuticular waxes isolated from *Calliphora vicina* larvae were fatty acids (Gołębiowski et al., 2008a). In contrast, the fatty acids in *D. pini* exuviae extracts made up only 2.0% of all waxes (Gołębiowski et al., 2010). Alcohols ranging from $C_{24:0}$ to $C_{34:0}$ constituted the major lipid fraction of the cuticular waxes from *Heliothis virescens* pupae (Buckner et al., 1996). Wax esters consisting of even-numbered-carbon compounds from $C_{38}$ to $C_{64}$ were the major compounds (86%) present in the cuticle of *Bemisia argentifolii* nymphs and exuviae (Buckner et al., 1999). Cuticular waxes of insects also contain aldehydes as minor constituents. This group of waxes were identified in *Heliothis virescens* pupae, *Helicoverpa zea* pupae (Buckner et al., 1996), *Aleurodes singularis* adults and exuviae (Nelson et al., 1998), *Bemisia argentifolii* nymphs and exuviae (Buckner et al., 1999), *Bemisia tabaci* and *Trialeurodes vaporariorum* adults (Buckner et al., 1994). Triacylglycerols are compounds of cuticular waxes but they could also be contaminants derived from body lipids. For example, triacylglycerols were identified in the waxes of *Melanoplus sanguinipes* and *M. packardii* adults (Soliday et al., 1974). Fatty acid methyl esters are not common constituents of insect cuticular waxes. Both methyl and ethyl esters were identified in the cuticular waxes of *Acanthoscelides obtectu*s Say (Gołębiowski et al., 2008b). Like fatty acid methyl esters, ketones are minor constituents of cuticular waxes. The cuticular lipid of *Ceutorrhynchus assimilis* contains 98% of $C_{29}$, smaller amounts of $C_{26}$ (<1%) and traces of $C_{28}$, $C_{30}$ and $C_{31}$ (Richter & Krain, 1980). The ketones of *C. assimilis* are very similar to those in the surface waxes of *Brassica napus*, which suggests that they could be of host plant origin. Sterols are also minor constituents of cuticular lipids. Cholesterol is a cell membrane component in insects and a precursor of steroid hormones such as ecdysone. Insects lack the capacity to synthesize sterols, so they convert phytosterols to cholesterol *via* dealkylation. Cholesterol is the most abundant sterol in the cuticular waxes of insects, occurring, for example, in the waxes of *M. sanguinipes* and *M. packardii* (Soliday et al., 1974).

### 2.3 Isolation and class separation of plant cuticular waxes

Plant cuticular waxes are usually extracted by dipping the plant material in an organic solvent of intermediate polarity for 10 - 60 sec (Bakker et al., 1998; Hamilton, 1995b; Jetter et al., 2006; Stammitti et al., 1996). The solvents used include chloroform, dichloromethane, hexane, petroleum ether and toluene. A two-step extraction with the use of two portions of solvent may improve recovery. The classical extraction method is usually carried out at room temperature but occasionally at the boiling point of the solvent for the complete extraction of insoluble polymeric forms of aldehydes (Haas et al., 2001; Szafranek et al., 2008a). The different sides of the same plant organ (the adaxial and abaxial sides of leaves) may be characterized by cuticular waxes with different compositions, so their extraction is more complicated (Jetter et al., 2000; Buschhaus et al., 2007a). Epicuticular waxes can also be isolated by mechanical methods (Ensikat et al., 2000; Jetter et al., 2000; Riedel et al., 2007), which distinguish the epicuticular waxes outside the plant cuticle from the cuticular waxes embedded in the cuticle matrix. The extraction of 1 to 10 $cm^2$ of the plant surface area should yield a sufficient amount of wax for a single GC-FID or GC-MS analysis (Jetter et al., 2006).

GC is the most commonly used method to selectively identify and quantify the components of cuticular waxes. A schematic diagram of the procedures used in the analyses of plant cuticular waxes is given in Figure 1. Because of the complex nature of plant and insect cuticular waxes, fractionation according to chemical class is usually required before identification of individual components can be achieved. Special care has to be taken in the case of quantitative analyses. Internal standards representing all compound classes should be added early during the wax extraction before fractionation. Traditionally, separation schemes have been developed by using preparative column chromatography (LC) or thin layer chromatography (TLC) (Hamilton, 1995b). A typical example might involve LC separation on silica gel or alumina and gradient elution with increasing concentrations of ethyl acetate in petroleum ether. The separation can then be monitored by TLC. A rapid form of LC is flash chromatography where solvent is forced down the column by air pressure (Still et al., 1978), a procedure that has recently been used for the fractionation of eggplant cuticular waxes (Haliński et al., 2009).

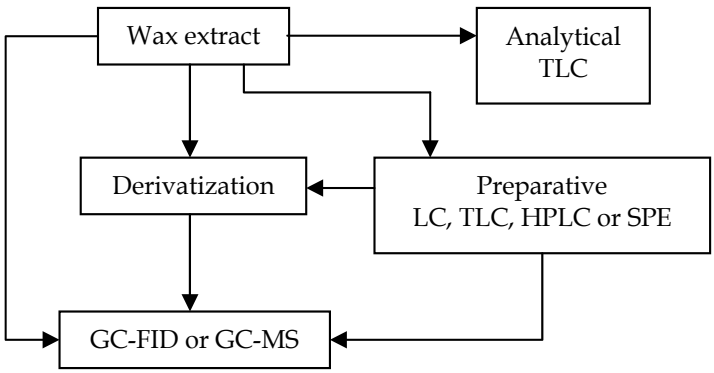


Fig. 1. Summary of typical procedures used in the analysis of plant cuticular waxes.

High-performance liquid chromatography (HPLC) has not been widely used in the analysis of cuticular waxes since these have no useful UV chromophores and because of the difficulties of separating components with different polarities (Hamilton, 1995b). Hwang et al. (2002) developed an HPLC method using a silica gel column and an evaporative light scattering detector (ELSD) to analyse sorghum wax. Szafranek & Synak (2006) and Szafranek et al. (2008b) used HPLC-ELSD for the preparative fractionation of potato cuticular waxes. HPLC separation of cuticular waxes has a better resolution and is more efficient than column chromatography.

Solid-phase extraction (SPE) is today the most popular sample preparation method but still hardly used in the fractionation of plant cuticular waxes. Nass et al. (1998) has developed a method involving the separation of the wax sample into two fractions using simple SPE in order to avoid artefacts in trimethylsilyl derivatization; the fraction containing aldehydes could be GC analysed without silylation. Perez-Camino et al. (2003) have described a procedure for the simultaneous determination of long-chain aliphatic aldehydes, and aliphatic and triterpenic waxes in olive oils. A fraction containing these compounds was isolated from the oil using SPE on silica-gel cartridges. Commercial SPE cartridges are convenient for the isolation of lipid classes, so they may be used more extensively in the

future. Solid phase microextraction (SPME) is a promising new technique for sample extraction/isolation before GC analysis, but as far as we know, it has been applied to the cuticular waxes of insects, but not yet to those of plants.

### 2.4 Isolation and separation of insect cuticular waxes

Extraction procedures aim to remove all of an insect's cuticular waxes. For this, a non-polar solvent is usually used, for example, pentane, hexane and petroleum ether for 10 seconds or chloroform for 1 min (Gołębiowski et al., 2011; Nelson & Blomquist, 1995). The use of a non-polar solvent minimizes the possible extraction of internal lipids, which are mostly free fatty acids and glycerides. Non-polar solvents are used for extracting waxes rich in hydrocarbons, whereas chloroform extracts the oxygenated compounds in cuticular waxes. Chloroform-methanol mixtures are used for short extractions to avoid the possible extraction of internal waxes. The choice of extraction solvent and extraction time depend on the quantity and quality of the waxes. For example, the cuticular waxes of the larvae and adults of khapra beetles *Trogoderma granarium* were obtained by immersing the insects in dichloromethane for 20 seconds (Maliński et al., 1986). A longer extraction was used to separate the cuticular waxes of four *Periplaneta* species (Said et al., 2005); in this case, insects anaesthetized in $CO_2$ were immersed in dichloromethane for 2 min. A 30-second immersion in chloroform was sufficient to obtain the cuticular waxes from *Tribolium destructor* larvae and beetles (Hebanowska et al., 1990). One immersion does not extract all of the cuticular waxes, however: two short extractions are more effective for the complete removal of the cuticular waxes without extraction of internal waxes. Sometimes, internal waxes are extracted because the cuticle is damaged or the extraction time is too long. Triacylglycerols are major components of internal waxes, but they are present in the cuticular waxes of insects, too. The presence of triacylglycerols in cuticular wax extracts can be a useful indicator that internal waxes have been extracted. A two-step extraction was used for the cuticular waxes of adults of *Frankliniella occidentalis* (Gołębiowski et al., 2007). The insects were first extracted in petroleum ether for 30 s, after which they were immersed in dichloromethane for 2 min.

Wax extracts are separated into classes of waxes by TLC, LC or HPLC. Table 1 gives examples of the methods for separating insect cuticular waxes.

| Insects | Separation | References |
|---|---|---|
| ***T. granarium*** | Hydrocarbons eluted with hexane on a column filled with silica gel activated at 150 °C for 24 hr | Maliński et al., 1986 |
| ***B. argentifolii*** | Lipid classes separated by high performance TLC using plates coated with silica gel. Developing solvent: hexane/diethyl ether/formic acid (80:20:1 v/v/v). Visualization: 5% concentrated sulphuric acid in 95% ethanol | Buckner et al., 1999 |
| ***A. obtectus*** | Lipid classes separated by HPLC-ELSD. The mobile phase consisted of petroleum ether (solvent A) and dichloromethane with the addition of 15% acetone and 1.5% isopropanol (solvent B). The gradient was programmed to change linearly from 100% A to 100% B within 20 min. | Gołębiowski et al., 2008b |

Table 1. Examples of the isolation and separation of insect cuticular waxes

Recently, SPME and solid injection (SI) have been used for extracting cuticular waxes of insects. SPME and SI can be used in combination with GC or GC–MS. SPME is a sample preparation technique using a fused-silica fibre coated on the outside with an appropriate stationary phase. The compound in the sample is directly extracted and concentrated on the fibre coating. The method saves preparation time and solvents, and it can improve detection limits. SPME can be used routinely in combination with GC, GC–MS, HPLC and liquid chromatography-mass spectrometry. SPME-GC and SPME-GC-MS are applicable to the extraction of volatile and semi-volatile organic compounds from biological samples. The following types of stationary phase can be used: non-polar polydimethylsiloxane (PDMS), polar polyacrylate (PA), Carboxen (Car)/divinylbenzene (DVB), Carbowax/templated resin (polar), mixed-polarity PDMS/DVB, Car/PDMS and DVB/Car/PDMS (Gołębiowski et al., 2011). Compounds of different volatility and polarity can be sampled simultaneously by carefully selection of the polarity and thickness of the fibre coating. The application of SPME to the analysis of insect cuticular hydrocarbons is based mainly on direct contact (DC-SPME) between the fibre and the cuticular surface. SPME adsorption from heated samples (headspace SPME, HS-SPME) is also used; in this technique the fibre is exposed to the vapour phase above a gaseous, liquid or solid sample. The cuticular fatty acid methyl esters from *Calliphora vicina* were analysed by HS-SPME (Gołębiowski, *data unpublished*). The photo of the sampling procedure of insect volatiles is given in Figure 2.

Fig. 2. Static headspace sampling of insect volatiles with a SPME device.

The cuticular hydrocarbons from *Bagrada hilaris* were analysed by SPME-GC-MS (Pasquale et al., 2007) having been sampled using fibres coated with polydimethylsiloxane (PDMS, 100 µm), polyacrylate (PA, 80 µm) and Carbowax–divinylbenzene (CW-DVB, 65 µm). The fibres were conditioned in the gas chromatograph injector port: PDMS at 250 °C for 30 min, PA at 300 °C for 2 h, and CW-DVB at 220 °C for 30 min. Sealed vials with the insects were thermostatted at temperatures of 130 and 150 °C for 10 min. The headspace volatiles were absorbed on the exposed fibre for 2 min. The loaded fibre was then desorbed in the gas chromatograph inlet port for 2 min. 12 hydrocarbons were identified in the cuticular waxes of *B. hilaris*: they consisted of a homologous series of n-alkanes ranging from $C_{17}$ to $C_{29}$. Although the hydrocarbon profiles of males and females were qualitatively similar, quantitative differences were observed. Two sampling techniques for the GC-MS analysis of free fatty acids from exocrine glands were used (Maile et al., 1998). Solvent-free sampling, either by SPME or SI, are the ideal conditions for the analysis of insect samples. The insect waxes are analysed without any danger of contamination. Small insects or their parts are first sealed in a soft soda glass capillary tube. This is inserted in the gas chromatograph injector port, then heated for 3 min, after which GC analysis commences. The SPME sampling of fatty acids from solutions has been examined with different fibres; the stationary phases used as coatings were polydimethylsiloxane (PDMS, 7 µm), polyacrylate (PA, 85 µm), and Carbowax-divinylbenzene (CW-DVB, 65 µm). The best results for saturated fatty acids were obtained with PDMS-coated fibres, whereas PA fibres are suitable for palmitoleic, oleic and linoleic acids. Solid injection was used to analyse the hydrocarbons of the small ant *Cardiocondyla wroughtonii* (Turillazzi et al., 2002) and can be used to analyse solid samples, including body parts, glands or even small size insects. The solid injector technique involves inserting sealed glass capillaries into the GC-MS injector. Samples are inserted into the groove, which is then moved to align with two holes that allow passage of the carrier gas over the sample in the GC once injected. 44 hydrocarbons were identified on the mass spectra obtained with solid injection. This method proved to be a superior means of extracting hydrocarbons from insects.

### 2.5 GC separation and detection of cuticular waxes

Gas chromatography was first applied to analyse cuticular waxes in the early 1960s, and its use has progressed with the introduction of capillary columns. Many of the more recently published papers deal with gas chromatography analysis (GC-FID or GC-MS) rather than with liquid chromatography (LC, TLC or HPLC). The aim of GC is to resolve the individual constituents of the complex wax mixture in order to obtain information on their qualitative and quantitative composition. This can be done in the following ways:

a. GC analyses of intact cuticular wax extracts or fractions;
b. chemical derivatization of functional groups containing active hydrogen and then GC analyses;
c. chemical hydrolysis of high-molecular-weight esters and then GC analyses of the liberated moieties.

The detectors most frequently used for GC analysis of cuticular waxes are the flame ionization detector (FID) and mass spectrometer (MS) (Evershed, 1992a; Hamilton, 1995b; Riederer & Müller, 2006). Occasionally, other detectors can be applied. For example, Grossi & Raphel (2003) used GC coupled to an atomic emission detector (AED) for the identification of a series of 1-chloro-n-alkanes in the leaf waxes of three halophytes.

GC analysis of cuticular waxes is carried out mainly on fused-silica capillary columns of different diameter (0.1-0.32 mm) and length (10-50 m). In our opinion, the most common stationary phases used for the analysis of cuticular wax are:

a. 100% dimethylpolysiloxane [for example, DB-1 equivalent to OV-1, RTX-1, HP-1, SE-30, CP-Sil 5CB, EC-1 (Grace, 2011)]
b. 5% phenyl/95% methylpolysiloxane [for example, DB-5 equivalent to OV-5, RTX-5, HP-5, SE-54, EC-5 (Grace, 2011)].

The direct analysis of wax extracts or, alternatively after the conversion of all hydroxyl-containing compounds into the corresponding trimethylsilyl (TMSi) derivatives, is often used to obtain GC profiles of plant cuticular waxes. For example, Buschhaus et al. (2007b) reported alkanes, primary alcohols, alkyl esters, triterpenoids and minor components in the waxes of rose leaves. GC analysis was carried out with the temperature programmed to rise from 50 °C to 320 °C and held for 30 min at 320 °C. Generally speaking, gas chromatographic elution with the oven temperature programmed to rise from 40 or 50 °C to 320 or 340 °C is the usual procedure in the analysis of plant cuticular waxes containing typical constituents such as hydrocarbons, fatty acids, alcohols, aldehydes, ketones, diols, esters and triterpenoids (Buschhaus et al., 2007a; Jetter, 2000; Jetter et al., 1996; Jetter & Riederer, 2000; Szafranek & Synak, 2006). Figure 3 shows the typical GC pattern of intact cuticular wax extract.

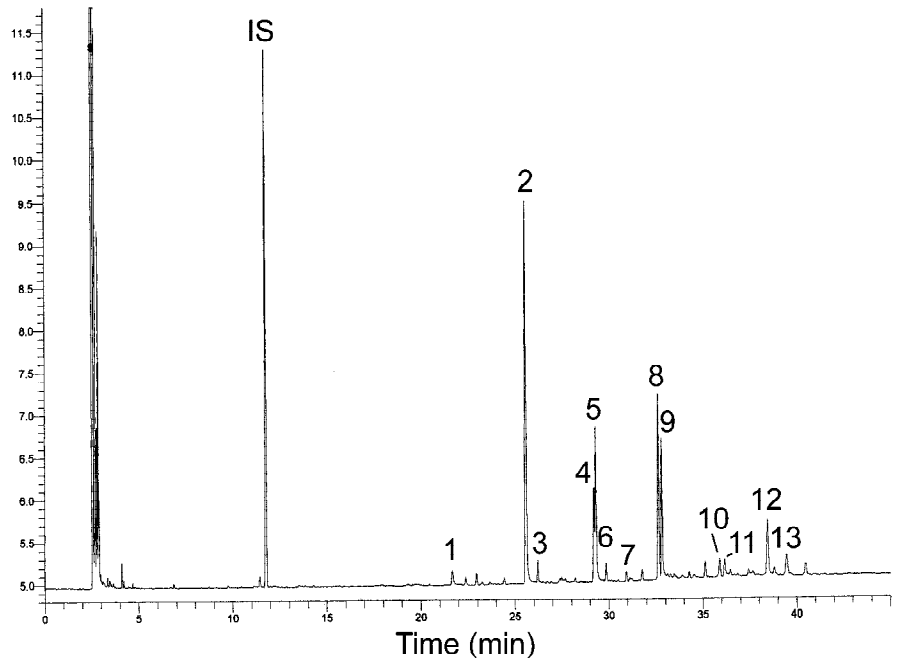


Fig. 3. GC-FID chromatogram of marigold (*Tagetes patula* L.) leaf cuticular waxes obtained on an RTX-5 capillary column with a temperature programme from 180 °C to 320 °C at 4 °C/min. (1) 1-Docosanol; (2) 1-tetracosanol coeluted with n-heptacosane; (3) tetracosanoic acid methyl ester; (4) n-nonacosane; (5) 1-hexacosanol; (6) hexacosanoic acid methyl ester; (7) n-triacontane; (8) n-hentriacontane; (9) 1-octacosanol; (10) n-tritriacontane; (11) 1-triacontanol; (12) β-amyrin; (13) α-amyrin; (IS) internal standard.

Insect cuticular waxes, consisting mainly of hydrocarbons, fatty acids, alcohols, aldehydes, and wax esters, have been analysed with the oven temperature programmed to rise from about 150 °C to 300 or 320 °C (Buckner et al., 1996; Nelson et al., 1994; Pasquale et al., 2007; Turillazzi et al., 2002) or from about 100 °C to 300 or 320 °C (Gołębiowski et al., 2007; Said et al., 2005). The initial temperature was significantly lower for the analysis of cuticular hydrocarbons, aldehydes, alcohols and wax esters in *Bemisia argentifolii* (50 °C to 320 °C) (Buckner et al., 1999), the fatty acids in *Dendrolimus pini* (50 °C to 310 °C) (Gołębiowski et al., 2010) and the surface waxes in *Acanthoscelides obtectus* (30 °C to 320 °C) (Gołębiowski et al., 2008b). The initial temperature depends on the molecular weights of the compounds to be analysed. For example, the fatty acids of *D. pini* ($C_{8:0}$ – $C_{34:0}$) were analysed by GC as methyl esters (Gołębiowski et al., 2010). Owing to their volatility even at relatively low temperatures, short-chain fatty acids ($C_4$ to $C_{10}$) should be GC analysed with special care (Evershed, 1992a): temperature-programming from the lowest possible temperature (ca. 30 °C) is necessary to prevent the shorter chain compounds co-eluting with the solvent.

Wax esters, steryl esters, triterpenoid esters or triacylglycerols are analysed at slightly higher temperatures. For example, wax esters from *Salix* leaves were GC–FID analysed and the oven temperature was programmed to increase from 200 °C to 380 °C, then held for 20 min at 380 °C (Szafranek et. al., 2008a). It should be noted that the high temperatures (generally above 300 °C) required for the elution of intact high-molecular-weight compounds can cause the loss of some thermally labile compounds (e.g. unsaturated compounds) (Evershed, 1992a). Wax esters can be GC analysed on non-polar capillary columns. Recently, Stránský et al. (2006) analysed a series of more than 200 wax ester standards using a temperature gradient programme from 240 to 340 °C. The GC analysis of n-alkanes up to $C_{54}$ and esters up to $C_{52}$–$C_{53}$ could be performed under these chromatographic conditions. The problem with wax ester analysis, however, is that separation largely occurs according to carbon number; hence, the chromatographic peaks represent mixtures of non-separated wax esters. Some ester isomers elute at nearly the same time: for example 18:0-20:0 (an 18:0 acid moiety with a 20:0 alcohol moiety) and 20:0-18:0. The study shows the influence of the number of double bonds in the ester molecule on chromatographic behaviour in comparison with the corresponding saturated ester. Saturated esters have the longest retention times. Esters with three double bonds are eluted before esters with one or two double bonds.

High-temperature gas chromatography (HTGC) and HTGC coupled to mass spectrometry has great potential for the determination of a great number of high-molecular-weight compounds, e.g. sucrose fatty acid esters, ethoxylated fatty alcohols, triglycerides and wax esters (Pereira & Neto, 1999; Pereira et al., 2004). HTGC non-polar and medium polar capillary columns can be used up to 400°C and in some cases 480 °C. On the other hand, high-resolution GC commercial capillary columns can be used up to 370 °C in the routine analysis of compounds of 1200 Da and more. This enables the direct analysis of extracts and fractions of natural products, in some cases without prior derivatization or clean-up. The injection method in HTGC is very important. Cold on-column or programmable temperature vaporizing (PTV) injection are the preferred techniques, as they eliminate discrimination against high boiling analytes. For example, high molecular weight alkanoates (three series of α-tocopherol, β-tocopherol and phytol esterified to fatty acids) in Amazonian plants were analysed by HTGC (Pereira et al., 2002). The cuticular waxes from ivy leaves

containing ester waxes (alkyl alkanoates and alkyl coumarates) were analysed directly by GC-MS, HTGC-MS and ESI-MS/MS by Santos et al. (2007). However, with ESI-MS/MS analysis the detection of a wider range of ester waxes was possible than with HTGC-MS.

Fatty acids are GC analysed after their conversion to non-polar derivatives such as methyl or silyl esters (Evershed, 1992a). Often, esters are prepared as derivatives of the fatty acids liberated from wax esters or sterol esters by hydrolysis (Sümmchen et al., 1995; Szafranek & Synak, 2006). The details and other important considerations of fatty acid analysis have been well summarized by Christie (1994). Generally speaking, fatty acid methyl esters (FAME) can be GC analysed employing non-polar stationary phases like other cuticular wax components. Unsaturated compounds should be analysed on polar stationary phases (e.g. Carbowax). The determination of FAME often requires high resolution for the separation of positional and configurational isomers. The elution order is as follows (Evershed, 1992a):

a. branched-chain FAME: increasing alkyl-chain branching reduces the retention time compared to a linear saturated counterpart and the elution order is independent of the polarity of the stationary phase;
b. positional isomers of unsaturated FAME: the ECL values increase with increasing distance of the double bond from the carboxyl group on both polar and non-polar phases;
c. geometric isomers of unsaturated FAME: the *E*-isomer elutes before the *Z*-isomer on a polar column. The order is reversed on a non-polar column.

Hydrocarbons are easily analysed by GC because of their chemical and thermal stability (Evershed, 1992a). The elution order of n-alkanes reflects the differences in their molecular weights. The effects of chain-branching and isomerism follow the same trends as for fatty acid methyl esters. Insects contain a variety of methyl-branched alkanes that have been widely investigated since the 1970s (Nelson & Blomquist, 1995). With certain combinations of the number and position of methyl branches, methyl-branched alkanes may have GC similar retention times to those of n-alkanes with one or two carbon atoms fewer in the chain. Elution times may overlap for methyl-branched alkanes that differ by one or even two carbons in the chain (Carlson et al., 1998). Since separation of branched-chain alkanes is difficult, it is usually necessary to perform GC-MS.

Fatty alcohols can be GC analysed as native compounds, but their separation is improved by preparing silyl or acetate derivatives (Szafranek & Synak, 2006; Shepherd et al., 1999; Stammitti et al., 1996). Ketones and aldehydes are analysed without derivatization (Szafranek & Synak, 2006; Nass et al. 1998), although aldehydes can be converted to more stable dimethyl acetal derivatives (Drozd, 1981) or other derivatives. See Table 2 for examples of derivatives used in the GC analysis of less common cuticular wax components.

### 2.6 Derivatives of cuticular wax components for GC analyses

The most common derivatives used in the GG analysis of cuticular waxes containing hydroxy-compounds are trimethylsilyl derivatives (TMSi ethers and esters). They are volatile but not stable, particularly in acidic or wet conditions. Analytes are derivatized with common silylation reagents such as BSA [N,O-bis(trimethyl-silyl)acetamide], BSTFA [N,O-bis(trimethyl-silyl)trifluoroacetamide] and others (Blau & Halket, 1995; Drozd, 1981). TMCS (trimethylchlorosilane) can be used as catalyst. TMSi derivatives should be prepared

immediately prior to the analysis because of their possible sensitivity to moisture. In the case of GC-FID analyses, silicone dioxide, produced by the decomposition of silyl derivatives in a flame, is deposited on the electrodes and slowly decreases the sensitivity of the detector. The reaction is simply carried out in a septum-closed vial by dissolving the sample of cuticular waxes in the reagent mixture and leaving it in the heating block for some time. For example, the reagent mixture of BSTFA-pyridine at 70 °C for 30 min (Jetter et al. 2000; Jetter & Riederer, 2000; Vermeer et al., 2003); BSTFA–TMCS (99:1, v/v) at 90 °C for 15 min (Haliński et al., 2009); BSA-TMCS (85:15, v/v) at 70 °C for 30 min (Szafranek & Synak, 2006); BSA-TMCS (85:15, v/v) at 100 °C for 60 min (Gołębiowski et al., 2008b; 2010). Little (1999) described the artefacts in TMSi derivatization reactions and the formation of several artefacts in the silylation of aldehydes and ketones. Aldehydes have been SPE separated and GC analysed without silyl derivatization (Nass et al., 1998).

Dimethyl acetals can be prepared by heating the aldehydes under reflux with 2% anhydrous methanolic HCl for 2 h, after which the methanolic solution is cooled and neutralized with a small excess of sodium carbonate. Finally, the acetals are extracted from methanol with light petroleum (Drozd, 1981); alternatively, an aldehyde mixture with 1% sulphuric acid in methanol is left overnight in a vial at 50 °C (Christie, 1994).

High-molecular-weight esters, such as wax esters, steryl esters, triterpenoid esters and triacylglycerols, are often hydrolysed, after which the liberated moieties are GC analysed; however, this procedure provides only limited compositional information. The very convenient one-pot small-scale hydrolysis–silylation procedure, applicable to ester-emulsifiers, oils, fats, wax esters and other hydrolysable lipids, has been proposed by the IUPAC (Brüschweiler and Hautfeune, 1990). The procedure involves the saponification of esters (ca. 10 mg) in 0.5 N ethanolic KOH solution (0.25 mL) at 80 °C for 3 h, evaporation to dryness in a stream of nitrogen and then silylation of the hydrolysis products. This enables the components of very small samples to be determined (< 0.1 mg). This procedure was used for the hydrolysis of wax esters and triterpenoid esters in potato and eggplant cuticular waxes (Szafranek & Synak, 2006; Haliński et al., 2009). A solution of 1-2% (v/v) concentrated sulphuric acid in methanol can be easily used for the transesterification of acyl lipids and the esterification of free fatty acids (Christie, 1994). More procedures for ester hydrolysis and transesterification and for fatty acid esterification are described in detail by Christie (1994).

The free hydroxyl groups of long-chain alcohols and other compounds are frequently acetylated prior to GC analysis. Acetic anhydride in pyridine (5:1, v/v) is a mild reagent for acetylation: the sample (up to 50 mg) is dissolved in acetic anhydride in pyridine (2 mL) and left at room temperature overnight, following which the excess reagents are removed in a stream of nitrogen (Christie, 1994). Another procedure was applied for alcohols in potato waxes (Szafranek & Synak, (2006) after Blau & Halket (1995)): the sample (ca. 0.1 mg) was dissolved in chloroform (0.5 mL), and acetic anhydride (0.1 mL) in acetic acid (0.2 mL) was added. The sample was left overnight in a glass vial at 50 °C, and the excess reagents were removed in a stream of nitrogen.

Dimethyl disulphide adducts are easily prepared derivatives for the GC-MS location of double bonds in alkenes and fatty acid methyl esters (Evershed, 1992b). The procedure is as follows: a small (ca. 0.5 mg) sample is dissolved in 50 μL dimethyl disulphide, and 50 μL

carbon disulphide is added with 300 μg iodine; the mixture is kept at 60 °C for 40 h in a sealed vial, after which the reaction is quenched with aqueous $Na_2S_2O_3$; the organic phase is extracted and evaporated in a stream of nitrogen (Vincenti et al., 1987).

See the monographs by Blau & Halket (1995), Christie (1994) and Drozd (1981) for more derivatization procedures prior to GC analysis.

### 2.7 GC identification of cuticular waxes

Gas chromatography (GC-FID and GC-MS) can be used for both qualitative and quantitative analyses. Complete identification can be effected if GC retention data and mass spectral data are together taken into consideration. GC-FID identification is based on the comparison of retention times of analytes and authentic standards determined under identical GC conditions or by their co-chromatography. Unfortunately, standards of cuticular wax constituents are rarely commercially available. Some standards may be produced in the laboratory from pure components or natural extracts. For example, the carbon number in the analyses of wax esters can be assigned by comparison of their retention times with that of a synthetic wax ester of known structure (Evershed, 1992a). Beeswax is well-characterized so it can be used as a standard mixture. In beeswax wax esters, the predominant fatty acid moiety is hexadecanoic acid and the chain lengths of the alcohol moieties range from $C_{26}$ to $C_{36}$.

For other compounds, the literature should be consulted for retention data. These are presented in a number of ways, usually as relative retention times, as Kovats retention indices (KI) or programmed-temperature retention indices (RI), or as equivalent chain length values (ECL). Kovats retention indices are based on a linear relationship between the logarithms of the adjusted retention times ($\log t'_R$) of a homologous series of compounds (saturated straight-chain alkanes) and their carbon chain lengths (Castello, 1999). The drawback of the Kovats retention index is related to the column temperature: KI values change with changing temperature. The Kovats retention index is determined under isothermal GC oven conditions and defined as

$$KI = 100z + 100\frac{\log t'_{R,X} - \log t'_{R,Z}}{\log t'_{R,Z+1} - \log t'_{R,Z}}, \quad (1)$$

where $t'_{R.X}$ is the adjusted retention time of unknown compound *X*; $t'_{R.Z}$ is the adjusted retention time of an n-alkane with *z* carbon atoms; $t'_{R.Z+1}$ is the adjusted retention time of an n-alkane with (*z+1*) carbon atoms; compound X is eluted between both n-alkanes; $t'_{R.Z} < t'_{R.X} < t'_{R.Z+1}$.

The programmed-temperature retention index is used when linear temperature programmes are carried out and defined as:

$$RI = 100z + 100\frac{t'_{R,X} - t'_{R,Z}}{t'_{R,Z+1} - t'_{R,Z}} \quad (2)$$

The retention times of the n-alkanes should behave linearly as a function of the carbon number, i.e. the difference between the retention time of two adjacent homologues is the same in the whole programmed analysis (Castello, 1999).

Similarly, ECL are used for the identification of an unknown FAME under isothermal GC oven conditions (Evershed, 1992a):

$$ECL = n + \frac{\log t'_{R,X} - \log t'_{R,n}}{\log t'_{R,n+1} - \log t'_{R,n}} \quad (3)$$

where $t'_{R.X}$ is the adjusted retention time of the unknown FAME *X*; $t'_{R.n}$ is the adjusted retention time of a saturated straight-chain FAME with *n* carbon atoms; $t'_{R.n+1}$ is the adjusted retention time of a saturated straight-chain FAME with (*n+1*) carbon atoms; compound X is eluted between both saturated straight-chain FAMEs; $t'_{R.n} < t'_{R.X} < t'_{R.n+1}$.

Recently, Stránský et al. (2006) determined programmed-temperature retention indices for a series of wax ester standards on a DB-1 (100% dimethylpolysiloxane) fused silica column.

Itoh et al. (1982) determined the relative retention times of 168 acetate derivatives of sterols and triterpene alcohols on non-polar OV-1 (dimethyl silicone) and slightly polar OV-17 (50% phenyl-50% methyl silicone) glass capillary columns. Values are relative to cholesterol acetate.

The equivalent chain-lengths (ECL) of the methyl ester derivatives of 79 unsaturated fatty acids have been determined by GC with fused silica columns coated with Carbowax 20M (polyethylene glycol), Silar 5CP, CP-Sil 84 (Chrompak/Varian) and a 5% phenyl-methyl silicone (Christie, 1988). CP-Sil 84 is equivalent to AT-SILAR-90, DB-23, Rtx-2330, SP-2330 [poly(80% biscyanopropyl)-20% cyanoproylphenyl siloxane] (Grace, 2011).

Insect cuticular hydrocarbons are commonly identified on the basis of retention indices (Nelson & Blomquist, 1995). Pomonis et al. (1989) determined the Kovats retention indices of monomethyl-pentacosanes, some internally branched dimethylalkanes and 2,*x*-dimethylheptacosanes on a cross-linked methyl silicone fused silica capillary column (Hewlett-Packard). Carlson et al. (1998) described a protocol for the identification of methyl-branched hydrocarbons in insect cuticular waxes. In this protocol, programmed-temperature retention indices are assigned to peaks, then the patterns in GC peaks that probably contain homologues are marked to assist subsequent GC-MS interpretation. The authors also included data from the literature covering most of the insect methylalkanes.

### 2.8 GC-MS identification of cuticular waxes

Combined GC-MS is the standard technique used for identifying cuticular waxes, and electron ionization (EI) is the most widely used MS ionization technique in their analysis. The most frequent and simple method of identification involves comparison of the recorded mass spectra with those in standard mass spectral libraries or with those of authentic standards. Unfortunately, the spectra of wax constituents are rarely published in MS libraries (e.g. NIST Chemistry WebBook, Wiley Registry of Mass Spectral Data, MSDC). For compounds that remain unidentified in the above MS libraries, the mass spectra found in the literature can be consulted. Table 2 gives the references of published mass spectra or fragmentation patterns for some less common cuticular wax components.

| Wax class | Examples of compounds (derivatives) | References |
|---|---|---|
| **Secondary alcohols** | 2-Heptacosanol (native, acetate derivative, TMSi ether) | Szafranek & Synak, 2006 |
| | Nonacosan-13-ol (TMSi ether) | Wen et al., 2006 |
| **Ketones** | 2-Heptacosanone | Szafranek & Synak, 2006 |
| | Nonacosan-10-one (methyl oxime) | Jetter & Riederer, 2000 |
| **Diketones** | Nonacosane-10,11-dione (TMSi ether, quinoxaline derivative) | Jetter & Riederer, 2000 |
| **Hydroxyaldehydes** | 3-Hydroxyhexacosanal (native, TMSi ether) | Vermeer et al., 2003 |
| | 5-Hydroxyoctacosanal (native, TMSi ether, acetate derivative) | Wen & Jetter, 2007 |
| **Esters** | Tetradecanoic acid isopropyl ester | Szafranek & Synak, 2006 |
| | Hexacosyl hexadecanoate Tetracosyl coumarate (TMSi ether) | Santos et al., 2007 |
| | Benzoic acid esters | Gülz et al., 1987 |
| **Phenylalkyl esters** | 3-(4′-Hydroxyphenyl)-propyl esters of fatty acids (TMSi ethers, acetate esters) | Jetter et al., 2002 |
| **Ketols** | 15-Hydroxynonacosan-14-one, 16-Hydroxynonacosan-14-one (TMSi ether) | Wen & Jetter, 2009 |
| | 10-Hydroxynonacosan-11-one/11-hydroxynonacosan-10-one (TMSi ether, methyloxime TMSi ether) | Jetter & Riederer, 2000 |
| **Diols** | Nonacosane-14,15-diol Nonacosane-13,15-diol (TMSi ethers) | Wen & Jetter, 2009 |
| | Hexacosane-1,7-diol (TMSi ethers) | Jetter et al., 1996 |
| | β-Alkanediols (TMSi ethers, isopropylidene ethers), γ-alkanediols (TMSi ethers) | Jetter, 2000 |
| | 1,5-Alkanediols (TMSi ethers, acetates) | Wen & Jetter, 2007 |
| | Hentriacontanediol and octacosanediol isomers (TMSi ethers, acetates) | Wen et al., 2006 |
| **δ-Lactones (1,5-Alkanolides)** | Hexacosanolide, octacosanolide | Jetter & Riederer, 1999 |
| **Triterpenoids** | Amyrin, lupeol (TMSi derivatives) | Gülz et al., 1987 |
| **Sterols** | Campesterol, stigmasterol (TMSi derivatives) | Gülz et al., 1987 |

Table 2. Literature data for mass spectra or fragmentation diagrams of less common cuticular wax components

The interpretation of mass spectra of common wax components are described elsewhere (Hamilton, 1995b; Evershed, 1992b; Christie, 1994). The mass spectra of fatty acids, alcohols, wax esters and other lipids can be found in an open access website (The AOCS Lipid Library, 2011). Briefly, the identification is performed on the basis of the characteristic fragment and molecular ions. For example, the mass spectra of saturated fatty acid methyl

esters generally exhibit characteristic fragment ions at *m/z* 74 and 87 (Evershed, 1992b). The relative abundance of *m/z* 74 decreases with increasing unsaturation, thereby complicating the interpretation of mass spectrum even more. The molecular ion $M^{+\cdot}$ confirms the number of carbons and the degree of fatty acid unsaturation. Christie (1998) summarized the use of picolinyl ester and dimethyloxazoline derivatives for the analysis of unsaturated fatty acids and the interpretations of their mass spectra.

The ions at *m/z* 73 and 75 are very common in the mass spectra of trimethylsilyl derivatives (TMSi) (Evershed, 1992b). TMSi esters of fatty acids possess characteristic ions at *m/z* 73, 75, 117, 129, 132, 145 and $[M\text{-}CH_3]^+$. TMSi ethers of long-chain alcohols are characterized by the ions at *m/z* 73, 75, 103 and $[M\text{-}CH_3]^+$. The mass spectra of long-chain alcohol acetates show the following ions – *m/z* 43, *m/z* 61 $[CH_3COOH_2]^+$ – together with the corresponding fragment at $[M\text{-}60]^{+\cdot}$ and $[M\text{-}60\text{-}28]^{+\cdot}$, but molecular ions are not always detected (Christie, 1994). In our opinion, acetate derivatives are less informative than TMSi derivatives, so it is more convenient to prepare the same TMSi derivatives for every component of the wax extract. Mass spectra of underivatized long-chain alcohols contain little structural information and are easily confused with those of monoalkenes (Christie, 1994). The characteristic ions are 55, 57, 69, 83, 97, 111 and weak $[M\text{-}18]^{+\cdot}$, $[M\text{-}46]^{+\cdot}$.

The mass of spectrum of an underivatized long-chain aldehyde is characterized by the ions *m/z* 82, 96, $[M\text{-}18]^{+\cdot}$ and a weak $M^{+\cdot}$ (Hamilton, 1995b; Christiansen et al., 1969).

The mass spectrum of the wax ester of the type RCOOR' show the important diagnostic ions in the alcohol or acid moieties: $[RCOOH_2]^+$, $[R'\text{-}1]^+$ and $[COOR']^+$ (Hamilton, 1995b).

Recently, the mass spectral characteristics of three classes of wax esters were presented by Zhang et al. (2010). The authors developed a system from GC/MS data for the identification of wax esters in complex samples.

Mass spectra of branched-chain alkanes: 2-methylalkanes are identified by peaks at $[M\text{-}43]^+$ and the less intense peak at $[M\text{-}15]^+$, whereas 3-methylalkanes have peaks at $[M\text{-}29]^+$ and a less intense peak at $[M\text{-}57]^+$( Hamilton, 1995b and the references therein).

## 3. Terpenoids

Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. These compounds belong to the isoprenoid group. Even though isoprene itself has not been found in nature, its polymers, terpenic hydrocarbons and their oxygen derivatives are very often present in large quantities in different species. The single isoprene unit, therefore, represents the most basic class of terpenes, the hemiterpenes. An isoprene unit bound to a second isoprene is the defining characteristic of terpene, which is also a monoterpene ($C_{10}$). Sesquiterpenes contain three isoprene units ($C_{15}$), while diterpenes ($C_{20}$) and triterpenes ($C_{30}$) contain two and three terpene units respectively. Tetraterpenes consist of four terpene units, polyterpenes more than four such units. In nature, terpenes occur predominantly in the form of hydrocarbons, alcohols and their glycosides, ethers, aldehydes, ketones, carboxylic acids and esters. The chemical diversity of plant terpenoids originates from often complex terpenoid biosynthetic pathways. McGarvey & Croteau (1995) reviewed terpene biosynthesis. Mapping strategies were used to determine the variance and composition of amino acids at terpene synthase active sites. In a more recent

review of terpene synthase genes, Zwenger & Basu (2007) performed an *in silico* analysis of publicly available microarray data using Genevesitgator software (Zimmerman et al., 2004). Apart from broad structural diversity, terpenes also exhibit a wide array of biological actions. Essential oils have antimicrobial, antiparasitic, insecticidal and antioxidant/prooxidant activities that often represent the combined bioactivity of multiple components. Many plant terpenoids are cytotoxic towards tumour cells and are applied as chemotherapeutic or chemopreventive compounds (Bhalla, 2003; Bifulco, 2005). Terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants. Many insects metabolize the terpenes they have obtained with their plant food to growth hormones and pheromones.

### 3.1 Terpene isolation from plants

Organic solvent extraction has long been used for isolating essential oils from natural products (Taylor, 1993). To isolate terpenes from plant material dichloromethane extraction (Bowman et al., 1997) and methanol extraction can be applied (Verma et al., 1990). A two-day extraction with a mixture containing pentane and dichloromethane was used to isolate guajava essential oil (Vernin et al., 1991). Water extraction has proved to be an effective technique for isolating essential oil from citrus fruits (Lancas & Cavicchioli, 1990). Also, sequential extraction with toluene, citric acid and benzene has been proposed as a step prior to the determination of vinblastine in rose essential oil (Volkov & Grodnitskaya, 1994). The most common technique used to obtain essential oils from plant materials is steam or hydro-distillation. Analysis of the terpene hydrocarbon fraction of the essential oil obtained by the steam distillation of the fruit of *Schinus molle* L. was described by Bernhard & Wrolstad (1963). This technique has been applied extensively as a step prior to GC-MS for compositional studies of essential oils, as in the case of valerian (Bos et al., 1997), curcuma (Zwaving & Bos, 1992), cinnamon (Jayaprakasha et al., 1997) and rosemary, sage and lavender (Guilleèn et al., 1996). Steam distillation was also applied to obtain potato alcohols (Szafranek et al., 2006) and sesquiterpenes from potato varieties (Szafranek et al., 2005). Kawamura et al. (1999) reported using pressurized-liquid extraction (PLE) methods for extracting taxol from the bark of *Taxus cuspidate*. An interesting result from this study was that although in conventional extraction methods the taxol content of the water extract was very low, this was improved by use of the elevated temperature and pressure conditions of PLE. The influence of some experimental parameters on the PLE of *P. gaudichaudianum* Kunth leaves was elucidated (Peres et al., 2006). The optimization of the main variables involved in the PLE process (extraction temperature and time) was done using the extraction yield and the GC-MS profile of the extracts. The best results were obtained for the following parameters: 10 min and one extraction cycle at 85 °C.

Currently, supercritical fluid extraction (SFE) represents a new separation technique that is very suitable for the isolation of volatile compounds from plant matrices (Sedlakova et al., 2011). α-Cellulose has been used as a model plant matrix for investigating the conditions required to optimize the SFE of typical plant constituents – limonene, caryophyllene, carvone, eugenol and santonin – using $CO_2$ as the extraction medium (Smith & Burford, 1992); 250 bar and 40 °C were chosen as the optimum conditions. The effects of adding modifiers to the supercritical fluid were also examined. The chemical composition and antimicrobial activity of essential oil-rich fractions obtained by supercritical $CO_2$ extraction from *Rosmarinus officinalis* L. were investigated (Santoyo et al., 2005). The most active

fraction was obtained using 4% ethanol as modifier (extraction pressure – 25 MPa; extraction temperature – 60 °C). SFE with $CO_2$ was used for natural essential oil extraction from a Portuguese-grown rose geranium (*Pelargonium* sp.) (Gomes et al., 2007); the best extraction conditions were: extraction time 15–30 min, temperature 40 °C, pressure 90–100 bar. The $CO_2$ geranium extract had a superior organoleptic quality, with a very fresh natural floral–fruity character and a pale yellow colour, most suitable for use in perfumery.

New approaches such as stir bar sorptive extraction (SBSE) have recently been used with interesting applications. SBSE is one of the most prominent techniques recently employed by some authors to determine volatiles from grapes (Caven-Quantrill & Buglass, 2006; Moreno et al., 2008; Zalacain et al., 2007; Pedroza et al., 2010).

### 3.2 Headspace methods

Headspace (HS) methods are used for the separation of volatile compounds from complex solid matrices such as plant materials. Reproducible and rapid identification of volatile compounds in aromatic plants can be achieved when static HS sampling is coupled to GC-MS (Esteban et al., 1996). More recently, the capabilities of automated HS sampling in the analysis of volatile compounds from *Origanum vulgare* was developed (Garciá & Sanz, 2001). The application of automatic HS–GC to the determination of the safrole content in different *Asarum* species from China and Europe was also described (Stuppner & Ganzera, 1998).

Solid-phase microextraction is a more recent solvent-free extraction method eliminating most of the drawbacks of other extractive methods, such as solvent use, thermal decomposition of compounds, excessive time preparation and high cost. To detect terpenes using SPME fibres, one of three sampling techniques is generally applied: (i) exposing fibres to the headspace of vials containing liquid or solid samples (Isidorov et al., 2003; Adam et al., 2005; Kos et al., 2006; Santos et al., 2006; Vichi et al., 2006), (ii) exposing fibres to air circulating over the sample (Zini et al., 2001; Isidorov et al., 2005), or (iii) inserting fibres into a vial containing the sample (Lopez et al., 2006). Because of the small dimensions of the sampling device and the simplicity and speed of the extraction procedure, HS-SPME is able to collect fragrances from live plants with minimum disturbance of the specimen, under both laboratory and field conditions. So far, there have been many qualitative experiments reporting the relative composition of individual compounds based on chromatogram peak areas (Isidorov et al., 2003; Santos et al., 2006; Vichi et al., 2006). The usefulness of eight available SPME fibres was elucidated to evaluate the recoveries of some terpene components with different polarities and structures present in the headspace of four aromatic and medicinal plants: rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.) and valerian (*Valeriana officinalis* L.) (Bicchi et al., 2000). The results showed that the most effective fibres were those consisting of two components, i.e. a liquid phase (polydimethylsiloxane) and a porous solid (carboxen or divinylbenzene, or both). PDMS-SPME was used to study volatile compounds released by intact and mechanically damaged leaves of *Abies fraseri* (Vereen et al., 2000). After 5 min of extraction, monoterpenes such as 3-carene predominate; after 3 h, the major component in the chromatograms is bornyl acetate, with minor amounts of heavier compounds, e.g. camphor and borneol. PDMS/DVB fibres were used to quantify emissions of methyl chavicol, an oxygenated terpene from live branches (*Pinus ponderosa*) (Bouvier-Brown et al., 2007). In order to check the intra- and interpopulational variability of the terpene pattern, a total of 74

*Juniperus communis* samples were investigated by HS-SPME, GC-MS and GC-FID (Filipowicz et al., 2009). SPME−GC-MS was also used to determine monoterpene compounds in *Mentha piperita* (Rohloff, 1999). Author also compared the results obtained using SPME and steam distillation. Compared to solvent-based samples from essential oil distillation, relatively higher amounts of high-volatile monoterpenes and smaller quantities of less volatile compounds such as menthol and menthone were detected in SPME sampling.

### 3.3 Separation and detection of terpenes

Gas chromatography possesses inherent advantages that make it particularly attractive for the characterization and quantitative analysis of terpene mixtures. These include high separation efficiencies, short residence times in the chromatographic column, and the use of an inert atmosphere during analysis, the lack of azeotropes, and applicability to very small samples. In most cases capillary columns with dimethyl polysiloxane (methyl silicone) non-polar and Carbowax 20M polar phases are used. Carbowax 20M phases include DB Wax, BP-20, PEG 20M and HP 20, while methyl silicone phases include SE-30, SF-96, OV-1, OV 101, BP 1, CPSIL 5CB, SP 2100, DB 1, DB 5 and HP 1 (Davies, 1990). Among these fused-silica capillary GC columns, DB 1 or DB 5 and CPSil 5 are usually preferred.

One of the most important developments in gas chromatography was the introduction of enantioselective capillary columns with high separation efficiency in the mid-1960s by Gil-Av et al. (1966). At first, phases based exclusively on chiral diamide structures were used. The first optimum performance was achieved with the use of Chirasil-Val, a methyl–polysiloxane phase containing about 6% branched aliphatic side chains with L-valine in a diamide linkage, and similar polymeric chiral diamide stationary phases (König, 1987) possessing excellent thermal stability. Finally, capillary columns using different hydrophobic cyclodextrin derivatives were introduced. The first GC separations of enantiomers using CDs were obtained by Koscielski & Sibilska in 1983; they separated β- and α-pinene, and the corresponding pinanes and δ-3-carene with a column packed with underivatized CDs. CDs are generally carried in non-polar to moderately polar polysiloxanes, as first proposed by Schurig & Novotny (1990). The main reasons for this are the wider range of operating temperatures, the inertness and efficiency of columns prepared by high temperature silylation, the possibilities of tuning column polarity by using different diluting phases, the small amounts of CD necessary to prepare columns, the shorter analysis times, and the possibility of measuring the thermodynamic parameters involved in enantiomer discrimination. Papers published over the period 1989-94 concerning applications of cyclodextrin derivatives (CDDs) to the GC separation of volatile racemates in the essential oil, extract, flavour and aroma fields were reviewed by Bicchi et al. (1995).

Enantioselective GC has found a wide variety of applications, for instance, studies of citronellol (Ravid et al., 1992) and α-terpineol (Ravid et al., 1995) in a variety of species and verbenone in rosemary oils (Ravid et al., 1997). Chiral phases were applied to the separation of linalool and linalyl acetate in a variety of plant species (König et al., 1992; Cassabianca et al., 1998), extracts of *Angelica* seeds and roots (Holm et al., 1997) and other monoterpenoids in geranium oils (Kreis & Mosandl, 1993). Separation of monoterpenes in Scots pine and juniper oils (Hiltunen & Laakso, 1995), *Abies* (Holm et al., 1994) and *Picea* (Persson et al., 1996) oils and limonene, linalool, citronellal and β-citronellol (*Cymbopogon winterianus*)

(Lorenzo et al., 2000) also relied on chiral analysis. Studies of tea tree oil and other members of the *Myrtaceae* (Leach et al., 1993), sesquiterpenes (König et al., 1999; Cornwell et al., 2000; Bülow and König, 2000) and diterpenes (Pietsch and König, 1997; Pietsch and König, 2000) were also carried out.

The most common detection method used in gas chromatography is FID. The nitrogen–phosphorus detector (NPD) can be used to identify nitrogen-containing compounds (Stashenko et al., 1996). Another possibility is the use of oxygen flame ionization detection (O-FID) for the selective determination of oxygenates (Betts, 1994; Schneider et al., 1982). Mass spectrometry is a very useful tool for detecting complex terpene mixtures.

### 3.4 GC identification of terpenes

Retention indices are fundamental to making retention a reliable identification tool for GC. Identification is based on the direct comparison of retention times with standards or a precise knowledge of retention indices (Davies, 1990; Stashenko et al., 1993; Stashenko et al., 1996). Some 900 Kovats indices of 400 individual compounds were summarized from the general literature (Davies, 1990). A compilation such as that of Adams (1995) reveals an enormous number of compounds that are present in essential oils. The programmed-temperature retention indices listed in Adams' monograph were obtained on a DB-5 column. Identification is hampered, because the retention indices of many related components are similar. The presence of unsaturated bonds, various branched and cyclic compounds, and oxygenated analogues (e.g. alcohols and ketones) further complicate the issue. Problems still surround the variation of stationary-phase polarity and mobile-phase characteristics as a function of temperature in programmed analysis. An effective approach is to combine the retention data from stationary phases of different polarity. Apart from comparing obtained retention indices with values published in the literature, terpenes can also be identified using co-injection with standards.

### 3.5 GC-MS identification of terpenes

Nowadays the combination of GC-MS in electron impact mode is a well-established technique for the routine analysis of essential oils. With this technique, additional information can be obtained from mass spectra. However, it has to be emphasized that identification of terpenes based only on mass spectra is also virtually impossible. Molecular rearrangement and isomerization processes in unsaturated hydrocarbons result in very similar mass spectra lacking characteristic fragmentation patterns. There are several ways of solving this problem. One is to use GC-MS-MS (tandem mass spectrometry), which analyses each component of such complex peaks separately. Another is single ion monitoring (SIM), a very selective method that has turned out to be the most reliable procedure for quantitative analysis. Apart from these techniques, combined data of retention times, Kovats indices (Richmond and Pombo-Villar, 1997) and mass spectral data enable the unambiguous identification of sesquiterpenoid constituents. Numerous papers have recently been published on GC-MS techniques used to analyse essential oils and volatile compounds (MacLeod & Ames, 1991; Elias et al., 1997; Szafranek et al., 1998; Szafranek et al., 2005; Szafranek & Szafranek, 2008; Gołębiowski et al., 2008c; Gołębiowski et al., 2009).

The mass fragmentation of terpenes was widely discussed by Budzikiewicz et al. (1963), and the features of the mass spectra of mono-, bi- and tri-cyclic terpenoids were investigated

(Yermakov et al., 2010). It was established that the mass spectra of these compounds are absolutely identical in the mass values of fragment ion peaks, although there are minor differences in their relative intensities. The characteristic ions in the spectra of all compounds were $[M\text{-}CH(CH_3)_2]^+$ and $[M\text{-}CH(CH_3)_2\text{–}H_2]^+$. Eleven terpenoid derivatives from the extract including three steroids and eight pentacyclic triterpenes were identified by GC-MS in an extract of the stem bark of *Ficus mucuso* (Djemgou et al., 2009). HS volatiles from flowers, as well as green and ripe mango fruit of cv. Ataulfo from Soconusco, Chiapas, were identified by GC-MS (Sandoval et al., 2007). Chemical identification was confirmed by comparison of the mass spectra pattern with the NIST 2002 computer library and the retention times of synthetics. A new *cis*-sabinene hydrate chemotype was detected in large thyme (*Thymus pulegioides* L.) by Groendahl & Ehlers (2008). Analyses were done using an GC coupled to an inert mass selective detector with an ion source of 70.0 eV at 230 °C. Two columns of different polarities were used: one was an EC-Wax capillary column with oven conditions that included an isothermal hold at 60 °C for 5 min, followed by a ramp of 10 °C/min to 250 °C. The other was a HP-5MS capillary column with an initial oven temperature of 60 °C, followed by a ramp of 3 °C/min to 246 °C. Terpenes were identified using retention times of standards, the NIST 2005 mass spectral library and/or programmed-temperature retention indices. Five chemotypes were detected: carvacrol, linalool, geraniol and thymol, all of which are known to occur in large thyme. In addition, essential oils containing the monoterpene *cis*-sabinene hydrate as the dominant component were found. No sabinene hydrate chemotype has previously been detected in large thyme, although it does occur in other thyme species.

Nevertheless, sometimes mass spectra measured in EI mode are problematic, because they miss the molecular ions, especially esters. In this case, the application of GC-MS in chemical ionization (CI) mode using various reagent gases often yields valuable additional information. Negative ion chemical ionization (NCI) with OH as the reactant ion (Hendriks et al., 1985; Cazaussus et al., 1988) is an additional method. However, there is no characteristic fragmentation pattern in the mass spectra of terpenes obtained by NCI. For sesquiterpenes with one or two non-conjugated double bonds this problem can be solved by using trioxo(*tert*-butylimido)osmium(VIII) (Rücker et al., 1990). This reagent forms cyclic osmate ester amides as intermediates which, after reduction, yield vicinal mono-amino alcohols, bis-amino alcohols and aminotriols. The derivatives formed in such a reaction have characteristic mass spectra that can be used as a fingerprint for the identification of the respective parent compound.

### 3.6 Two-dimensional GC

While GC-FID is the traditional method for essential oil quantification, GC-MS is the most common analytical method for component identification. However, the wide concentration range of the analytes (from ppb to percentage levels), as well as the presence of numerous isomers (terpenes and oxygenated terpene structures), make qualitative analysis difficult. In addition, the mass spectra of these compounds are usually very similar, so peak identification often becomes very difficult and sometimes impossible.

Recently, comprehensive, two-dimensional gas chromatography (GC×GC) has been extensively used in studies of essential oils. Comprehensive GCxGC enhances the peak capacity for a chromatographic run, allowing better separation in complex sample analysis. GCxGC technology allows the use of two different separation mechanisms in order to increase the

separation power of the chromatographic system. The two directly coupled columns in GCxGC provide orthogonal separation of compounds, and importantly, enable the simultaneous two-column separation of the whole sample. Thus a combination of a non-polar column with a polar column may be a good first choice for a suitable dual column set for essential oils. The principles, practical and theoretical aspects, and the most significant developments of GC×GC were reviewed by Mondello et al. (2008). There are several reports of GCxGC used for determining essential oils (Dimandja et al., 2000; Shellie et al., 2003; Shellie et al., 2001).

The high resolution GC×GC separation of an essential oil and the identification of selected separated components by time-of-flight mass spectrometry (TOF-MS) was reported by Shellie et al., (2001). These authors showed that GC×GC allows orthogonal separation mechanisms on the two columns to achieve separation of components (e.g. borneol and terpinen-4-ol, and *cis*-caryophyllene and β-farnesene) that would otherwise be unresolved on a single column. The authors concluded that peak compression led to the generation of fast second-dimension GC peaks and a ca 25 times better detection sensitivity than with conventional GC elution. This allows many more compounds to be detected when using the GC×GC approach. GC-MS and GC×GC with FID were used in the analysis of peppermint (*Mentha piperita*) and spearmint (*Mentha spicata*) essential oils. Components including acetates, alcohols, furans, ketones, sesquiterpenes and terpenes were detected. The GC×GC chromatogram of peppermint essential oil displays 89 peaks compared to the 30 peaks in the GC-MS chromatogram; likewise, 68 peaks were detected in the GC×GC chromatogram of spearmint compared to 28 in GC-MS. This technique has been successfully used in the industrial analysis of plant materials to improve component separation and identification. In addition, analysis of *Artemisia annua* L. volatile oils using multi-dimensional gas chromatography has indicated that this technique can achieve the complete separation of a wide range of terpenes (Ma et al., 2007). These authors found nearly 700 components, the majority of which were terpenes. The investigation of the volatile compounds of dried rhizomes of *Coptis chinensis* was carried out by Gao et al. (2011). Volatile profiles were established and compared after headspace solid-phase microextraction using a PDMS/DVB fibre coupled to comprehensive 2D GC×GC-TOFMS. Analyses were performed and compared on two column-phase combinations (non-polar/polar and polar/non-polar). Terpenoids represented the most numerous group of compounds identified.

## 4. Conclusion

Modern analytical techniques can provide accurate and precise profiles of plant and insect cuticular waxes as well as terpenoids. For structural studies the most effective procedures are those based on a combination of mass spectra and GC retention index data.

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5

# Major Volatile Compounds Analysis Produced from Mezcal Fermentation Using Gas Chromatography Equipped Headspace (GC–HS)

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## 1. Introduction

Alcoholic beverages are produced from a variety of raw materials, principally fruits and cereals. Various microorganisms are used to ferment the sugars present in the must. Fermentation can be making using either the addition of specific microorganisms or spontaneous fermentation from naturally occurring microorganisms. Both, the microorganisms and the raw material affect the volatile compounds that are produced, and these volatile compounds are responsible for the aroma and unique taste of these beverages (Nykanen. 1983, Moreno. 2009).

In Mexico, alcoholic beverages produced during prehispanic times were used in religious rituals, as food, and for medicine (Lappe . 2008). *Agave* or "magueys" are used as principal raw material to produced alcoholic beverages. Since agaves grow best in arid and semiarid areas, they thrive in Mexico, where 45.3% of the land is semiarid. The Agave includes 20 genera, and the agave genus has 300 species, of which 200 species are indigenous to Mexico. The Mexican state of Oaxaca has the highest number of agave species (Valenzuela. 2007, Vázquez. 2007, Peña. 2004, SAGARPA 2004).

Agave species are used to produce both distilled and non-distilled alcoholic beverages, including: Tequila, mezcal, bacanora and raicilla, the first three has origin appellation mean only some places has the license to the production (www.impi.gob.mx). Another beverage is pulque a non-distilled alcoholic beverage (Lappe. 2008, Lachenmeier. 2006).

## 2. Mezcal process

Alcoholic beverages distilled from agave are produced in 6 stages: agave harvesting, cooking, milling, fermentation, distillation and aging, in the case of tequila, mezcal and bacanora in the official Mexican laws, the stages and other characteristics in the process are mentioned and most be followed for the factories (NOM-006-SCFI-1994 1994, SCFI 1997, SCFI 2004). The length of aging determines the type of mezcal: silver, reposado or añejo. While mezcal, tequila, raicilla and bacanora are produced using a similar process, they differ in the raw material used and the processing materials (equipment) and conditions. The next section briefly explains the mezcal process (figure 1).

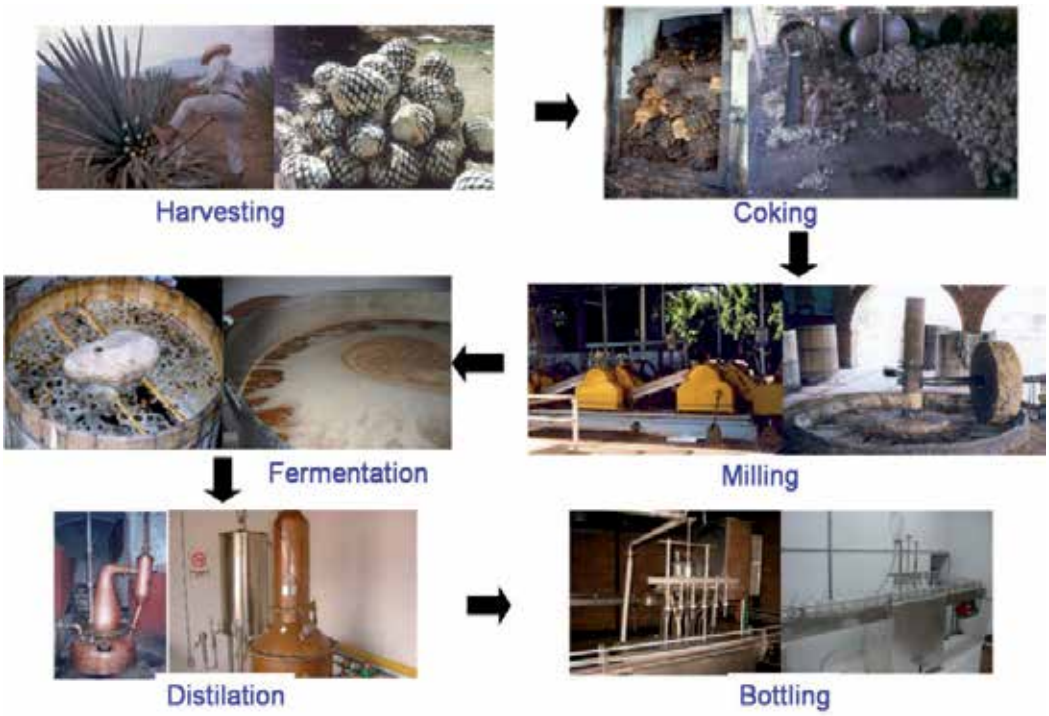


Fig. 1. Mezcal processes.

### 2.1 Harvesting the agave

The raw material for mezcal is *Agave*. In Mexico, different alcoholic beverages are produced from different agave species. Tequila is produced from *Agave tequilana* Weber blue variety, bacanora from *A. angustifolia* and raicilla from *A. inaequidens*. Mezcal is produced from a variety of different species, depending on the location where it is produced. *A. angustifolia*, *A. potatorum* and *A. karwinskii* are principally used in Oaxaca State; *A. durangensis* in Durango State; *A. salmiana* in San Luis Potosí, Guanajuato and Zacatecas States; *A. cupreata* in Guerrero; and finally, *A. angustifolia* is used in Tamaulipas State (SCFI 1997, Lappe. 2008).

The agave plant is grown for 5 to 9 years before it is harvested. After harvesting, the leaves stripped off in a process called "jimado" collecting the heart or "piña", which weighs from 25 to 100 kg depending on the agave species. Finally the agave piñas are transported from the fields to the mezcal factory.

### 2.2 Cooking

Since agaves contain oligofructans (López. 2003), they must be hydrolyzed to obtain fermentable sugars. This is done by cooking the agave piñas in ovens, after they have been cut into two to four pieces. There are different types of ovens: autoclaves, room ovens (or "mamposteria") and soil ovens. In the soil oven, the cooking time and temperature are not controlled, which affects the amount of hydrolysis. For room ovens and autoclaves, the temperature and time are controlled and the hydrolysis is uniform.

### 2.3 Milling

After cooking, the agave piñas are crushed with wood or steel mallets, or in a rudimentary mill using a large stone wheel 1.3 m in diameter and 0.5 m thick. The stone wheel is pulled by animals around a circular pit, crushing the cooked agave. The agave juice and the bagasse are collected and placed in fermentation tanks. Some factories have recently begun to use mills similar to those used in the sugar industry but smaller. In this case, water is added and the bagasse is separated, and only the agave juice is placed in the fermentation tank.

### 2.4 Fermentation

Mezcal factories start fermentation after the agave juice and the bagasse have been put into the fermentation tank; either by adding specific yeast, or by allowing the microorganisms present in the wort to ferment the juice spontaneously. Water is added as the temperature increases or an ethanol smell is detected (the quantity of water in the majority of factories is not precise). When yeast is added the fermentation last between 2 to 4 days, and spontaneous fermentation requires 2 or 3 weeks. The fermentation tanks are open and are fabricated from wood, stone, animal skins, or steel.

### 2.5 Distilling

After fermentation, the wort (agave juice and bagasse) is collected and put into the distillation equipment. Distillation separates ethanol and volatile compounds. The distillation is done in a pot still and rectifying column consisting of a kettle to hold the fermented wort and a condenser or a plate heat exchanger. Some factories use a steam coil to heat the wort, but most heat the kettle in a stove. Mezcal is distilled twice. In the first distillation, the alcohol concentration is between 20 to 40% by volume, after removing the first (heads) and last extraction (tails). The second distillation brings the concentration to 45 to 60 % by volume.

### 2.6 Aging

The maturation step is not mandatory and the white mezcal (silver) may be bottle at 35 to 50% alcohol volume. But, some factories put the mezcal in oak barrels for at least 2 months then the mezcal is called “mezcal reposado”, and if the time is more than a year is called “mezcal añejo”. The alcoholic degree is adjust to the level wanted.

## 3. Volatile compounds produced in mezcal

The aroma and taste of alcoholic beverages are critical to their acceptance by consumers, and the primary determinants of aroma and taste are volatile compounds. For mezcal, some of the volatile compounds are specified by Mexican laws (SCFI 1997), including methanol, higher alcohols (fusel alcohols), and the compounds that influence volatile acids like acetic acid. These specifications are shown in table 1.

Volatile compounds are produced throughout all mezcal production stages. Some derived directly from the raw material, but most of them are produced during fermentation, and, to

a lesser extent, during maturation, distillation and cooking (table 2). As was mentioned, the higher alcohols and methanol are produced in greater amounts than other volatile compounds like esters, aldehydes, ketones, carbonyls, acids, furans, and terpenes. More than 150 compounds have been found in tequila, and together they give tequila its characteristic aroma (Benn and Peppard. 1996). While the interaction between agave and yeast is very important, it is not completely understood, and more work is needed to elucidate how the agave juice influences the behavior of the yeast during fermentation. This will facilitate better control of the aroma in distilled agave beverages (De León. 2006, Molina. 2007, Alcázar-Valle. 2011). Since these volatile compounds are very important, some of their characteristics will be discussed.

| Specification | **Minimum concentration** | **Maximum concentration** |
|---|---|---|
| % alcohol by volume at 20ºC | 36.0 | 55.0 |
| Dry extract g/l | 0.2 | 10.0 |
| Milligrams per 100 cubic centimeters referred at alcohol anhydrous | | |
| Total acid (as acetic acid) | 0.00 | 170.0 |
| Higher alcohols mg/100 ml | 100.0 | 400.0 |
| Methanol mg/100 ml | 100.0 | 300.0 |

Table 1. Mezcal specifications under Mexican Laws (SCFI 1997).

| | *Agave* | **Cooking** | **Fermentation** | **Distillation** | **Aging** |
|---|---|---|---|---|---|
| **Mezcal** | Terpenes, fatty acids, saponins. | no data | Higher alcohols, esters, aldehydes, terpenes, furans. | Acetals, acids, alcohols, ketones, aldehydes, esters, phenols, furans, terpenes. | no data |

Table 2. Some of the volatile compounds generated in mezcal production (Peña. 2004, Molina. 2007, Alcázar-Valle. 2011).

### 3.1 Higher alcohols

The higher alcohols have strong aroma including: 1-propanol, 2-methyl-1-propanol (isobutanol), 1-butanol, 2-methyl-1-butanol (amyl alcohol), and 3-methyl-1-butanol (isoamyl alcohol). These compounds are frequently found in alcoholic beverages, and can be produced from amino acids by catabolic or anabolic reactions. The catabolic reaction starts with a deamination reaction followed by a decarboxylation to produce an aldehyde, which is finally catalyzed to alcohol by an alcohol dehydrogenase enzyme (Table 3). The other way is during the amino acid anabolism, when there are not enough amines in the media to produce amino acids. The α-ketoacids are decarboxylated producing aldehydes, which are then transformed to higher alcohols (Arrizon. 2001).

| Amino acid | α-ketoacid | Aldehyde | Higher alcohol |
|---|---|---|---|
| Threonine or metionine | α-ketobutyrate | Propilaldehyde | 1-propanol |
| Valine | α-ketoisovalerate | α-hidroxy-isovaraldehyde | Isobutylic |
| Leucine | α-ketoisocaproate | Isovaraldehyde | Isoamylic |
| Isoleucine | α-keto-β-methyl-valeric | α-hidroxy-isocaprylaldehyde | Amilic |

Table 3. Biosynthesis of higher alcohol (Berry. 1987, Arrizon. 2001).

### 3.2 Methanol

Methanol is produced during the cooking stage of the mezcal process. Agave contains pectin with methoxyl groups, which are broken by the high temperature in the ovens. Some yeast has a pectin-methyl-esterase enzyme, which splits the methoxyl groups from the pectins and produces methanol during the fermentation (Berry. 1987).

### 3.3 Esters

Esters give fruitlike flavors and aromas to beverages. They have a low odor threshold: only few milligrams can be detected. Production of esters is controlled by the enzyme, acyl-coenzyme A, and depends on the fermentation conditions and the yeast species used. In tequila, 47 different esters compounds have been identified, and the majority is ethyl acetate. In mezcal, 18 different esters compounds have been identified, primarily ethyl acetate, ethyl hexanoate, ethyl octanoate, and 2-phenethyl acetate (Gshaedler. 2004, Molina. 2007, Nykanen. 1983).

Ethyl acetate is produced by the yeast, *Saccharomyces cerevisiae,* using the acetyl alcohol transferase enzyme. It links the acetate from the acetyl-coA with an ethanol molecule. The esters produced can be affected by esterases enzymes, because they hydrolyze the aliphatic and aromatic esters. These enzymes can hydrolyze lipids to obtain medium chain fatty acids (Moreno. 2009). Production of esters is related to the quantity of amino acids. When the nitrogen from the amino acids is high, fatty acids decrease and acyl transferase is not inhibited (Arrizon. 2001).

### 3.4 Carbonyls

Among the most important carbonyls are the ketoacids, which are essential for amino acid synthesis and for the higher alcohols, since the carbonyls are intermediaries when the aldehydes are produced by the yeast (Nykanen. 1983). When the yeast viability or the cell activity is decreased, the yeast produces more aldehydes, because the metabolic reactions stop and fermentation cannot be completed (Nykanen. 1983, Moreno. 2009).

Acetaldehyde is the primary compound produced during alcoholic fermentation; the main biosynthesis is during the anabolic process by the pyruvate decarboxylase enzyme. Aldehydes are also produced during the maturation stage by oxidation of alcohols (Nykanen., 1983; Berry. 1987).

### 3.5 Terpenes

Terpenes are found in *Agave* in two classes: free or glycosylate (monoterpenes or sesquiterpenes). The monoterpenes are compounds with 10 carbon atoms. They have a strong aroma with a fruity or herbal odor. Previous works in agaves have found nine different terpenes in *A. salmiana*, eight in *A. angustifolia,* and 32 in *A. tequilana* Weber blue variety. During fermentation, the sesquiterpenes are hydrolyzed to monoterpenes by β-glycosidase enzyme produced by yeast. Moreover, some yeasts like *Saccharomyces cerevisiae*, *Torulaspora delbruckii* and *Kluyveromyces lactis*, have enzymes that transform a monoterpenes into one another. During wine and beer production, these changes give aromatic differences to the beverages (Peña. 2004, King. 2000, Takoi. 2010).

Mezcal studies have found terpenes including: α-terpineol, cintronellol, linalool, oxide trans-linalool, farnesol, a-nerolidol, α-terpinene and limonene, principally, but only in white mezcal, not during the process (Molina. 2007, De León. 2006).

### 3.6 Furans

The furans are a family of compounds that are produced during a thermal process. In mezcal they can be produced during either the cooking stage or distillation. The majority of the furan compounds are 2-furfuraldehyde and 5-hydroxymethhyl-2-furfuraldehyde. These are produced during thermal degradation of sugars (De León. 2006, Molina. 2007).

## 4. Identification and quantification of volatile compounds in mezcal fermentation by gas chromatography

Chromatography is a laboratory technique for separating mixtures in order to analyze their components. The chromatography bases are as follows: a sample is dissolved in a mobile phase and then the mobile phase is forced through an immobile, immiscible stationary phase. The compounds in the sample interact differentially with the stationary phase and are eluted with different retention time, separating the compounds in the sample (Skoog Doublas A. 1992). In gas chromatography (GC), the sample is vaporized and injected into the chromatographic column head. The elution is with an inert gas like helium or nitrogen which is non-reactive with the sample. In this method, there is no interaction between the sample compounds and the mobile phase. The sample characteristics must be understood in order to obtain the best separation of the compounds. All the compounds must be volatile at the injection temperature, because the gas chromatography column has a small diameter and a small particle can cover the injection system and obstruct the column (Kolb. 1997, Skoog. 1992).

For complex samples with solids or non-volatile compounds, it is necessary to first extract the components to others that can affect the system, but the sample needs to retain the original compounds as much as possible. Various methodologies have been developed to

analyze complex samples including distilling, liquid-liquid separation and head-space injection systems. However, it must be understood how these techniques effect the sample in order to determine the composition of the original sample.

In mezcal fermentation, as was mentioned, the wort includes agave juice which contains sugars, fibers (bagasse), yeast, brown color and dusty. Therefore, it is not possible to do a direct injection. Some investigators have used different methods to analyze fermented agave juice to evaluate its composition during the fermentation.

### 4.1 Extraction methodologies

Extraction methodologies are described in the following sections.

#### 4.1.1 Solvents extraction

The methodology most used in the analysis of food and beverages is solvents extraction. Solvents are added to the sample which does not react with the compounds in the complex mix (table 4). However, there are disadvantages to this method: it takes a long time to dissolve the components in the sample; and some non volatile compounds can be retained in the solvent. As a result, it is not possible to achieve 100% extraction (Kolb. 1997, Núñez. 1986).

The selection of the solvents is very important, because the compounds to be extracted must all be miscible in them. The solvents properties including: dielectric constant in order to obtain the desired polarity, the boiling point, the miscibility and the purity must all be known. Sometimes it is important to use azeotropic solvents to obtain a more complete extraction process.

#### 4.1.2 Steam distillation

This method is used when a pollution- free extraction is needed. It is often used when the compounds in the sample are volatile, immiscible with water, and have low steam pressure and high boiling point. The steam distillation is used principally for temperature sensitive materials like essential oils (table 4).

#### 4.1.3 Batch distillation

Batch distillation is used when the components in the sample are water soluble and are more volatile than water. The sample is heated until it is near its boiling point. Then the volatile compounds vaporize in the distillation system and condensed with a refrigerant. With this methodology, it is possible to separate only the non-volatile compounds (left in the flask with the sample) and obtain the same volume initial adjusted with water. Solvents with a low boiling point may be used to reduce the temperature required (table 4).

#### 4.1.4 Head-space extraction

There are other methods for analyzing compounds that don't require solvents for extraction. A sample of known weight and volume (liquid, solid, or liquid and solid mix), is put it in a closed vial, heated to the desired temperature, and maintained at that temperature while the

volatile compounds are vaporized, until equilibrium is reached between the sample and the free space. Then the volatile compounds are taken and injected into the GC (static head-space), or an inert gas may be added to the vial to increase the pressure and a sample in the volatile section is taken (dynamic head-space) and injected in the gas chromatograph (figure 2). This method is known as head-space extraction (Kolb. 1997).

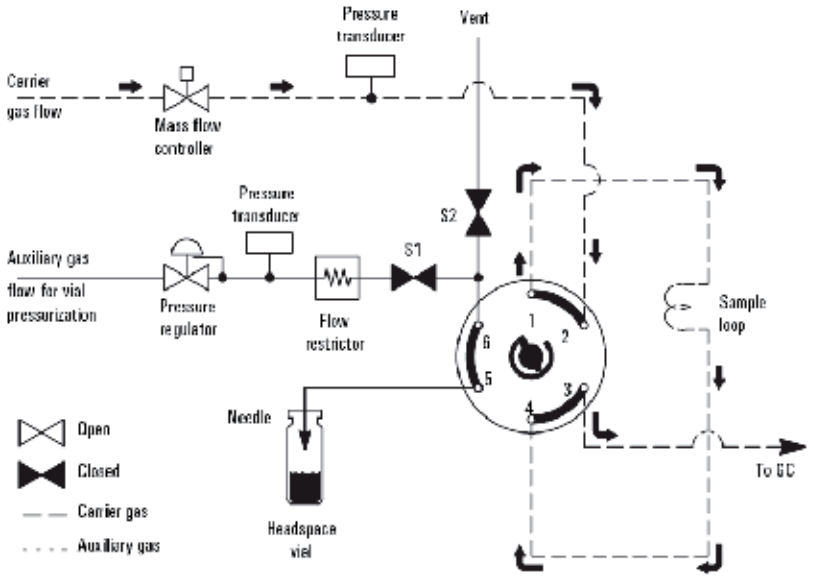


Fig. 2. Automatic **s**ample injection into headspace (Agilent 2000)

| Method | Advantage | Disadvantage |
|---|---|---|
| **Head-space** | Allows direct evaluation of the volatile compounds responsible for the odor in foods at the vapor phase. The analysis can be performed quickly at a low cost. | Its use is restricted to samples where the volatile compounds to be evaluated are not retained in the sample matrix.. |
| **Solvent extraction** | Ideal for the extraction of compounds that are neither lipids nor contain lipophilic substances | This technique is time consuming, the analyte generally is more diluted in the extraction and it is hard to avoid some non-volatile compounds in the extraction. |
| **Steam stripping** | Effective in the extraction and condensation of volatile compounds in water | Produces a large number of compounds by decomposition of the pH and temperature |
| **Batch distillation** | Requires small sample amounts, also, presents excellent yield of the extraction compounds | The appearance of compounds that are not part of the sample, from different phenomena (hydrolysis, oxidation, thermal reactions) |

Table 4. Comparison of extraction methods (Martín del Campo. 2011, Gshaedler. 2004).

The dynamic head-space technique is recommended, because thermodynamic equilibrium can be reached with all if the volatile compounds, decreasing the risk of not detecting important components in the samples. Moreover, it avoids having non-volatile compounds in the chromatograph (Núñez. 1986). To obtain better results, it is necessary to control the operating conditions, like agitation time, vial temperature, heat time, sample volume take in the head space, sample injected in the GC, etc. (Bylaite. 2006).

Studies of other alcoholic beverages like wine have used dynamic head-space to quantify and identify volatile compounds (Leino. 1993, Mestres. 1998). More than 45 volatile compounds have been identified, some of which had very low concentrations. Head space analysis is a good method for separating, quantifying, and identifying compounds and for determining the authenticity of the wines being tested (Etievant. 1986, Savchuk. 2001).

### 4.2 Volatile compounds produced during the fermentation stage in the mezcal process

The volatile compounds in mezcal and other alcoholic beverages have been determined in the final product (white and aged mezcal) (Molina. 2007, De León. 2006, Lachenmeier. 2006). However, to control the composition of volatile compounds in white mezcal, it is necessary to understand how each stage in the mezcal process influences the volatile compounds generation. More than 80% of the volatile compounds present in mezcal beverages are produced during fermentation (Lachance. 1995, Tellez-Mora. 2001), so it is important to understand how the different volatile compounds are produced and which factors influence the amount and types formed. In the case of mezcal there have been few studies are available related to the synthesis of the volatile compounds during fermentation (Segura. 2010, Alcázar-Valle. 2011). The next section will be explained the methodologies used to evaluate the volatile compounds.

#### 4.2.1 Batch distillation for quantifying volatile compounds in fermented agave juice

Arellano, et al. (2008) used batch distillation to evaluate the volatile compounds in fermented agave juice. The agave juice was distilled prior to the chromatographic evaluation. Their procedure was as follows: add 5 mL of agave juice to 5 mL of water in a boiling flask, then connect the flask to micro distiller with a vigreaux column to increase the reflux, next the vapor is condense by a refrigerant at 0°C, and the first 5 mL are recover. With this method, is recovered 5 mL volume of the sample separate to the color, sugars, fiber and other non-volatile compounds. Next, 0.5 μL of distilled agave juice is injected into a Hewlett Packard GC, 6890 series, using a DB-wax chromatography column 30 m. x 0.25 mm x 0.25 μm with an ionization flame detector (Arellano. 2008). The volatile compounds identified and quantified were: acetaldehyde, ethyl acetate, methanol, ethanol, 1-propanol, isobutanol, amyl alcohol and ethyl lactate using external standards. This methodology was used to compare the volatile compounds produced for different indigenous tequila yeast at two fermentation temperatures. The results showed that different volatile compounds were produced depending on the yeast strain and the fermentation temperature.

Batch distillation methodology has been applied in other works to evaluate the variability of volatile compounds produced in spontaneous fermentation. This has proved to be a good method to for evaluating the fermentation stage, and it is now used in the quality control systems of some tequila factories (Arellano. 2009).

It is important to know that the distilled sample contain high level of water, because the ethanol produced is about 8% by volume (63 g/L of ethanol). The water will contribute to column stationary phase degradation and thus the column's life span. Using this technique, it is possible to determine the levels of those volatile compounds which are governed by the Mexican quality laws for mezcal (SCFI 1997), tequila (NOM-006-SCFI-1994 1994) and sotol (NOM-159-SCFI-2004 2004) during the fermentation stage, prior to distillation. This enables the distillers to modify the distillation conditions to ensure that those compounds are at the appropriate levels.

### 4.2.2 Solvent extraction for quantify volatile compounds in fermented agave juice

Pentane is a non-polar solvent commonly used because extract ethanol at low levels, and it has an affinity for esters and fatty acids. Dichloromethane is often used because is less dangerous than pentane and can be easily purified, but is moderately polar. While there are no studies for mezcal covering this method for evaluating the volatile compounds in fermented agave juice, it has been used successfully for tequila (Martín del Campo. 2011, Prado-Jaramillo. 2002, Pinal. 2001).

The procedure used was as follows: 15 mL of a mixture of pentane and dichloromethane 3:1 v/v was added to 70 mL of fermented agave juice and agitated for 5 min. This solution was centrifuged to separate two phases. The supernatant was recovered and sodium sulphate was added in order to remove the water. Then, the extract was concentrated in a Kuderna – Danish flask with a vigreaux column. The system was heated to 40°C in a water bath, and the solution was evaporated up to 0.4 mL sample remain. Finally, it was injected in a GC-MS Hewlett Packard 5890 series II to separate the compounds using a DB-wax column 30 m. x 0.25 mm x 0.25 μm. The total ion chromatograms were obtained with an electron impact mode ion source working at 70 eV and tracked at 1.6 scans/sec. The compound identifications were made with a 5970 MS chemstation G1034 C version C 01.05, comparing the spectrums with a Wiley library or with the Kobats index (Prado-Jaramillo. 2002). To quantify the volatile compounds, the samples were injected again in a Gas Chromatograph with Flame Ionization Detector (GC-FID), using the same column and conditions as were used in Gas Chromatograph with Mass Detector (CG-MS) explained above.

Using this method 29 higher alcohols, 11 aldehydes, 16 organic acids, 18 ketones, 58 esters, 9 phenols, 12 furans, 26 terpenes, 4 sulfuric compounds, 5 hydrocarbons, 2 lactones, 2 pyrans and 15 others compounds were identified. In total, 207 volatile compounds were found. The higher alcohols had the high concentration and the esters were the most diverse compounds found (Prado-Jaramillo. 2002).

The solvent extraction methodology is a powerful tool for identifying and quantifying the volatile compounds in fermented agave juice, but it requires sufficient laboratory equipment and enough time to process all the information.

### 4.2.3 Headspace for quantify volatile compounds in fermented agave juice

The head-space methodology to analyze the volatile compounds in agave juice is another technique that can be used, since it does not require handling of samples and it is feasible to add solids in the vial which can influence in the sample volatile composition.

As was mentioned previously, in producing mezcal from fermented agave juice, the agave bagasse is added to the fermentation tanks during the fermentation stage. If only a liquid sample is taken, this will affect the composition. Therefore it is necessary to take a sample that contains solids in the same proportion as was in the wort (agave bagasse – water) at initial fermentation (Arellano. 2009).

To separate the volatile compounds, the sample quantity must be large enough that the minor components can be detected. With many samples, the more volatile compounds could fill the head space, making it difficult to detect the minor volatiles. Vials of different volumes can be used, depending on the separation desired. In the head space system it is necessary to heat the sample to vaporize the volatile compounds. The temperature must be regulated to obtain the kind of compounds that are wanted. With low temperatures, only the most volatile compounds will be vaporized, and if the temperature is near to the boiling point of water, a high level water vapor will get into the chromatography column.

Head-space methodology was used to evaluate samples of agave juice. Samples weighing 2 grams were put in a 20 mL vial, then they were put it in a head-space HP7694E programmed for these conditions: vial temperature 80°C, loop temperature 110°C, transfer line 115°C, vial equilibrium time 5 min, pressurization 2 min, loop filling 0.2 min, loop equilibrium time 0.5 min, injection time 1 min, injection volume 1 mL. The GC HP6890 with a FID detector was programmed as follow: the oven sloop was 55°C for 5 min, next increased at rate for 5°C/min to reach 160°C, next increased 25°C/min to 220°C, finally the temperature was maintained for 8 min. The chromatography column was an HP Innowax 60 m x 0.32 mm x 0.25 μm. The injector and detector temperature were 250°C. The injection used a 1:50 split. The analysis time was 45 min, including the extraction by head-space and GC.

The volatile compounds quantified were: acetaldehyde, ethyl acetate, methanol, 1-propanol, isobutanol, amyl alcohols, isobutanol, ethyl valerate, ethyl lactate, ethyl caprylate, ethyl caproate, 2-furfuraldehyde and 2-phenyl ethanol (figure 3). Two yeasts isolated from fermentation stage in the mezcal processes were used to evaluate the volatile production in different agave juice species (table 5 and table 6). Both yeasts are *Kluyveromyces marxianus* and the volatile compounds were produced at different levels, those results show the importance to select the yeast used in the process.

| Compound | Juice | | | | |
|---|---|---|---|---|---|
| | *A. angustifolia* | *A. cupreata* | *A. durangensis* | *A. salmiana* | *A. tequilana* |
| Acetaldehyde (ppm) | 296.03±3.32 | 305.26±0.50 | 23.08±1.71 | 54.23±1.54 | 39.84±0.16 |
| Ethyl acetate (ppm) | 58.12±7.83 | 22.72±2.76 | 155.03±6.02 | 80.32±0.43 | 53.65±1.34 |
| 1-propanol (ppm) | 20.98±4.32 | 35.51±0.41 | 14.28±0.28 | 22.84±1.65 | 15.89±0.46 |
| Isobutanol (ppm) | 316.91±5.432 | 116.63±1.90 | 113.92±14.86 | 267.15±12.43 | 136.24±4.41 |
| Amylic alcohol (ppm) | 242.67±13.26 | 170.36±2.54 | 90.06±0.05 | 170.22±22.31 | 157.95±2.90 |

Table 5. Volatile compounds produced during mezcal fermentation using different agave juices with the yeast OFF1 (*K. marxianus*)

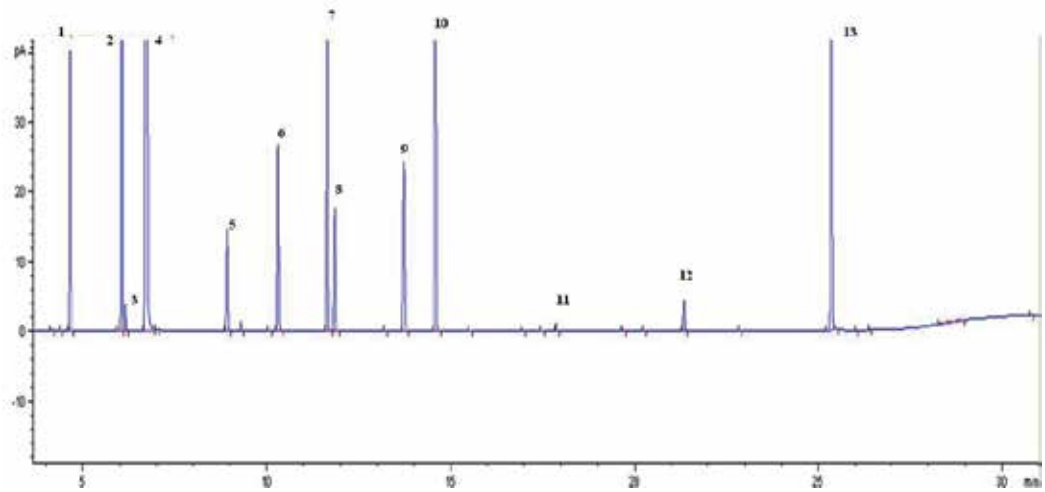

Fig. 3. Gas chromatogram of the volatile compounds in a fermented agave juice using headspace injection system: (1) Acetaldehyde, (2) ethyl acetate, (3) methanol, (4) ethanol, (5) 1-propanol, (6) Isobutanol, (7) ethyl valerate, (8) 1-butanol, (9) amyl alcohols, (10) ethyl caproate, (11) ethyl lactate, (12) 2-furfuraldehyde, (13) ethyl caprate.

Table 1 shows the Mexican law governing the contents of mezcal, which specifies the concentration of higher alcohols and methanol. The law for tequila specifies the levels of acetaldehyde, 2-furfuraldehyde and esters. Using head-space methodology it is possible to evaluate during the fermentation stage the volatiles compounds according to the Mexican laws for all alcoholic distilled agave beverages (NOM-006-SCFI-1994 1994, Arellano. 2009). It is also possible to quantify other compounds, but that requires changing the head-space and GC-FID conditions.

| Compound | Juice | | | | |
|---|---|---|---|---|---|
| | *A. angustifolia* | *A. cupreata* | *A. durangensis* | *A. salmiana* | *A. tequilana* |
| Acetaldehyde (ppm) | 74.95±11.14 | 82.05±0.04 | 30.49±6.10 | 84.23±9.21 | 33.73±1.02 |
| Ethyl acetate (ppm) | 88.85±6.81 | 34.01±0.05 | 205.93±0 | 100.71±19.86 | 60.82±5.49 |
| 1-propanol (ppm) | 19.73±0.82 | 14.32±2.93 | 16.40±1.89 | 24.12±4.65 | 16.51±0.30 |
| Isobutanol (ppm) | 186.79±2.06 | 30.89±6.30 | 70.74±8.12 | 235.84±19.21 | 66.93±1.94 |
| Amylic alcohol (ppm) | 171.79±3.05 | 88.76±0.05 | 101.43±0.05 | 287.83±6.73 | 109.20±9.79 |

Table 6. Volatile compounds produced during mezcal fermentation using different agave juices with the indigenous yeast SLP1 (*K. marxianus*).

Others studies using head space methodology have observed a difference in the volatile composition based on both agave species and yeast species (Segura. 2010, Alcázar-Valle. 2011). Terpenes have been found in mezcal and could be used to identify which agave was used as the raw material, but under the head-space conditions they could not be detected (Alcázar Valle. 2011). It is necessary change the conditions to look other volatile compounds that are important for these beverages (Molina. 2007). The head-space methodology can have less sample handling time and may be less costly than the others methodologies.

## 5. Conclusion and perspectives

The chromatographic systems to evaluate the fermented wort are a powerful methodology to indentify and quantify volatile compounds. The correct selection of the volatile compounds analysis is very important because the time and equipment needed is different for each methodology. Batch distillation is the most common system used in a laboratory since it is easy and cheap. However the life span of the chromatography column can be reduced by samples that have high water content. The solvent extraction allows obtain many volatile compounds, but is necessary a GC-MS to identify the desired compounds and GC-FID to quantify them, it is needed much sample to the extraction and also the solvents are dangerous to the operator and to the environment. With the dynamic head-space methodology is possible to identify and quantify the volatile compounds in the Mexican laws regulated and other more, but it is better has an automatic equipment to avoid the sample handling.

Finally, there are other methodologies that can be used to evaluate the volatile composition in fermented agave juice for the mezcal production, like the membranes to solid phase micro extraction (SPME). This method avoids separating the specific compounds, but it is necessary find the correct conditions in order to obtain the desired results.

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# Part 2

## Wine Technology

# 6

# Using Odorant Series as an Analytical Tool for the Study of the Biological Ageing of Sherry Wines

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## 1. Introduction

By using analytical techniques, the number of characterised compounds in white wine has been increased to more than 800 (Etievant, 1991; Bayonove et al., 2000). However, only a limited number of them have any significance in the determination of flavour. A method is therefore needed in order to measure the aromatic potency of the different volatile compounds so as to distinguish those compounds which contribute significantly to the overall aroma of wine. With this in mind, several authors (Guth, 1997; Audoin et al., 2001; Aznar et al., 2001; Moyano et al., 2002; Lopez et al., 2003; Rocha et al., 2004; Moreno et al., 2005; Chaves et al., 2007; Zea et al., 2007, 2008; Moyano et al., 2009, 2010; Ruiz et al., 2010; Zea et al., 2010) propose an estimation of the importance of a flavour compound on the basis of its Odour Activity Values (OAVs). Using the calculated OAVs and odour descriptors, we study the odour profile of sherry wines in order to identify differences in the typical aroma compounds ascribable to the ageing procedure used (whether biological or oxidative). However, the large number of compounds involved in the aroma fraction of wine, and the differential odour impact of each one, makes any conclusions difficult and speculative. As such, the aroma fraction of sherry wines was studied by grouping into odorant series (OSs) the odour activity values of the compounds exhibiting similar odour descriptions. This method has the advantage that it greatly reduces the number of variables to be interpreted, preserving their relative importance according to the OAV of each compound. The OS, as an analytical tool, allows the comparison of the aroma fractions of Fino wines with different ageing times so as to study the changes in the aromatic fingerprints during the ageing stage of wines.

## 2. Sherry wines

The well-known sherry wines are principally produced in two southern regions of Spain, Jerez and Montilla-Moriles. The climate of these regions largely determines the typical composition of these wines and includes moderately low average minimum temperatures (lows of 5 ºC in winter and highs of 17 ºC in summer) and high temperatures in the summer (above 35 ºC or even above 40ºC during the grape-ripening stage). The region is a dry zone, where rainfall is unevenly distributed throughout the year and at times is around 400 mm

below average. These favourable climatic conditions allow the white grape varieties used to obtain sherry wines (Palomino Fino and Pedro Ximenez) to reach a high reducing sugar concentration, above 250 g $L^{-1}$ in some areas. Due to this, fortification is not necessary after the alcoholic fermentation of the base wine since it has a high ethanol content, of about 15 % (v/v), which is obtained in a natural manner. The musts have a low acidity (3-4.5 g $L^{-1}$ as tartaric acid) which is generally increased by the winemakers. Moreover, the musts are added to a $SO_2$ concentration of around 100 mg $L^{-1}$.


Fig. 1. Sherry-type wines: Fino, Amontillado, Oloroso and Pedro Ximénez

Following harvesting, the grapes are stemmed and pressed. Plate presses are most widely used for this purpose, even though pneumatic ones are more efficient (as they apply pressure in a more uniform manner). The must obtained from the first pressing – called "yema" – and that obtained in subsequent ones, is centrifuged or racked to remove suspended solids. It is then pumped into stainless steel containers or concrete cones and allowed to ferment. During the process, special care is exercised to prevent the temperature from rising above 25-26 $^{o}$C. The refrigeration systems currently available can efficiently maintain even lower temperatures.

So-called Fino, Amontillado and Oloroso wines (Figure 1) are three typical types of dry sherry wines obtained under identical fermentation conditions (with same wine base) but with different ageing conditions and procedures as part of the "criaderas" and "solera" system. A more detailed description of this system can be found in the next section. Thus, while Fino wines are aged exclusively by biological ageing, Oloroso wines are obtained by chemical ageing only. However, Amontillado wine is aged by involving both methods sequentially, so that the wine initially undergoes biological ageing – as with a Fino wine –

and then chemical ageing, like an Oloroso wine. The first type of sherry wine has a very light yellow colour, with an almond flavour and pungent notes, while the second type has a very dark colour that results from the oxidation of phenolic compounds, and a flavour with distinct notes of oak and walnut. Finally, the third type of sherry wine has a colour in between the two previous wines, but closer to the Oloroso wine, and it has a flavour with hazelnut notes that is the most complex among the three (Medina et al., 2003).

A special sherry is the famous Pedro Ximenez sweet wine (Figure 1) which is produced from raising grapes containing more than 400 g $L^{-1}$ of sugars. The musts obtained are fortified to an ethanol content of 13.5% (v/v) and are afterwards subjected to oxidative ageing in a "criaderas" and "solera" system.

### 2.1 The "Criaderas" and "Solera" ageing system

Essentially, this industrial ageing method involves storing the wine in 500 L American oak casks, which are stacked in rows called "escalas". The casks in each "escala" contain wine of the same degree of ageing. The first "escala" – called the "solera" – is that closest to the ground and it contains the oldest wine. A fraction of its volume is withdrawn periodically for bottling. After each withdrawal, the casks of the "solera" are replenished with wine from the second "escala" – also called the first "criadera" – which in turn is replenished with wine from the third "escala" (the second "criadera") and so forth. The topmost "escala" – called the "añada" – contains young wine from the year's vintage. More detailed information about this traditional ageing method can be found in the papers by Casas (1985), Domecq (1989), and Zea et al. (1996, 2008).

## 3. The biological ageing of sherry wines

The production of Fino type sherry wine involves a long biological ageing process (5–7 years) that is carried out by "flor" yeasts. More than 95% of these yeasts are identified as S*accharomyces cerevisiae* races (Martinez et al., 1997). These microorganisms grow spontaneously on the wine surface after the alcoholic fermentation of the must and develop an aerobic metabolism that endows the resulting product with typical sensory properties as regards the aroma (Cortes et al., 1998; Moyano et al., 2002; Moreno et al., 2005). The biosynthesis of acetaldehyde and the consumption of ethanol, glycerine and volatile acids by the "flor" yeasts are the most typical and best known reactions which take place in wine during biological ageing. Acetaldehyde is synthesised by means of the enzyme alcohol dehydrogenase in the presence of $NAD^+$ (Garcia-Maiquez, 1988). According to Zea et al. (2001), the acetaldehyde content allows the differentiation of Fino wines from other types of sherry wines produced by oxidative ageing. Acetaldehyde is responsible for the pungent character typical of Fino wine and it directly contributes to the ethereal and ripe apple notes of its aroma. Likewise, this compound is a precursor for the synthesis of other odorant products, thereby indirectly contributing to several distinctive notes to the aroma profile of wine. "Flor" yeast also increases the contents of other aroma compounds such as higher alcohols and acetates, ethyl esters, lactones and terpenes (Zea et al., 1995). Consequently, the aroma composition of the biologically aged wine is rather different of young wine (Moyano et al., 2002).

To facilitate the ageing of the wine, cellars must be conditioned to maintain a constant temperature of 15-18 ºC, with a relative humidity as high as possible and efficient ventilation throughout the year. This procedure provides the wine with similar sensory properties year after year and allows the yeasts to be brought into contact with younger wines, which supply the nutrients required for the "flor" (yeast film) to develop and grow and thereby ensure the desired homogeneity and inalterability in the ageing of the Fino wines. In addition, "flor" yeasts protect the wine from chemical browning, preserving its pale colour throughout the ageing period (Baron et al., 1997). Moreover, the compound released by the wood of the casks and used in the ageing is the second most important way of contributing to the aroma of the wine. The type of wood, in addition to the ethanol content of the wine and the temperature of the cellar, are the main factors that influence the efficiency of extraction of such compounds (Singleton, 1995; Arapitsas et al., 2004). As a result, the Fino type is a dry white wine and is considered to be the world's finest sherry.

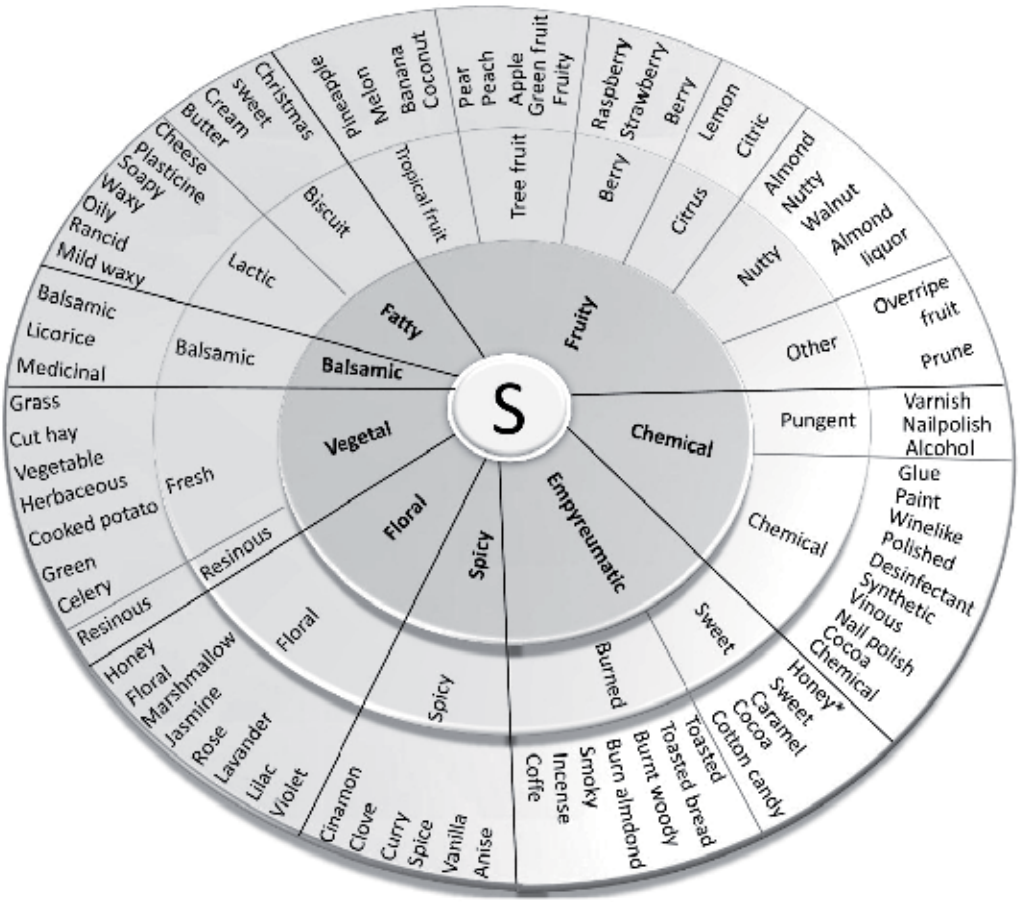
## 4. The sherry aroma wheel

With improved analytical methods there has been a large increase in the number of compounds identified in wines. Volatile compounds from different families (such as alcohols, esters, aldehydes, terpenes, etc.) play an important role in the organoleptic characteristics of wines. This wide variety of compounds with different chemical properties and with different concentrations makes the flavour profile of wines very complex. Therefore, it is necessary to standardise the terminology so as to facilitate the knowledge of the aromatic profile of wines.

In an article by Noble et al. (1984), the authors proposed an aroma wheel to standardise the terminology of the aromas of wines and so facilitate the communication among the members of the wine industry. The "Wine Aroma Wheel" provides a graphical representation of the different categories and aromatic components that can be found in the wine. Subsequently, a modified version of the wine aroma wheel (Noble et al., 1987) was constructed in order to clarify and improve the proposed list of standardised terminology. This version has been widely used to describe the flavour profiles of many types of wines in the world. However, many of the descriptive terms for wines on the wheel are not characteristic of sherry wines, for which some are not even listed. For this reason, it is necessary to define a wheel for sherry wines due to the characteristic flavour profile that they present. As far as we are aware, no aroma wheel has been developed for sherry wines.

The aim of this section is, therefore, to demonstrate a "Sherry Aroma Wheel" (Figure 2) in order to standardise the terminology for the aromatic description of these types of wines.

As can be seen, at the centre of the wheel are the eight odorant series (OSs) which group the odorant terms that define the flavour profile of these wines: fruity, chemical, empyreumatic, spicy, floral, vegetal, balsamic and fatty. By reading outwards first, from the odorant terms to the odour descriptors, a more precise description of the sherry aroma can be offered.




Moyano, L., Ruiz, M.J. & Zea, L., 2011 ©
Fig. 2. Sherry Aroma Wheel showing the odorant series (OSs), odorant terms and odour descriptors

## 5. Sherry wines studied

Very pale sherry wines, (Fino-types) obtained from the grape cultivar Pedro Ximenez – which were subjected to biological ageing in aerobic conditions in American oak casks – were used. Because the concept of "vintage" is not applicable to sherry wines, for better precision the ageing times were calculated following commercial criteria (by considering the age and volume of the mixed wines in the "solera" and "criaderas" system), resulting in times of 0 (without ageing), 1.5, 2.5, 4.5 and 6 years (named "solera") under veil yeasts. These last samples are commercially considered to be high quality typical Fino sherry-type wines. Three different samples for each ageing time were used, and each sample was obtained by mixing the wine extract from 20 casks. Also, the samples were selected by expert tasters as being more representative among the wines produced in 21 cellars from the Montilla-Moriles region.

## 6. Aroma compounds analysis

### 6.1 The identification and quantification of aroma compounds

Each of the aromatic compounds analysed was identified by means of its retention time, coeluted with a standard solution of the commercial product and confirmed by mass spectrometry (Hewlett-Packard 5972 MSD, Palo Alto, CA, USA). Positive ion electron impact mass spectra were acquired in scan mode, with a range of m⁄z 39–300 and a scan-rate of 1.6 scan $s^{-1}$. For each compound the mass spectra was confirmed by comparison with the Wiley mass spectral library. The chromatographic column, injector and oven temperatures, carrier gas and its flow, were the same as those used for the quantification, as described below.

The volatile compounds were quantified by capillary column gas chromatography after continuous extraction of 100 mL of a wine sample with 100 mL of freon-11 for 24 h. Previously, the wine was adjusted to pH 3.5 and 5 mL of internal standard (30 mg $L^{-1}$ of 2-octanol) was added. The freon extract containing the volatile compounds was concentrated to 0.2 mL in a Kuderna-Danish microconcentrator and 3 µL was injected into a Hewlett-Packard-5890 series II gas chromatograph equipped with an HP-INNOWax fused silica capillary column (60 m x 0.32 mm ID, 0.25 µm film thickness), with a FID and a sniffing port connected by a flow-splitter to the column exit. The oven temperature programme was as follows: 5 min at 45 $^{o}$C, 1$^{o}$C $min^{-1}$ ramp to 185 $^{o}$C and 30 min at 185 $^{o}$C. The injector and detector temperatures were 275 $^{o}$C and 300 $^{o}$C, respectively. The carrier gas was helium at 70 kP and split 1:30. The quantification was made using chromatographic response factors, calculated for each compound in relation to the internal standard in standard solutions of commercial products supplied by Sigma-Aldrich (Munich, Germany). The acetaldehyde was quantified by using the enzymatic test from R-Biopharm (Darmstadt, Germany).

### 6.2 Threshold, odour description and odorant series

Although the odour descriptions and thresholds of the compounds can be obtained from previous research and bibliographic sources (Singleton, 1995; Kotseridis & Baumes, 2000; Lambrechts & Pretorius, 2000; Aznar et al., 2001; Lopez et al., 2003), the high content of ethanol in the studied wines advised their determination by a taste panel. The taste panel consisted of 20 judges of both sexes (between 20 and 55 years old), trained but not selected. The threshold is defined as the lowest concentration capable of producing a sensation. This sensation must be detected by at least 50% of the judges in a taste panel. Five solutions of ascending concentration of each compound (supplied by Sigma Aldrich, Germany) were used. Starting from the solution with the lowest concentration, the judges indicated an odorant sensation different to that perceived by the control (14% v/v ethanol/water). Likewise, the judges were asked for the aroma descriptors, and these were fixed by comparing with bibliography. The non-descriptive or abstract terms were eliminated. The coincident (or not very dissimilar) terms provided by at least 50% of the judges were considered to be similar and grouped in the eight OSs. The series used in this work grouped compounds with similar odour descriptors and represented the main constituents of the aroma profile of the Fino wine: fruity, chemical, balsamic, vegetal, fatty, empyreumatic, floral and spicy odours. Because of the high complexity of olfactory perceptions, some aroma compounds were included in two or more OSs, according to the findings of certain authors (Charles et al., 2000; Cliff et al., 2002; Rocha et al., 2004). Table 1 shows the odour descriptions, OSs and thresholds of the aroma compounds studied.

| Compound | Odour description | Odorant series(*) | Threshold ($mg\,L^{-1}$) |
|---|---|---|---|
| Acetaldehyde | overripe apple | 1 | 10 |
| Ethyl acetate | pineapple, varnish, balsamic | 1, 2, 3 | 7.5 |
| 1,1-Diethoxyethane | green fruit, liquorice | 1, 3 | 1 |
| Methanol | chemical, medicinal | 2, 3 | 668 |
| Ethyl propanoate | banana, apple | 1 | 5 |
| Ethyl isobutanoate | strawberry, melon | 1 | 0.015 |
| Propyl acetate | glue, celery, Christmas sweet | 2, 4, 5 | 65 |
| 2,3-Butanedione | butter | 5 | 0.1 |
| Methyl butanoate | strawberry, glue, cheese | 1, 2, 5 | 1 |
| Isobutyl acetate | apple, banana | 1 | 6.14 |
| 2-Butanol | vinous | 2 | 1000 |
| 1-Propanol | ripe fruit, alcohol | 1, 2 | 830 |
| Ethyl butanoate | banana, pineapple, strawberry | 1 | 0.020 |
| Butyl acetate | banana, ripe pear, glue | 1, 2 | 4.6 |
| Isobutanol | alcohol, wine like, nail polish | 2 | 40 |
| Isoamyl acetate | banana | 1 | 0.030 |
| 1-Butanol | medicinal | 3 | 820 |
| Isoamyl alcohols | alcohol, nail polish | 2 | 65 |
| Ethyl hexanoate | banana, green apple | 1 | 0.005 |
| 1-Pentanol | bitter almond, synthetic, balsamic | 1, 2, 3 | 676 |
| *p*-Cymene | citric, solvent, synthetic | 1, 2 | 66 |
| Ethyl pyruvate | vegetable, caramel | 4, 6 | 100 |
| Acetoin | buttery, cream | 5 | 30 |
| Octanal | green, honey, soapy | 4, 5, 7 | 0.64 |
| 4-Methyl-1-pentanol | almond, toasted | 1, 6 | 50 |
| 3-Methyl-1-pentanol | vinous, herbaceous, cocoa | 2, 4, 6 | 50 |
| Ethyl lactate | strawberry, raspberry, buttery | 1, 5 | 100 |
| 1-Hexanol | grass, resinous, cream | 4, 5 | 8 |
| 3-Ethoxy-1-propanol | overripe pear | 1 | 50 |
| Z-3-hexenol | grass, green | 4 | 100 |
| Ethyl octanoate | pineapple, pear, soapy | 1, 5 | 0.002 |
| 1-Heptanol | oily | 5 | 2.5 |
| Isobutyl lactate | banana, pear, balsamic, vegetal | 1, 3, 4 | 340 |
| Furfural | burn almond, incense, floral | 6, 7 | 15 |
| Ethyl 3-hydroxybutanoate | grape, marshmallow | 1, 7 | 67 |
| Benzaldehyde | bitter almond, nutty, smoky | 1, 6 | 5 |
| 1-Octanol | varnish, soapy, waxy, jasmine, rose | 2, 5, 7 | 10 |
| 5-Methylfurfural | bitter almond, spice | 1, 8 | 16 |
| Isobutanoic acid | rancid butter | 5 | 20 |
| *γ*-Butyrolactone | coconut, caramel | 1, 6 | 100 |
| Butanoic acid | rancid, cheese | 5 | 10 |
| Furfuryl alcohol | medicinal | 3 | 15 |
| 3-Methylbutanoic acid | parmesan cheese, rancid | 5 | 3 |
| 2,3-Butanediol | buttery, creamy | 5 | 668 |
| Diethyl succinate | overripe, lavender | 1, 7 | 100 |
| Neral | lemon, citric | 1 | 1 |
| Ethyl 3-hydroxyhexanoate | rubber | 3 | 0.045 |
| α-Terpineol | lilac | 7 | 38 |
| Methionol | cooked potato, cut hay | 4 | 0.5 |

**(*)** 1= fruity, 2= chemical, 3= balsamic, 4= vegetal, 5= fatty, 6= empyreumatic, 7= floral, 8= spicy

Table 1. Odour descriptions, odorant series and threshold of the aroma compounds in sherry-type wines.

| Compound | Odour description | Odorant series(*) | Threshold (mg $L^{-1}$) |
|---|---|---|---|
| 1-Decanol | pear, polished, waxy, violet | 1, 2, 5, 7 | 5 |
| β-Citronellol | rose | 7 | 0.1 |
| Phenethyl acetate | rose, honey | 7 | 0.25 |
| Hexanoic acid | cheese | 5 | 3 |
| Benzyl alcohol | disinfectant | 2 | 900 |
| E-Oak lactone | coconut, burn woody, vanilla | 1, 6, 8 | 0.122 |
| Phenethyl alcohol | rose, honey | 7 | 10 |
| Z-Oak lactone | coconut, burn woody, vanilla | 1, 6, 8 | 0.035 |
| Pantolactone | liquorice, toasted bread | 3, 6 | 500 |
| 4-Ethylguaiacol | toasted bread, smoky, clove | 6, 8 | 0.046 |
| Diethyl malate | overripe peach, prune | 1 | 760 |
| Z-Nerolidol | apple, vegetal, waxy, rose | 1, 4, 5, 7 | 64 |
| Ethyl myristate | mild waxy, soapy | 5 | 494 |
| Octanoic acid | rancid oily | 5 | 8.8 |
| γ-Decalactone | peach | 1 | 1 |
| Eugenol | cinnamon, clove | 8 | 0.005 |
| 4-Ethylphenol | disinfectant | 2 | 140 |
| Decanoic acid | rancid, waxy | 5 | 15 |
| Phenethyl octanoate | synthetic, waxy, rose | 2, 5, 7 | 10 |
| Monoethyl succinate | caramel, coffee | 6 | 1000 |
| Lauric acid | waxy, soapy | 2, 5 | 10 |
| Sotolon | walnut, cotton candy, curry | 1, 6, 8 | 0.005 |
| Ethyl furoate | glue, paint | 2 | 1 |

**(*)** 1= fruity, 2= chemical, 3= balsamic, 4= vegetal, 5= fatty, 6= empyreumatic, 7= floral, 8= spicy

Table 1. (continued). Odour descriptions, odorant series and threshold of the aroma compounds in sherry type wines.

### 6.3 Statistical procedures

The OAVs and the basic statistics of the OSs were presented as the mean ± SD of three samples for each ageing time. ANOVA and linear regression analysis were performed on the triplicated samples by using the Statgraphics 5.0 computer program (STSC Inc., Rockville, MD, USA).

## 7. Odorant series as analytical tool in sherry-type wines

The OSs used as an analytical tool for the study of Fino wines have already been defined in Table 1 and were calculated by adding the OAVs of all the corresponding compounds (including those with OAVs < 1). From a theoretical point of view, these compounds did not directly contribute to the aroma profile, although some authors consider that these compounds may enhance some aromatic notes due to their synergistic effects with other odorant compounds (Freitas et al., 1999; Lopez et al., 1999).

It should be noted that the values obtained in the OSs do not necessarily represent arithmetic sums of the aroma perceptions. However, this method largely facilitates the comparison of the aroma profiles of wines of the same type, because the OSs used always included the same compounds.

The aroma compounds studied during the biological ageing of Fino wines belong to different chemical families. The higher alcohols determined were methanol, 2-butanol, 1-propanol, isobutanol, 1-butanol, isoamyl alcohols, 1-pentanol, 3- and 4-methyl-1-pentanol, 1-hexanol, 3-ethoxy-1-propanol, *Z*-3-hexenol, 1-heptanol, 1-octanol, furfuryl alcohol, 2, 3-butanediol, 1-decanol, benzyl alcohol and phenethyl alcohol.

Esters constituted the largest family and included acetates (ethyl, propyl, isobutyl, butyl, isoamyl, and phenethyl), ethyl esters of fatty acids (propanoate, isobutanoate, butanoate, hexanoate, octanoate, 3-hydroxybutanoate, 3-hydroxyhexanoate and furoate), ethyl esters of organic acids (pyruvate, lactate, ethyl myristate, diethyl malate and, mono- and diethyl succinate) and various other esters, such as methyl butanoate, isobutyl lactate and phenylethyl octanoate. The acids quantified included isobutanoic, butanoic, hexanoic, octanoic, decanoic, lauric and 3-methylbutanoic. The lactones included γ-butyrolactone, pantolactone, γ-decalactone and *E*- and *Z*-oak lactone; and the terpenes included neral ά-terpineol, β-citronellol and *Z*-nerolidol. The aldehyde family comprised acetaldehyde, benzaldehyde, furfural, 5-methylfurfural and octanal, and the phenol family included eugenol, 4-ethylphenol and 4-ethylguaiacol. Finally, 1, 1-diethoxyethane, acetoin, sotolon, 2, 3-butanedione, *p*-cymene and methionol were also determined.

The next four sub-sections show the different ways of using the OSs as analytical tools for the study of the biological ageing of sherry wines.

### 7.1 Using the odorant series to estimate the degree of biological ageing of sherry wines

In this section, we study changes in the OSs during the biological ageing of Fino sherry-type wines. Likewise, those OSs exhibiting a high correlation with the ageing time could be used as indicators for the degree of ageing of this type of wine.

As can be seen in Figure 3, all of the series showed OAVs>1 during the ageing process with the exception of the vegetal at 1.5 years. In addition, the OAVs for the eight series were higher after 6 years of ageing than at the beginning, providing evidence of an enrichment of the wine aroma. However, the floral OAVs, for the whole period studied, and the chemical OAVs, after 2.5 years, remained relatively constant, suggesting that these aroma series are unrelated to the biological ageing process. The major series throughout the process was fruity, with a maximum average OAV of 421 at 6 years of biological ageing (51.6% of the total aroma). The fatty and spicy series, with OAVs of 163 and 130 respectively, accounted for 20.0% and 15.9% of the total wine aroma at the end of the ageing process. The remaining series studied showed OAVs below 50 and, as a whole, accounted for only 12.5% of the aroma profile of the final aged wine.

By examining the changes of the OSs during the ageing period, one can identify those that are most closely related to the process and that can thus be reasonably used as aroma indicators for the degree of ageing of Fino wines. With this purpose in mind, some simple regression models were applied to the OAVs for the series at different ageing times. In most cases, the differences among the correlation coefficients were too small to justify a clear selection for a specific model. From a practical point of view, the linear regression model was chosen because interpretation is more intuitive and simple. The results thus obtained are shown in Table 2. Nevertheless, the chemical, balsamic, and floral series exhibited

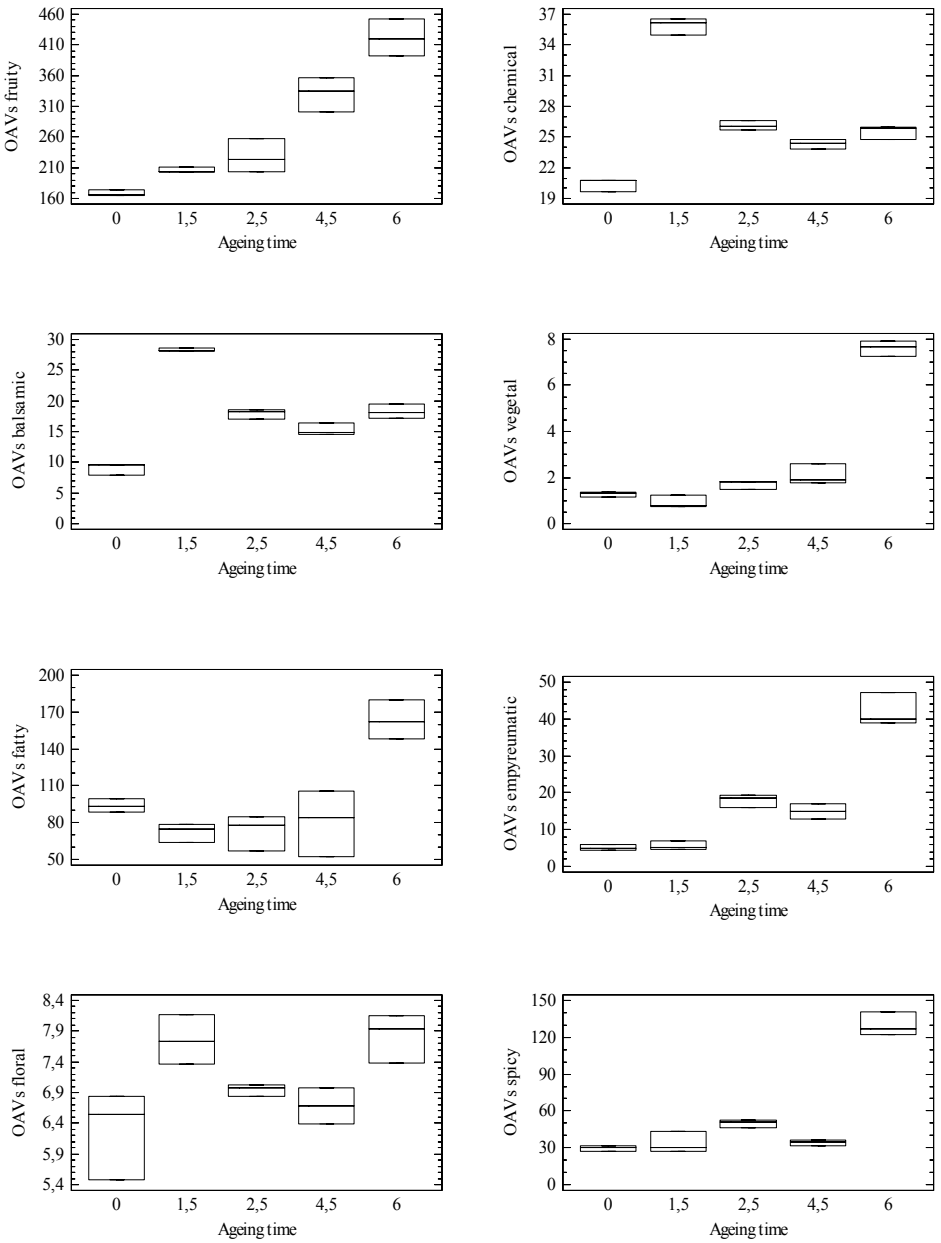



Fig. 3. Odour activity values (OAVs) of the odorant series (OSs) in sherry-type wines during their biological ageing.

$p$>0.05, so their fittings to a linear regression were not calculated. As can be seen, the OAVs for the fruity, empyreumatic and vegetal series exhibited very high significance ($p$<0.001), corresponding to a high $R^2$, particularly for the fruity series (0.9278). Based on the slopes of the regression lines obtained, the production-rate of compounds in the fruity series was much higher than those for the vegetal and empyreumatic series. This suggests that changes

in the fruity series are highly dependent upon the biological ageing process. Taking into account that the most of the contributors to this series are related with the "flor" yeasts activity, the fruity OAVs can be used as an objective indicator of the contribution of these microorganisms to the process.

| **Series** | **p** | **R-squared** | **slope** |
|---|---|---|---|
| Fruity | 0.0000 | 0.9278 | 42.5 |
| Chemical | 0.9093 | nc | nc |
| Balsamic | 0.6675 | nc | nc |
| Vegetal | 0.0003 | 0.6421 | 0.935 |
| Fatty | 0.0190 | 0.3556 | 10.2 |
| Empyreumatic | 0.0000 | 0.7399 | 5.47 |
| Floral | 0.1231 | nc | nc |
| Spicy | 0.0013 | 0.5617 | 13.4 |

nc: not calculated for the series with $p>0.05$

Table 2. Linear regression analysis of the OAVs of the series versus time in sherry-type wines during their biological ageing

### 7.2 Use of odorant series to shorten the biological ageing of sherry wines

In this study, commercial Fino sherry wines (5 years of biological ageing) which were selected by expert tasters as more representative were used. Also, selected strains of *S. cerevisiae* and *S. bayan*us (Kurtzman & Fell, 1998) were used. These yeast strains, corresponding to the S. cerevisiae capensis and bayanus races in the Kreger-van Rij classification (1984), were isolated from a velum of industrial wine produced in the Montilla-Moriles region. The criteria and tests for their selection have been reported in previous papers (Guijo et al., 1986; Moreno et al., 1991).

Figure 4a provides a plot of the values obtained for the OSs of the commercial wines studied. The figure shows the mean value for each series and those calculated, taking into account the standard deviations. In this mode, any value included between the highest and lowest in each series would be acceptable for a typical Fino wine. As can be seen, the fruity, balsamic, chemical and spicy series were those that contributed most markedly to the aroma profile.

To examine the utility of the analytical profile based in OSs, two experiments on the potential acceleration of the biological ageing of sherry wine were carried out. For their development, selected strains of S. cerevisiae and S. bayanus (two typical "flor" yeasts) were inoculated on un-aged wine contained in glass vessels, it being maintained at a constant temperature of 20 $^{o}$C for 9 months. Figure 4b shows the OSs of the wines aged with two "flor" yeasts, determined in the same way as for the above-mentioned commercial wines. For the better clarity of the results obtained, this figure only shows the mean value for each aroma series, and those that resulted in significant differences at $p<0.05$ revealed by ANOVA analysis have been marked with an asterisk. As can be seen, both yeast strains showed fruity and balsamic as major series. Likewise, the OAVs for the 'floral' and

'chemical' series were also higher with the *S. bayanus* strain than with the *S. cerevisiae*. In opposition, the OAVs for the fatty and empyreumatic series were higher with *S. cerevisiae* strain. Finally, neither of the yeast strains used was found to contribute to the spicy series.

On the other hand, in comparing the results obtained in the ageing experiments with the selected strains and the data for commercial wines, each OS was also subjected to an ANOVA. Only the fruity and balsamic series with the *bayanus* strain exhibited no significant differences with the commercial wines. All of the other series exhibited significantly lower OAVs than the commercial wines, particularly the floral, chemical and spicy series. The former series is mainly contributed to by terpenic compounds, and some authors (Zea et al., 1995; Cortes et al., 1998) have noted the ability of these "flor" yeasts to synthesise small amounts of these compounds, so the yeast strain used and cell autolysis in long time ageing (corresponding to the commercial wines), which may give rise to differences in their concentrations. Also, one should note that the duration of the experiments with the selected strains (9 months) may have been insufficient for the accumulation of terpenes, which could be reached with an ageing period of over 5 years, such as with the commercial wines used. Regarding the chemical and spicy series, the absence of 4-ethylguaiacol and eugenol may account for the differences observed. These compounds are slowly extracted from cask wood (Etievant, 1991; Aznar et al., 2001; Ferreira et al., 2001) and, therefore, they were present in the commercial wines aged in oak wood but absent in the wines aged in glass vessels.

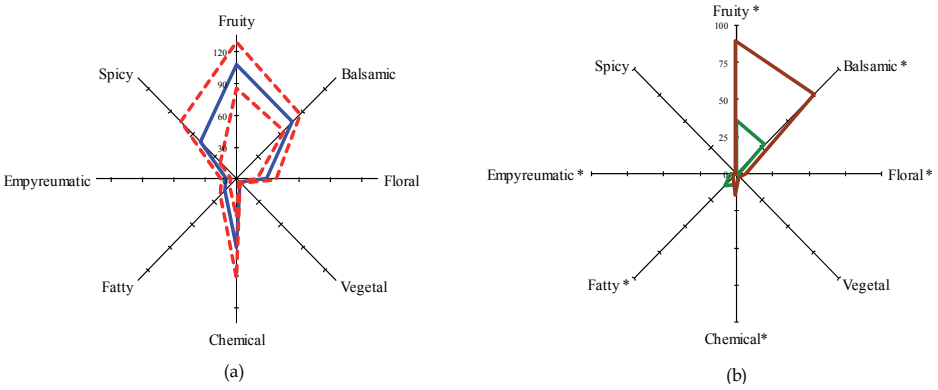



Fig. 4. (a) Aromatic profile of commercial wines: — means, - - - standard deviation. (b) Aromatic profile of the wines aged with two flor yeasts: — cerevisiae strains and — bayanus strains

On the whole, the results reveal that the main effects of the yeast strain used are exerted on the major OSs (fruity and balsamic). In this respect, the higher production of the *bayanus* strain in relation to the main compound contributors to these series confers characteristics similar to those of the commercial wines in a much shorter time (9 months *versus* 5 years).

On the other hand, contact with wood casks is necessary for the aroma profile of the wines obtained with selected strains to resemble that of commercial wines. In this sense, wine could be aged with selected strains of yeast in the wood casks themselves. However, maintaining the conditions required by the selected yeast cultures (mainly an appropriate temperature and the absence of contamination during the process) on an industrial scale is rather difficult. Therefore, it is reasonable to think that wine could be aged in two steps,

firstly under controlled conditions (yeast and temperature) intended to achieve most of the sensory profile of the sherry wines, and secondly under less strictly controlled conditions, in wood casks. Taking into account the efficiency showed by *S. bayanus*, the overall duration of the two steps could be shorter than that of the traditional ageing process, which would result in decreased costs.

### 7.3 Use of odorant series to estimate the relative contribution of the activity of "flor" yeasts and the extraction from wood

The contribution of wood to the aroma of aged Fino wine has been clearly shown in earlier experiments carried out to shorten the biological ageing time (Cortes et al., 1999; Moyano et al., 2002).

As mentioned already, the OSs which included most of the compounds from the wood were the empyreumatic and spicy. The empyreumatic series has a higher $p$ and $R^2$ values than the spicy series (see section 7.1). Nevertheless, the spicy series was significant at $p<0.01$, exhibiting a $R^2$ of 0.5617 and a slope of 13.4. According to Wonnacott & Wonnacott (1995) and Silva-Ferreira et al. (2003), a model can be assumed to fit experimental data well if it provides a correlation coefficient higher than 0.7 ($R^2$ = 0.4900). In addition, the spicy series includes eugenol (a compound extracted from wood and strong contributor in this series), in addition to the odorant compounds of interest present in the empyreumatic series. Therefore, the spicy series should better represent the contribution of wood to the biological ageing process.

Figure 5 shows the OAVs for the fruity series (indicator of the "flor" yeasts activity) versus those for the spicy series. The statistical program adjusted the data to a straight line obtaining a high correlation coefficient (0.7888) and a slope of 1.95. The points within the line represent the sensory balance between the two OSs, whereas the zones outside it represent an imbalance in favour of the compounds related to the yeasts' activity (above) or those provided by the wood (below). In addition, the plot allows an estimate of the ageing time needed to obtain a determined quality (which has a direct impact on the marketing price of the wine), because the higher points on the line represent a higher degree of ageing.

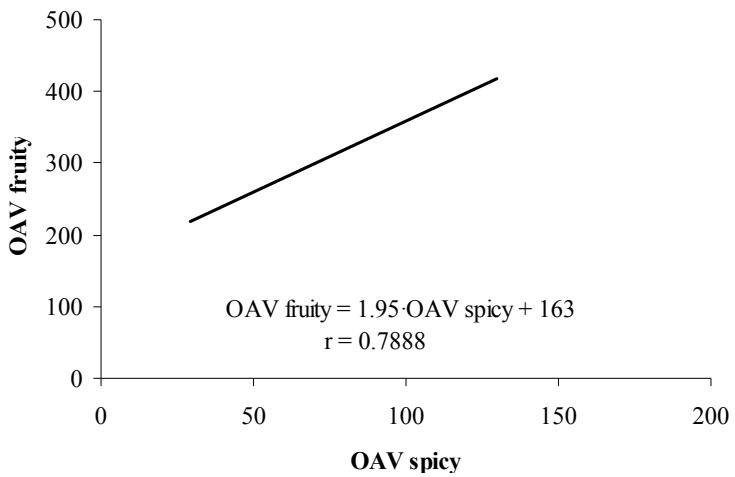



Fig. 5. Odorant Activity Values (OAVs) for the fruity series versus those of the spicy series in sherry-type wines during their biological ageing.

### 7.4 Odorant series as fingerprints for biological ageing in sherry wines

In this study, the aroma compounds detected by Gas Chromatography Olfactometry (GC-O) and with OAV>1 in at least one of the wine samples were considered. The use of OSs allows one to not only identify those series that contribute to the aroma profile of a wine, but also to rank them in terms of their odorant capacity and obtain its aromatic fingerprint.

To determine and compare the aromatic fingerprint of Fino sherry wines aged for different periods, the OAVs for the compounds with similar olfactory features were classified into the eight OSs identified as those best representing the aroma of this type of wine. Figure 6 shows the aromatic fingerprint of the young wine (0 years) and in those subjected to biological ageing for 2.5 and 6 years by arranging the mean values for each OS from high to low.

With the exception of the floral series and – to an extent – the fatty series, the results exhibit an increase in OAV with ageing time, and this is especially marked for the empyreumatic and fruity series. It can be seen that this pattern is preserved during the ageing time, except for the empyreumatic and vegetal series, as a result of the increased OAVs for sotolon and methionol respectively.

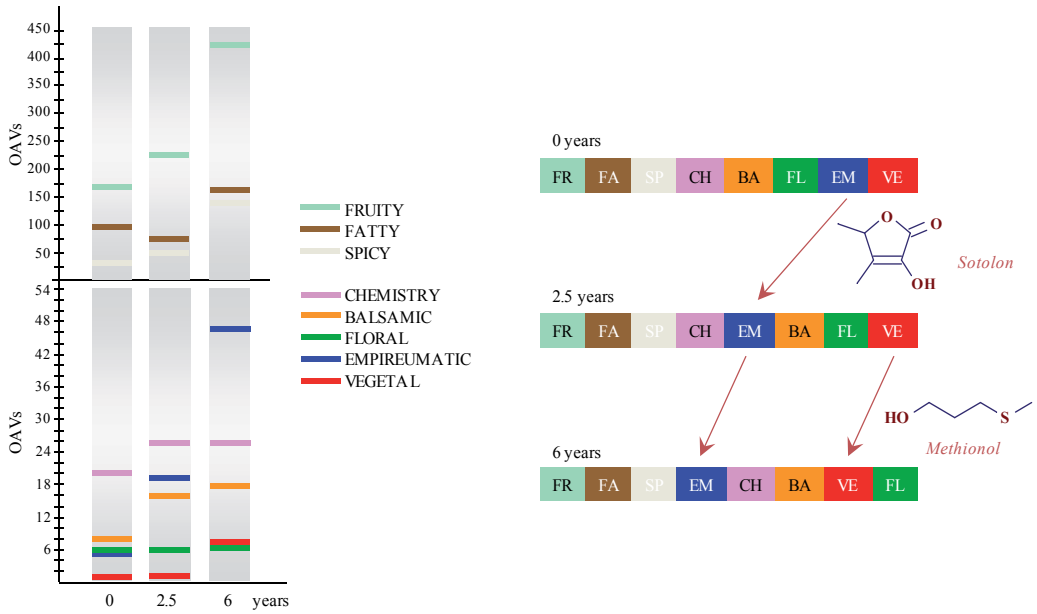



Fig. 6. The fingerprints of sherry wines during their biological ageing.

In conclusion, the aroma fingerprint for the oldest wine ("solera") can be quantitatively defined as fruity, fatty, spicy, empyreumatic, chemical, balsamic, vegetal and floral, in that order. A comparison of the OAVs for these series allows one to estimate the relative contribution of aroma compounds during the biological ageing of Fino sherry wines. In addition, and taking into account that the oldest wines are more valued by consumers, the aroma fingerprint can be advantageously used with a view to improving their sensorial quality.

## 8. Conclusion

The use of OSs as analytical tool for the study of the ageing of sherry wines is very useful; however, some aspects if the grouping of the compounds in the OSs established by this study admit of some criticism. Accordingly, the addition of the compounds' OAVs to calculate a series cannot be interpreted as an arithmetical addition of odorant sensations. Moreover, the assignment of some compounds to a particular series – or several series – may be arguable. In any case, the proposed method is valid for comparing wines of the same type (very pale sherry wines in this work) because the OSs are always comprised by the same compounds. However, this method for aroma fraction study has the advantage that it strongly reduces the number of variables to be interpreted, preserving their relative importance according to the OAVs of the compounds assembled.

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7

# Application of Gas Chromatography on the Evaluation of Grape and Wine Aroma in Atlantic Viticulture (NW Iberian Peninsula)

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## 1. Introduction

The volatile fraction of wine determines to a great extent its aroma, which is one of the most important characteristics influencing wine quality and consumer preferences. The volatile compounds are able to stimulate the sensorial organs that are responsible for the olfaction. These compounds correspond to small molecules, of medium hydrophobicity and molecular weight generally, between 30 g/mol and 300 g/mol (Morrot & Brochet, 2000).

Therefore, flavour is the sensation perceived by the brain when the olfactory epithelium is punched by a fraction of molecules, which were vaporized in the glass – orthonasal route – or put in contact with the mouth – retronasal route (Portmann, 2000). Consequently, the intensity of the olfactory sensation is not simply dependent on the concentration of the volatile compound in the liquid phase, but depends also on its volatility, its vapour pressure and its perception threshold (Meilgaard et al., 1999).

The olfactory perception threshold could be defined as the minor stimulus which could be able to promote an olfactory sensation in at least 50 % of a jury of a sensory panel. If the tasters are able to identify the odour, a recognition threshold is specified. On the other hand, if the volatile compound is already present in the tasting solution, a difference threshold could be defined as the minor addition of the substance susceptible to promote a change in the sensory stimulus (Dubois, 1993; Meilgaard et al., 1999).

### 1.1 Classification and origin of volatile compounds

The huge complexity of wine aroma may be attributed to the higher number of volatile constituents which are the result of a longer biotechnological sequence and the broad variability of concentrations from few ng $L^{-1}$ to hundreds of mg $L^{-1}$. Furthermore, each volatile compound presents its own olfactory perception threshold which is, in turn, influenced by the other constituents of the wine.

Depending on the origin, and considering the biotechnological sequence of winemaking, wine flavour can be classified into four different groups (Bayonove et al., 1998): varietal aroma, typical of grape variety, which depends essentially on soil, climate, phytotechny, sanitary conditions and degree of ripeness; pre-fermentative aroma, originated during grape processing and subsequent operations, namely transport, pressing, maceration, and clarification; fermentative aroma, produced by yeasts during alcoholic fermentation and lactic acid bacteria during malolactic fermentation, which depends mainly on fermentation temperature and microorganism species; post-fermentative aroma, which results from transformations occurred during conservation and ageing of wine.

More than 1000 volatile compounds could be found in wine (Poláskova et al., 2008), but only less than 10 % may contribute to the flavour. The participation of each compound varies considerably by qualitative reasons, positive or negative olfactory impact, or by quantitative reasons, linked to the perception threshold. Additionally, the antagonism or the synergism between compounds should be also taking in account. Table 1 shows some relevant volatile compounds contributing to wine aroma and the respective perception thresholds and descriptors. It must be noted that, for each compound, a broad range of perception thresholds could be found in literature. This could be explained by the purity of the compounds in test, the composition of the matrix used in the essays (water; hydroalcoholic solution and its ethanol content; characteristics of the model wine solution; type of wine) and the adopted tasting method, orthonasal or retronasal.

| **Family/Compound** | **Perception Threshold,** ***PT*** [a] / (μg $L^{-1}$) | | **Descriptor** [b] | **Reference** |
|---|---|---|---|---|
| ***Monoterpenes*** | | | | |
| Z-rose oxide | 0.2 | hydro-alcoholic solution; retronasal | green, floral | [a] Guth, 1997; [b] Ong & Acree, 1999 |
| Nerol oxide | 100 | water | fragrant | [a,b] Simpson, 1979 |
| Linalool | 25.2 | model wine solution; orthonasal | lemon | [a] Ferreira et al., 2000; [b] Escudero et al., 2004 |
| HO-trienol | 110 | water | linden | [a] Simpson, 1979; [b] Ribéreau-Gayon et al., 2000 |
| α-terpineol | 250 | model wine solution; orthonasal | pine | [a] Ferreira et al., 2000; [b] Meilgaard, 1975 |
| Nerol | 400 | water | lime, roses | [a] Ribéreau-Gayon et al., 2000; [b] Meilgaard, 1975 |
| Geraniol | 36 | model wine solution; orthonasal | rose-like, citrus-like | [a] Escudero et al., 2004; [b] Czerny et al., 2008 |
| Wine lactone | 0.01 | hydro-alcoholic solution; retronasal | sweet, coconut | [a] Guth, 1997; [b] Guth, 1996 |

| Family/Compound | Perception Threshold, *PT* [a] / (μg $L^{-1}$) | | Descriptor [b] | Reference |
|---|---|---|---|---|
| ***Methoxipyrazines*** | | | | |
| 3-isobutyl-2-methoxypyrazine | 0.0039 | water; orthonasal | bell pepper-like | [a,b] Czerny et al., 2008 |
| 3-isopropyl-2-methoxypyrazine | 0.0062 | water; orthonasal | earthy, pea-like | [a,b] Czerny et al., 2008 |
| ***$C_{13}$-norisoprenoids*** | | | | |
| β-damascenone | 0.05 | hydro-alcoholic solution; retronasal | sweet, apple | [a] Guth, 1997; [b] Escudero et al., 2004 |
| β-ionone | 0.09 | model wine solution; orthonasal | flowery, violet-like | [a] Ferreira et al., 2000; [b] Czerny et al., 2008 |
| ***Thiols*** | | | | |
| 4-mercapto-4-methyl-2-pentanone | 0.0008 | model wine solution; orthonasal | box tree, broom | [a,b] Tominaga et al., 1998a |
| 4-mercapto-4-methyl-2-pentanol | 0.0055 | model wine solution; orthonasal | citrus zest | [a,b] Tominaga et al., 1998b |
| 3-mercaptohexyl acetate | 0.0042 | model wine solution; orthonasal | box tree, passion fruit | [a] Tominaga et al., 1996; [b] Tominaga et al., 1998a |
| 3-mercapto-1-hexanol | 0.06 | model wine solution; orthonasal | passion fruit, grapefruit | [a, b] Tominaga et al., 1998b |
| ***$C_6$-compounds*** | | | | |
| *Z*-3-hexen-1-ol | 400 | hydro-alcoholic solution; retronasal | lettuce-like | [a] Guth, 1997; [b] Czerny et al., 2008 |
| ***Alcohols*** | | | | |
| 2-methyl-1-butanol | 1 200 | water; orthonasal | alcohol, solvent | [a] Czerny et al., 2008; [b] Meilgaard, 1975 |
| 3-methyl-1-butanol | 30 000 | hydro-alcoholic solution; retronasal | alcohol, solvent | [a] Guth, 1997; [b] Meilgaard, 1975 |
| 2-phenylethanol | 14 000 | model wine solution; orthonasal | roses, perfumed | [a] Ferreira et al., 2000; [b] Escudero et al., 2004 |
| ***Esters*** | | | | |
| Ethyl butyrate | 20 | hydro-alcoholic solution; retronasal | fruity | [a] Guth, 1997; [b] Czerny et al., 2008 |
| Ethyl hexanoate | 14 | model wine solution; orthonasal | fruity, apple | [a] Ferreira et al., 2000; [b] Meilgaard, 1975 |
| Ethyl octanoate | 5 | model wine solution; orthonasal | fruity, fresh | [a] Ferreira et al., 2000; [b] Escudero et al.,2004 |
| Ethyl decanoate | 200 | model wine solution; orthonasal | fruity, fatty acid | [a] Ferreira et al., 2000; [b] Meilgaard, 1975 |
| Ethyl 2-methylbutyrate | 18 | model wine solution; orthonasal | fruity | [a] Ferreira et al., 2000; [b] Czerny et al., 2008 |

| Family/Compound | Perception Threshold, *PT* [a] / (μg L$^{-1}$) | | Descriptor [b] | Reference |
|---|---|---|---|---|
| Ethyl 3-methylbutyrate | 3 | model wine solution; orthonasal | fruity, blueberry-like | [a] Ferreira et al., 2000; [b] Czerny et al., 2008 |
| 3-methylbutyl acetate | 30 | hydro-alcoholic solution; retronasal | banana | [a] Guth, 1997; [b] Meilgaard, 1975 |
| 2-phenylethyl acetate | 250 | hydro-alcoholic solution; retronasal | flowery | [a] Guth, 1997; [b] Escudero et al., 2004 |
| ***Fatty acids*** | | | | |
| 3-methylbutyric acid | 33.4 | model wine solution; orthonasal | fatty, rancid | [a] Ferreira et al., 2000; [b] Escudero et al., 2004 |
| 2-methylbutyric acid | 3 300 | hydro-alcoholic solution; retronasal | sweaty, cheesy | [a] Guth, 1997; [b] Czerny et al., 2008 |
| Hexanoic acid | 420 | model wine solution; orthonasal | sweaty, cheesy | [a] Ferreira et al., 2000; [b] Meilgaard, 1975 |
| Octanoic acid | 500 | model wine solution; orthonasal | fatty, unpleasant | [a] Ferreira et al., 2000; [b] Escudero et al., 2004 |
| ***Phenols*** | | | | |
| 4-ethylguaiacol | 33 | model wine solution; orthonasal | smoky, gammon-like | [a] Ferreira et al., 2000; [b] Czerny et al., 2008 |
| 4-vinylguaiacol | 1100 | model wine solution; orthonasal | clove-like, smoky | [a] Ferreira et al., 2000; [b] Czerny et al., 2008 |
| ***Sulphur Compounds*** | | | | |
| Dimethyl sulphide | 10 | hydro-alcoholic solution; retronasal | asparagus-like, putrid | [a] Guth, 1997; [b] Czerny et al., 2008 |
| 3-(methylthio)-1-propanol | 500 | hydro-alcoholic solution; retronasal | cooked potato-like | [a] Guth, 1997; [b] Czerny et al., 2008 |
| ***Carbonyl Compounds*** | | | | |
| Acetaldehyde | 10 000 | hydro-alcoholic solution; orthonasal | fresh, green | [a] Moreno et al., 2005; [b] Czerny et al., 2008 |
| 3-hydroxy-2-butanone | 30 000 | hydro-alcoholic solution; orthonasal | fruity, moldy, woody | [a] Moreno et al., 2005; [b] Meilgaard, 1975 |
| 2,3-butanedione | 100 | hydro-alcoholic solution;retronasal | buttery | [a] Guth, 1997; [b] Czerny et al., 2008 |

Table 1. Perception thresholds and odour descriptors of relevant wine volatile compounds

### 1.2 Varietal compounds

The wine constituents linked to grape variety are the monoterpenols, abundant in Muscat varieties, the methoxypyrazines, which characterize the *Cabernet* family, the $C_{13}$-norisoprenoids, numerous in *Chardonnay*, volatile thiols in *Sauvignon*, volatile phenols in *Traminer aromatico* and dimethyl sulphide in *Syrah*, but these compounds could also contribute significantly to the aroma of several other varieties (Allen et al., 1991; Sefton et al., 1993; Segurel et al., 2005; Tominaga & Dubourdieu, 2000; Versini, 1985). Except for the methoxypyrazines, these constituents occur in grapes in the form of non-volatile precursors like unsaturated fatty acids, glycosides, carotenoids, cysteine S-conjugates and phenolic acids, which can originate flavour compounds during or after the technological sequence of winemaking (Bayonove et al., 1998). However, monoterpenols are also abundant as free odorants in some grape varieties, like *Muscat* or *Gewürztraminer*.

Monoterpenes are $C_{10}$-terpenoids which are formed in the plant, by the fusion of two molecules of isopentenylpyrophosphate by the so-called ispoprene-rule, and subsequent enzymatic reaction. Considering flavour properties, monoterpenols are the most interesting terpenoids, namely linalool, HO-trienol, α-terpineol, nerol and geraniol, and two monoterpenic oxides, rose oxide and nerol oxide (Ribéreau-Gayon et al., 2000). These compounds have low perception thresholds, in the range of $\mu g\ L^{-1}$, and may contribute to the floral notes of wines (Table 1). It is well established that the concentration of monoterpenols increase during the maturation period, but the optimum date could be attained before commercial maturation; they are mostly located in grape skin, depending on grape variety however. Consequently, the adopted technology to extract grape juice may have a huge influence in the final wine. Some non-odoriferous monoterpenes may undergo chemical transformations during wine storage and ageing, leading to the appearance of interesting odoriferous monoterpenes. For example, *wine lactone* may be formed from *E*-2,6-dimethyl-6-hydroxy-2,7-octadienoic acid (Bonnländer et al., 1998) and the possible precursors of rose oxide could be 3,7-dimethylocta-5-en-1,7-diol and 3,7-dimethylocta-7-en-1,6-diol (Rapp et al., 1984). On the other hand, monoterpenic polyols (*e.g.* 3,7-dimethylocta-1,5-dien-3,7-diol, 3,7-dimethylocta-1,7-dien-3,6-diol and 3,7-dimethylocta-1-en-3,6,7-triol) may undergo chemical transformations at the acidic conditions present in wines, resulting in low perception threshold monoterpenic compounds (Williams et al., 1980).

Methoxypyrazines are nitrogen heterocyclic compounds originated probably from aminoacids catabolism (Bayonove et al., 1998; Ribéreau-Gayon et al., 2000), although their origin was not completely established. The most referred compounds that influences wine aroma are 3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine and 2-methoxy-3-*sec*-butylpyrazine. They contribute to the earthy and vegetable notes – green pepper and asparagus – of wines; the perception thresholds are very low, in the range of some $ng\ L^{-1}$ (Czerny et al., 2008; Ribéreau-Gayon et al., 2000). Although the methoxypyrazines were detected in *Merlot* and *Semillon* cultivars, they seem to be characteristic of the *Sauvignon* family, namely *Sauvignon blanc* and *Cabernet-Sauvignon* (Allen et al., 1994; Lacey et al., 1991). Methoxypyrazines are located mainly in the skin and their content decrease during grape maturation (Lacey et al., 1991). It must be noted that the "green pepper" character of *Cabernet-Sauvignon* wines could be considered as positive or negative, depending on the concentration of these volatile compounds (Ribéreau-Gayon et al., 2000).

The main volatile thiols identified in wines are 4-mercapto-4-methyl-2-pentanone, 4-mercapto-4-methyl-2-pentanol, 3-mercapto-1-hexenol, 3-mercapto-3-methyl-1-butanol and 3-mercaptohexyl acetate (Tominaga et al., 1996; Tominaga & Dubourdieu, 2000). These compounds are present in grapes in the form of cysteine S-conjugates being enzymatically liberated during winemaking or during tasting of a wine. Vegetal notes –box tree and broom– as well as fruit notes –passion fruit, grapefruit– are associated to these compounds; additionally, perception thresholds are extremely low, of few ng $L^{-1}$ (Table 1). Although these compounds were initially referred as typical of *Sauvignon blanc* wines, the actual knowledge indicates they are widespread among cultivars, *e.g. Gewürztraminer*, *Riesling*, *Pinot blanc*, *Semillon*, *Cabernet-Sauvignon*, *Merlot*, etc. (Tominaga & Dubourdieu, 2000).

$C_{13}$-norisoiprenoids derive from carotenoids, by oxidative degradation (Enzell, 1985), and are usually divided in two groups: megastigmanes and non-megastigmanes. For megastigmanes, ionone series (oxygenation in $C_9$) and damascone series (oxygenation in $C_7$) are reported. These compounds are present in grapes as glycoconjugates, although they could appear episodically in the free form. Nevertheless, they are abundant in wines because glycoconjugates are susceptible of being hydrolysed, enzymatically or in the acidic conditions of wine. The megastigmanes β-ionone and β-damascenone are the most cited $C_{13}$-norisoprenoids, with floral and fruit notes and low perception thresholds (Baumes et al., 1986; Sefton et al., 1993); although β-damascenone is important for the aroma of the majority of white and red wines, β-ionone seems to be only significant on the aroma of some red wines (Etiévant et al., 1983). Two non-megastigmanes are also frequently cited: vitispirane –camphor odour– and mainly 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN), with perception threshold of 20 μg $L^{-1}$, which contributes to the kerosene and petroleum character of old *Riesling* wines (Simpson, 1978). Some non-megastigmanes derive from megastigmanes by diverse chemical reactions (Sefton et al., 1989). It must be noted, that solar exposition favours the synthesis of carotenoids in grapes before *véraison* and its degradation to $C_{13}$-norisoprenoids after this date (Razungles et al., 1993).

Glycosidic precursors are of greater importance as they can be hydrolysed to a certain extent during winemaking, wine conservation and ageing, chemically or by microorganisms endogenous enzymes, and also by the addition of exogenous enzymes. It makes possible the production of aromatic wines, with varietal characteristics, from non-aromatic varieties (D'Incecco et al., 2004; Günata et al., 1993). Respecting monoterpenic compounds, the glycoconjugated fraction of varietal flavour compounds is usually more abundant than free fraction (Günata et al., 1985; Oliveira et al., 2000). Glycosidic precursors are constituted by a glycone part formed by one or two sugar molecules, linked to a volatile aglycone. The sugars could be β-D-glucopyranoside, 6-0-α-L-rhamnopyranosil-β-D-glucopyranoside (rutinoside), 6-0-α-L-arabinofuranosil-β-D-glucopyranoside, 6-0-β-D-apiofuranosil-β-D-glucopyranoside or, less frequently, α-D-glucopyranoside (Voirin et al., 1990; Watanabe et al., 1997); the volatile aglycones could be monoterpenic alcohols and oxides, $C_{13}$-norisoprenoids, volatile phenols, linear alcohols, etc. (Oliveira et al., 2000). These glycoconjugates could be enzymatically hydrolysed by the action of the enzymes α-rhamnosidade, α-arabinosidase, β-apiosidase and β-glucosidase; in a first step, the first three enzymes hydrolyse disaccharides at the 1,6 linkage releasing rhamnose, arabinose and apiose and then β-glucosidase liberates the volatile aglycon from glucose (Dupin et al., 1992; Günata et al., 1988). Contrarily to varietal volatile compounds in the free form, the

glycoconjugates are more equitably distributed between skin and pulp (Günata et al., 1985; Wilson et al., 1986). Solar exposition favours the synthesis of glycoconjugates which accumulate in grape during the maturation period (Razungles et al., 1998).

Some phenolic acids like caffeic acid, *p*-coumaric acid and ferulic acid can act as precursors of volatile phenols, which could contribute positively to wine aroma, when they are present at low concentrations; associated descriptors are smoky, clove-like and leather (Table 1). Yeasts can conduct the decarboxylation of phenolic acids to volatile phenols, as well as esterase activities present in enzymatic preparations used in winemaking. During wine storage and ageing, volatile phenols may be further transformed.

These varietal compounds were usually used to classify grape varieties in Muscat, aromatic non-muscat and neutral or, at the limit, to discriminate them or even to distinguish *terroirs* (Genisheva & Oliveira, 2009; Marais et al., 1992; Oliveira et al., 2000).

### 1.3 Pre-fermentative compounds

Pre-fermentative compounds are formed during harvesting, transport, crushing and pressing, as well as during eventual must heating or grape maceration (Cabaroglu et al., 1997; Nicolini et al., 1996). This group comprises $C_6$-alcohols and $C_6$-aldehydes derived from grape lipids (linoleic and linolenic acids), in the presence of oxygen, by a sequence of enzymatic reactions (Crouzet et al., 1998). Volatile compounds produced are hexanal, *Z*-3-hexenal, *E*-2-hexenal and the corresponding alcohols (Moio et al., 2004; Ramey et al., 1986). During winemaking, aldehydes are reduced to the respective alcohols by yeasts. Another $C_6$-compound is usually present in wines, *E*-3-hexenol, but references about its formation mechanism aren´t found. The herbaceous flavour characterise this group of compounds.

### 1.4 Fermentative compounds

Fermentative compounds are alcohols, fatty acids, esters, carbonyl compounds, sulphur compounds and some volatile phenols (Bayonove et al., 1998); they contribute to the vinous character of wine and are, quantitatively, the majority of volatile compounds. These compounds are produced mainly during alcoholic fermentation and the minor part, but not less important, if it occurs, during malolactic fermentation. Therefore, all wines present a similar pattern of fermentative compounds.

Alcohols having more than two carbons and only one alcohol function are usually named higher alcohols. They are practically absent in grapes and musts but they are found in wines at relatively higher concentrations, reaching together values greater than 100 mg $L^{-1}$. The main alcohols are 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol (isoamyl alcohol) and 2-phenylethanol, frequently above 50 mg $L^{-1}$, and 1-propanol, 1-hexanol, between 1 mg $L^{-1}$ and 50 mg $L^{-1}$; another frequently cited alcohol, cooked potato-like odour, is 3-(methylthio)-1-propanol also known as methionol (Czerny et al., 2008; Ferreira et al., 2000). However, only 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol and methionol seem to contribute to wine aroma. Nevertheless, some authors report about 300 mg $L^{-1}$ as the upper limit at which the overall concentration contribute positively to wine aroma (Rapp & Mandery, 1986; Rapp & Versini, 1995); this value depends on the rest of composition, however. The formation of higher alcohols is linked to the amino acids

metabolism by yeasts, through the Erhlich mechanism (catabolic pathway) where amino acids undergo successively a deamination, a decarboxylation and a reduction; they could also be synthesised through the metabolism of the sugars (anabolic pathway), via pyruvate, having the keto acids as intermediates. The contribution of each of these pathways depends on the higher alcohol and on the yeast assimilable nitrogen present in the medium (Henschke & Jinarek, 1993).

Volatile fatty acids present in wine may derive from the anabolism of lipids, resulting in compounds with even number of carbon atoms, by oxidative decarboxylation of α-keto acids or by the oxidation of aldehydes. Volatile fatty acids synthesised from α-keto acids are mainly propanoic acid, 2-methyl-1-propanoic acid (isobutyric acid), 2-methyl-1-butanoic acid, 3-methyl-1-butanoic acid (isovaleric acid; 3-methylbutyric acid) and phenylacetic acid. From lipid metabolism, the following fatty acids are reported: butanoic acid (butyric), hexanoic acid (caproic), octanoic acid (caprylic) and decanoic acid (capric) (Dubois, 1994). Although fatty acids are characterized by unpleasant notes (Table 1), only few compounds of this family attain its perception threshold. However, their flavour is essential to the aromatic equilibrium of wines (Etiévant, 1991).

Ethyl esters are formed from the reaction between ethanol and fatty acids, while acetates results from the esterification of a higher alcohol with acetic acid. Esters present, generally, fruity pleasant flavours, except ethyl acetate which is not well accepted at concentrations above 100 mg $L^{-1}$. Although, as longer is the chain, lesser pleasant is the volatile ester (Table 1). As olfactory notes are similar, a synergic effect is reported (Dubois, 1994). The synthesis of esters is dependent on the need of yeasts to form fatty acids, which is also correlated with the amount of assimilable nitrogen in must (Bell et al., 1979; Nykänen, 1986). There are a huge number of esters found in wines since, in theory, each fatty acid may react with each alcohol to form an ester. Among them, ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, 3-methylbutyl acetate (isoamyl acetate) and 2-phenylethyl acetate are the main contributors to the aroma of young wines (Dubois, 1994; Oliveira et al., 2008a). For this group of compounds, the reported synergic effect may reduce individual perception threshold (Dubois, 1994); for this reason, other esters such as hexyl actetate, 3-methylpropyl acetate (isobutyl acetate) may also contribute to the fruity character of wines. Esters of fixed acids like diethyl succinate and ethyl lactate are present at higher levels in wines but, since their perception thresholds are also very high, only ethyl lactate may contribute, occasionally, to wine aroma (Dubois, 1994).

Sulphur compounds produced by yeasts are mainly thiols, mono and polysulphides, and thioesters. They are regularly associated with powerful and undesirable odours; nevertheless, as molecular weight increases, the negative perception is reduced. Accordingly, two distinct groups are usually reported: low molecular weight sulphur compounds (boiling point < 90 $^{o}$C) and high molecular weight sulphur compounds (boiling point > 90 $^{o}$C). The first group is usually related to organoleptic defects while the second one participates on wine aroma in a very complex way (Darriet et al., 1999; Dubois, 1994; Etiévant, 1991). Light sulphur compounds present at the end of alcoholic fermentation apart from sulphur dioxide are methyl mercaptans (*e.g.* methanethiol), ethyl mercaptans (*e.g.* ethanethiol) and respective thioacetates, sulphides (*e.g.* hydrogen sulphide, carbonyl sulphide) and disulphides (*e.g.* carbon disulphide). Although these compounds are associated to bad descriptors like rotten eggs, onion and rubber, they could participate

positively to wine aroma if the concentration is near de perception threshold (Bayonove et al., 1998; Darriet et al., 1999). Main heavy sulphur compounds are secondary products of amino acids metabolism (cysteine, methionine and homomethionine). The most common cited are 2-mercaptoethanol, 2-methylthioethanol, 3-(methylthio)-1-propanol (methionol), methionyl acetate, methional and 3-methylthiopropanoic acid.

A vast number of carbonyl compounds could be formed by α-keto acids decarboxylation. Nevertheless, as they are reduced by yeasts and/or by the presence of $SO_2$, they exist in wines at levels which are not easily detectable. The compounds susceptible of influencing wine aroma are basically acetaldehyde, 3-hydroxy-2-butanone (acetoin) and 2,3-butanedione (diacetyl) (Bayonove et al., 1998).

### 1.5 Post-fermentative compounds

It is well known that during wine storage and ageing there are many chemical changes in the volatile composition. These reactions depend on wine composition, pH, storage time and temperature (Marais & Pool, 1980; Usseglio-Tomasset, 1983). The majority of fatty acid ethyl esters is hydrolysed during conservation and, ethyl esters of fatty acids related to yeast nitrogen metabolism (*e.g.* ethyl 2-methylbutyrate and ethyl 3-methylbutyrate) and esters of organic acids (*e.g.* diethyl succinate) increase during this period (Díaz-Maroto et al., 2005; Dubois, 1994; Oliveira et al., 2008a); however, diethyl succinate do not influence wine aroma. Also, the terpenic profile may change, with the disappearance or strong decline of the compounds initially present, with the simultaneous formation of other terpenic compounds with higher oxidation state; temperature and pH have a decisive influence (Di Stefano & Castino, 1983; Marais et al., 1992). Dimethyl disulphide increases during wine storage (Marais & Pool, 1980). Some norisoprenoids may appear or increase their concentration during the ageing period, *e.g.* β-damascenone, TDN and vitispirane (Marais et al., 1992). The acidic medium also favours the hydrolysis of glycosidic precursors and the transformation of aglycon moieties (Dugelay, 1993; Sefton et al., 1993).

## 2. Extraction of grape and wine volatiles

When dealing with grapes, a blender is usually used to liquefy the matrix, being the analytes dissolved from pulp and skin into the must. Particle size of the fragmented skin can be an important parameter to obtain reproducible results as the extent to which the grapes are broken up can influence the extraction rates. Furthermore, prior to extraction, the must should be clarified by centrifugation and filtration to assure a clean final solution (Oliveira el al., 2008a). Respecting wines, only the clarification step should be implemented if necessary. After this initial step, samples (grape juices, musts and wines) could be treated as liquid samples.

The wine volatile fraction is extremely complex, mainly because of the great number of compounds, which are from different chemical classes, covering a wide range of polarities, solubility and volatilities. Moreover, the concentration range of these compounds is from a few ng $L^{-1}$ to hundreds of mg $L^{-1}$. Furthermore, volatile compounds are contained in complex and compositionally very variable matrices where they can be associated and therefore their volatility modulated by other wine macro-components (polyphenols, ethanol, polysaccharides) (Andujar-Ortiz et al., 2009; Pozo-Bayón & Reineccius, 2009). Finally, but

also of importance, is the fact that many aroma compounds are chemically very unstable and can be easily oxidized or thermo degraded (Castro et al., 2008). Although grape volatile composition is simpler than that of wines, analyses may deal with small concentrations, equal or below the μg $L^{-1}$ level.

Accordingly, the determination of volatile compounds in grapes and wines often requires extensive sample extraction and preparation regimes prior to instrumental analysis. The amount of sample preparation needed depends on the sample matrix and the properties and level of analyte to be determined (Ridgway et al., 2007). The typical steps within sample preparation include sampling/homogenisation, extraction, clean-up and concentration followed by the final analysis. Another step that can be included at several points is derivatisation. For the determination of volatile compounds, the final analysis is invariably achieved using a powerful separation technique, gas chromatographic, combined with an appropriate detector (Ridgway et al., 2007).

There are available a wide range of analytical tools for the extraction of volatile compounds. These methodologies are essentially based on the solubility of the analytes in organic solvents, based on the adsorptive capacity of polymeric phases and based on their sorptive capacity on polymeric phases or solvents. Moreover, since the techniques deal with volatile compounds, the headspace approach is typically associated whenever appropriate (Andujar-Ortiz et al., 2009; Ridgway et al., 2007).

Currently, the most commonly used methods are *liquid-liquid extraction* (LLE), *solid phase extraction* (SPE) and *solid phase microextraction* (SPME). LLE is a versatile technology which may possibility simultaneous extractions using solvents with distinct polarities; since earlier works used large solvent volumes, the tendency to reduce costs and environmental impacts conducted to *liquid-liquid micro extraction* (LLME) and *dispersive liquid-liquid micro extraction* (DLLME). SPE also uses diminutive volumes of solvent and as the advantage of being a selective technique by using appropriate adsorbent phases. The SPME technique, as well as *stir bar sorptive extraction* (SBSE), is a solvent-free approach with very low limits of detection; SPME, mainly, starts to dominate the field by replacing the ancient methods. Applications of the most common methodologies used for the extraction of grape and wine volatiles are illustrated in Table 2.

Other two methodologies are described in literature: *supercritical fluid extraction* (SFE) and *liquid-phase microextraction* (LPME). SFE was successfully applied, using $CO_2$ or methanol-modified $CO_2$, respectively to analyse wine volatile compounds (Blanch et al., 1995; Karásek et al., 2003) and to extract grape glycosides (Palma et al., 2000). LPME is an alternative miniaturized sample preparation approach which makes use of only a very small amount of solvent for concentrating analytes (Liu & Dasgupta, 1996; Jeannot & Cantwell, 1997). It overcomes many of the disadvantages of LLE as well as some of those of SPME (*e.g.* independence of a commercial source and sample carryover). Extraction normally takes place between a small amount of water-immiscible solvent (*e.g. n*-octanol, ethylene glycol) and the bulk aqueous phase containing the analytes of interest. The volume of the receiving phase is in the microliter or submicroliter range conducting to high enrichment factors. Since the extraction medium is in the form of a single drop at the tip of a micro-syringe needle, this type of LPME has been termed *single-drop microextraction* (SDME). In the case of

| Extraction technique | Matrix/Analytes | Method | *LOD*/ ($\mu g\ L^{-1}$) | Reference |
|---|---|---|---|---|
| ***Solvent-based*** | | | | |
| LLE | Wine, 52 volatile compounds | 50 mL wine, 2×5 mL $CH_2Cl_2$; extract final volume = 500 μL; GC-MS | 2 to 534 | Perestrelo et al., 2006 |
| | Synthetic wine, 30 volatile compounds | 50 mL wine, 10 mL $CH_2Cl_2$; extract final volume = 300 μL; GC-MS | 1.0 to 34.1 | Andujar-Ortiz et al., 2009 |
| | Wine and grapes, β-ionone | 100 mL wine (250 mL grape juice), 10 mL $CH_2Cl_2$; extract final volume = 300 μL; GC-MS | | Kotseridis et al., 1999 |
| | Wine, 44 volatile compounds | 100 mL wine, 50 mL diethyl ether-*n*-pentane; 30 min sonication, 25 °C (under $N_2$); GC-MS | | Hernanz et al., 2008 |
| LLME | Wine, 3 $C_6$-compounds | 8 mL wine, 400 μL $CH_2Cl_2$; 15 min; GC-FID and GC-MS | 3.3 | Oliveira et al., 2006 |
| | Wine, 35 volatile compounds | 8 mL wine, 400 μL $CH_2Cl_2$; 15 min; GC-FID and GC-MS | | Vilanova et al., 2009 |
| | Wine, 40 volatile compounds | 3 mL wine + 7 mL $H_2O$, 200 μL $CH_2Cl_2$; 2500 $min^{-1}$, 10 min; GC-FID | | Ortega et al., 2001 |
| DLLME | Wine, 5 chlorophenols and 7 haloanisoles | 5 mL wine, 1 mL acetone (disperser) + 30 μL $CCl_4$ (extraction solvent); GC-MS | 0.004 to 0.108 | Campillo et al., 2010 |
| | Wine, 8 volatile compounds (cork and Brett taints) | 5 mL wine, 1.43 mL acetone (disperser) + 173 μL $CHCl_3$ (extraction solvent); GC-MS/MS | 0.05 to 0.75 | Pizarro et al., 2011 |
| | Wine, geosmin and 2-methylisoborneol | 12 mL wine, 8 μL $C_2Cl_4$; ultrasound 3 min, 20 °C; GC-MS | 0.002 to 0.009 | Cortada et al., 2011 |
| ***Adsorptive-based*** | | | | |
| SPE | Synthetic wine; 30 volatile compounds | 50 mL wine, LiChrolut-EN cartridge (200 mg), 1.3 mL $CH_2Cl_2$; GC-MS | 0.1 to 46.5 | Andujar-Ortiz et al., 2009 |
| | Wine; glycosylated volatile compounds | 15 mL wine; LiChrolut-EN (200 mg), Amberlite® XAD-2 (280 mg) and Lichrolut RP-18 (200 mg) cartridges; 4 mL pentane-dichloromethane (2 :1); 7 mL ethyl acetate; final volumes = 200 μL; GC-MS | | Ibarz et al., 2006 |
| | Wine; free and glycosylated terpenes | 25 mL wine; C-18 column; 35 mL dichloromethane; 30 mL methanol; final volumes = 1 mL | | Karagiannis et al., 2000 |

| Extraction technique | Matrix/Analytes | Method | *LOD*/ (μg $L^{-1}$) | Reference |
|---|---|---|---|---|
| | Wine; volatile compounds | 100 mL wine; 10 mL Amberlite® XAD-2 bed; 50 mL pentane-dichloromethane (2:1), final volume = 2 mL; 50 mL ethyl acetate, final volume = 200 μL; GC-MS | 0.3 | Oliveira et al., 2008b |
| | Grapes; free and glycosylated volatile compounds | 150 mL grape juice; 10 mL Amberlite® XAD-2 bed; 50 mL pentane-dichloromethane (2:1), final volume = 200 μL; 50 mL ethyl acetate, final volume = 200 μL; GC-MS | 0.1 | Genisheva & Oliveira, 2009 |
| ***Sorptive-based*** | | | | |
| SPME | Synthetic wine; 30 volatile compounds | 8 mL wine, StableFlex 85 μm CAR-PDMS fibre, 5000 $min^{-1}$; GC-MS | 1.1 to 270.4 | Andujar-Ortiz et al., 2009 |
| | Grape juice medium; 9 off-flavours | 10 mL grape juice medium, pH = 3.4, NaCl = saturation; DVB/CAR/PDMS fibre, HS-SPME, 30 min, 50 °C; GC-MS | 0.0047 to 3 | Morales-Valle et al., 2010 |
| | Wine; 34 volatile compounds | 40 mL wine, 50 mL vial; DVB/CAR/PDMS fibre, HS-SPME, 15 min, 37 °C; GC-FID and GC-MS | | Tat et al., 2005 |
| | Wine, 8 halophenols and haloanisoles | 10 mL wine, 20 mL vial; PA fibre, HS-SPME, 60 min, 70 °C; derivatization with MSTFA 25 °C, 25 min; GC-ECD | 0.0004 to 0.0038 | Pizarro et al., 2007 |
| SBSE | Grape juice, 34 volatile compounds | 100 mL prepared juice; PDMS bar (10 mm × 0.5 mm), SBSE; 6 h, room temperature; TD 290 °C, 4 min, trap = - 30 °C; GC-MS | | Salinas et al., 2004 |
| | Wine; 13 oak related volatiles | 25 mL wine; PDMS bar (10 mm × 0.5 mm), SBSE; 90 min, room temperature, 700 $min^{-1}$; TD 290 °C, 4 min, trap = - 30 °C; GC-MS | 0.0001 to 38.93 | Marín et al., 2005 |
| | Wine; 39 volatile compounds | 0.5 mL wine, 1.5 g NaCl, 20 mL vial; PDMS bar (10 mm × 0.5 mm), HSSE; 1 h, 23 °C, 1200 $min^{-1}$; TD; GC-MS | | Weldegergis & Crouch, 2008 |

Table 2. Most common methodologies used for the extraction of grape and wine volatiles (*LOD* – limit of detection)

volatile compounds, the acceptor phase is usually suspended above the sample (headspace extraction; HS-SDME), but direct immersion (DI-SDME) either in static or in dynamic mode is also applicable. The operation of this technique is somewhat similar of SPME, being the syringe, after the extraction procedure, transferred to the injection port of a gas chromatograph (Xu et al., 2007). An alternative concept of LPME is based on the use of a single, low-cost, disposable, porous, hollow-fibre made of polypropylene – HF-LPME (Rasmussen & Pedersen-Bjergaard, 2004). In this hollow fibre-based LPME device, the micro-extract is contained within the lumen of a porous hollow fibre, so the micro-extract is not in direct contact with the sample solution. As a result, samples may be stirred or vibrated vigorously without any loss of the micro-extract. Several applications of LPME were published concerning food analysis (Asensio-Ramos et al., 2011), but the majority refers to the extraction of non-volatile analytes (*e.g.* fungicides and pesticides). In spite of the possibilities of this technique, there are reported only few papers respecting volatile compounds. However, the extraction of the taints 2,4,6-trichloroanisole and 2,4,6-tribromoanisole in wines (Márquez-Sillero et al., 2011; Martendal et al., 2007), and alcohols and volatile sulphur compounds in beer (Tankeviciute et al., 2001; Xiao et al., 2006) should be referred.

### 2.1 Solvent-based techniques

In *liquid-liquid extraction* (LLE), analytes are extracted by direct partitioning with an immiscible solvent. This technique is based on the relative solubility of an analyte in two immiscible phases and is governed by the equilibrium distribution/partition coefficient. Extraction of an analyte is achieved by the differences in solubilizing power (polarity) of the two immiscible liquid phases. Typically a separating funnel is used and the two immiscible phases are mixed by shaking and then allowed to separate. To avoid emulsions, in some cases, salt may be added and centrifugation can be used if necessary. Alternatively a *matrix solid phase dispersion* (MSPD) approach can be used to avoid emulsions. Either layer can be collected for further analysis. To ensure the complete extraction of an analyte into the required phase, repeated extractions may be necessary. The major disadvantage of bulk liquid–liquid extraction is the need for large volumes of organic solvents. Also, due to the limited selectivity, particularly for trace level analysis, there is a need for clean-up or analyte enrichment/concentration steps prior to instrumental analysis (Ridgway et al., 2007). Although LLE is being replaced by more manageable and solvent-free techniques, this type of extraction is still a reference for the analysis of wine aroma compounds (Andujar-Ortiz et al., 2009; Kotseridis et al., 1999; Perestrelo et al., 2006; Cabredo-Pinillos et al., 2006; Hernanz et al., 2008; Jofré et al., 2010). Organic phases commonly used are dichloromethane, freon-11, *n*-pentane, diethyl ether, hexane–diethyl ether and diethyl ether–*n*-pentane, among others. The main advantages of this technique are its capacity to extract a wide range of compounds of different volatilities (as long as they have an affinity to the solvent), the high repeatability and the possibility of carrying out simultaneous extractions (Andujar-Ortiz et al., 2009). Table 2 show some examples of application of LLE technique.

Some variants of LLE technique were also referred to extract grape and wine volatile compounds. *Simultaneous distillation-extraction* (SDE) was applied to extract volatile compounds of grape juice (Caven-Quantrill & Buglass, 2006) and wine (Blanch et al., 1996). Carro et al. (1997) used *microwave-assisted extraction* (MAE) to analyse monoterpenes in must

samples. Also, *ultrasound-assisted extraction* (UAE) was successfully applied to must and wine samples (Cabredo-Pinillos et al., 2006; Peña et al., 2005; Hernanz et al., 2008; Cocito et al., 1995).

The necessity to reduce solvent volumes and therefore the costs, as well as the consumable time, conducted to the miniaturization of LLE techniques. In this way, the *liquid-liquid microextraction* – LLME – technique (Ortega et al., 2001; Oliveira et al., 2006) corresponds to a direct miniaturization of LLE, employing only a few hundreds of μL of solvent and a few mL of wine (table 1); the extraction is obtained by stirring the mixture in a sealed glass tube during about 10 min to 15 min; then the organic phase is detached by centrifugation before being collected by a syringe or a Pasteur pipette. This approach permits multiple parallel extractions, in short time period, using ordinary labware, and restricting the use of toxic solvents.

Recently, a novel and powerful microextraction technique, named *dispersive liquid–liquid microextraction* (DLLME) was suggested (Rezaee et al., 2006). In this method, the appropriate mixture of extraction solvent and disperser solvent, the extractant, is injected into the aqueous sample by a syringe, rapidly. This turbulent regimen gives rise to the formation of small droplets, which are dispersed throughout the aqueous sample, with very large interfacial area. Thereby, a cloudy solution is formed and the equilibrium state is achieved quickly and, therefore, the extraction time is very short (Rezaee et al., 2010). In fact, this is the principal advantage of DLLME. After centrifugation of the cloudy solution, a sedimented phase is settled in the bottom of a conical tube and used with the most appropriate analytical technique. Other advantages of DLLME include simplicity of operation, rapidity, low cost, high recovery and high enrichment factor. Campillo et al. (2010) and Pizarro et al., (2011) applied the technique to analyse some compounds responsible for the cork taint and the *Brett* character of wines, namely chlorophenols, chloroanisoles and volatile phenols. The use of ultrasound energy to disrupt the extractant phase may reduce even more the consumption of organic solvent because the disperser solvent is not needed. It is the achievement of the new *ultrasound-assisted dispersive liquid–liquid microextraction* (Cortada et al., 2011).

### 2.2 Adsorptive-based techniques

Solid phase extraction (SPE) involves a liquid–solid partition, where the extracting phase is a solid sorbent and it has been used extensively to remove and concentrate trace organic materials from liquid samples or solutions. The possibility of using different sorbent phases and eluents makes SPE a very selective technique, using different mechanisms for extraction/retention of analytes. A change in pH can be used to enable extraction. A wide range of sorbents have been used including $C_8$ and $C_{18}$ bonded phases on silica, polymeric resins (polystyrene/divinylbenzene copolymer), Florisil (activated magnesium silicate), polar sorbents such as alumina, charcoal, silica and cyano and amino-bonded. Ionic functional groups, such as carboxylic acid or amino groups can also be bonded to silica or polymeric sorbent to create ion-exchange sorbents. Mixed-mode sorbents are also available using both the primary and secondary mechanisms for selective retention of analytes; also, specific selective sorbents have been designed. These different phases enable interactions based on adsorption, H-bonding, polar and non-polar interactions, cation, anion exchange or size exclusion (Castro et al., 2008; Ridgway et al., 2007).

SPE has been extensively used to analyse grape and wine volatiles, either in the form of resin cartridges or even in hand-prepared bed columns. The most used sorbents are $C_{18}$ bonded phases, polystyrene/divinylbenzene copolymers and hydrophobic cross-linked polystyrene copolymer resins under the commercial names Bond Elut C18, Discovery DSC-18, Strata® C18, Lichrolut® RP-18, Lichrolut® EN, Chromabond® easy, Strata® SDB-L and Amberlite® XAD-2, among others.

This technique has been used to extract volatile compounds of wines either indistinctly or selectively, *e.g.* polyfunctional mercaptans, methoxypyrazines and terpenoids (Culleré et al., 2003; López et al., 2002, 2011; Mateo-Vivaracho et al., 2009; Weldegergis et al., 2011). However, its major advantage is the application to determine the nature and concentration of free and glycosidically bound volatile compounds in grape juices and wines. In summary, the methodology initially proposed by Günata et al. (1985), involves a previous activation of the resin (*e.g.* successively few mL of methanol and hydroalcoholic solution), followed by the percolation of about 25 mL to 150 mL of the liquid sample previously spiked with a known amount of an internal standard; then, the resin is washed with water and the free volatile compounds are extracted with few mL of an appropriate solvent (*e.g.* dichloromethane or azeotropic mixture pentane-dichloromethane 2:1, v/v); after that, the glycosidically bound volatile compounds are eluted with few mL of another solvent, *e.g.* ethyl acetate, methanol or ethyl acetate-methanol mixture 9:1 (Canosa et al. 2011; Genisheva & Oliveira, 2009; Ibarz et al., 2006; Oliveira et al., 2008b; Schneider et al., 2004). The extract containing glycosylate volatile compounds should be further treated. The solvent is removed and the glycosides ressuspended in an appropriate buffer before enzymatic or acidic hydrolysis; the liberated volatile compounds are then extracted with the same solvent used for free volatile fraction. Finally, the extracts are concentrated to about 100 µl to 200 µL in the case of grape juices and musts; when analysing wines, depending on the compounds of interest, the "free" extract may be concentrated to 1 mL, only.

Recently, a novel methodology has been developed which corresponds to a miniaturization of the conventional SPE (Abdel-Rehim, 2010; Altun et al., 2004). It was named *microextraction in packed syringe* (MEPS) and the major achievement is the considerably reduction of sample, as well as the extraction and washing solvent volumes. MEPS consist of two parts: the syringe and the barrel insert and needle assembly containing the SPE phase. The extraction phase of MEPS is based on a double pass system where the sample solvent both enters and exits from the bottom of the same bed volume. After extraction the bed volume can be washed with solvent before elution of the target compounds. However, until now, the application of this methodology to wine volatile compounds is limited to the analysis of the taints 2,4,6-tricholoanisole and 2,4,6-tribromoanisole (Jönsson et al., 2008).

### 2.3 Sorptive-based techniques

Sorptive extraction techniques are based on the distribution equilibria between the sample matrix and a non-miscible liquid phase. Matrices are mostly aqueous and the non-miscible phase is often coated onto a solid support. Analytes are extracted from the matrix into the non-miscible 'extracting' phase. Unlike adsorption techniques, where the analytes are bound to active sites on the surface, the total volume of extraction phase is important. Extraction of analytes depends on the partitioning coefficient of solutes between the phases (Ridgway et al., 2007). Two extraction techniques are commonly employed: *solid phase microextraction* (SPME) and *stir-bar sorptive extraction* (SBSE).

SPME is a solvent free sample preparation technique, originally developed by Pawliszyn and co-worker (Arthur & Pawliszyn, 1990). The initially developed fibre-SPME device continues to be the most widely used format. It consists of a fibre holder and a fibre assembly, the later containing a 1 cm to 2 cm long retractable fibre. The SPME fibre itself consists of a thin fused silica fibre coated with thin polymeric coating. The process involves the performance of two basic steps: (i) partitioning of analytes between the extraction phase and the sample matrix and then (ii) desorption of the concentrated extracts from the fibre into the analytical instrument, *e.g.* a gas chromatograph (Risticevic et al., 2009). The utilization of this technique offers the following benefits: short sample preparation times; small sample volumes; analyte concentration from liquid, gaseous and solid samples; solvent-free technique and; easily automated to allow the high-throughput analysis. The complete automation, one of the initially objectives of the invention, could achieve by the use of a commercial autosamplers (*e.g.* CombiPAL™, TriPlus™).

Generally SPME extraction of the analyte from the matrix is not an exhaustive extraction technique but is an equilibrium technique. The maximum sensitivity is obtained at the equilibrium point; however, it is not necessary to reach this point and the extractions can instead be performed for a defined period of time (Kataoka et al., 2000; Risticevic et al., 2009). When developing a SPME method, the following parameters should be optimized: type of coating; sampling mode (direct immersion or headspace); agitation conditions (speed, time and temperature); ionic strength; pH; sample volume; vial shape and headspace volume and; desorption conditions (Pawliszyn, 2009). The presence of high concentrations of matrix components or other compounds can result in competitive binding and displacement and potentially large errors can occur. Therefore, matrix effects can be an issue and quantitation should consider the use of the method of standard additions or the use of an isotopically labelled internal standard.

Currently, various types of stationary phases are commercially available, with different thickness and polarities, showing different affinities for different analytes. The commercially available single coatings and blending materials, sorting for increasing order of polarity, are: PDMS, CAR/PDMS (CAR = carboxen), DVB/CAR/PDMS (DVB = divinylbenzene), PA, PDMS/DVB, CW/DVB (CW = carbowax = polyethylene glycol) and CW/TPR (TPR = templated resin). The apolar phase PDMS presents affinity for apolar compounds (*e.g.* esters) and the polar phase PA is appropriate for polar compounds (*e.g.* alcohols). Furthermore, coatings utilizing the sol-gel technology have been developed (Kataoka et al., 2000; Liu et al., 2005). Recently, Ho et al. (2011) proposed the application of ionic liquids (ILs) and polymeric ionic liquids (PILs) as sorbent materials for SPME. Because of their unique physico-chemical properties, these compounds can be structurally-designed to selectively extract target analytes based on unique molecular interactions.

The need to use high throughput applications requires robust and reliable SPME assemblies. However, some disadvantages of SPME include batch to batch variation and robustness of fibre coatings which could only be used for 50 to 100 cycles (Risticevic et al., 2009). To overcome this problem, a special type of insert, flexible nickel-titanium alloy was used to construct the assembly needle, fibre core and plunger components of the metal SPME assembly. These materials permit more than 600 extraction/desorption cycles (Giraudel et al., 2007; Setkova et al., 2007a, 2007b).

More than one hundred papers reporting diverse applications to analyse wines were published until know. The majority of the referred methodologies use the *headspace* mode (HS-SPME) instead of the *direct immersion* mode (DI-SPME). In terms of performance, SPME showed comparable results to LLE or SPE. However, SPME is simpler and solvent-free, and uses smaller volumes of sample; nevertheless, on the other hand, LLE had the possibility of carrying out simultaneously the extraction of several samples (Bohlscheid et al., 2006; Castro et al., 2008). When the interest is to obtain the maximum information about the volatile fraction of a wine, the coating DVB/CAR/PDMS seem to be the most suitable (Tat et al., 2005). On the other hand, for specific applications, the choice of a suitable solid-phase, depends on the class of compounds be analyzed, *e.g.* CAR/PDMS for volatile sulphides and disulphides (Mestres et al., 1999), on-fibre derivatization (PA) for the determination of haloanisoles and halophenols (Pizarro et al., 2007).

Respecting grape juices and musts, the number of published works is much lower. For example, Sánchez-Palomo et al. (2005) studied 16 varietal compounds of grapes by HS-SPME and Morales-Valle et al. (2010) have determined the concentration of geosmine and fungal "off" volatiles metabolites in musts inoculated with *Botrytis cinerea* and *Penicillium expansum*.

*Stir bar sorptive extraction* (SBSE) was introduced in 1999 (Baltussen et al., 1999) as a solventless sample preparation method for the extraction and enrichment of organic compounds from aqueous matrices. In this sorptive-based method, the solutes are extracted into a polymer coating (polydimethylsiloxane – PDMS) on a magnetic stirring rod. Stir bar sorptive extraction of a liquid sample is performed by placing a suitable amount of sample in a vial. The stir bar is added and the sample is stirred, typically for 30 min to 240 min. The extraction time is controlled kinetically, determined by sample volume, stirring speed, and stir bar dimensions and must be optimized for a given application (David & Sandra, 2007). Stir bars of 1 cm to 2 cm long coated with 500 µm to 1 mm PDMS have been commercially available. Sampling could be also carried out in the headspace mode (*headspace sorptive extraction* – HSSE), placing the stir bar above the liquid sample using a special devices (David & Sandra, 2007).

After extraction, the stir bar is removed, dipped on a clean paper tissue to remove water droplets, and introduced in a thermal desorption unit (TD). In some cases, it is recommended to rinse the stir bar slightly with distilled water to remove adsorbed sugars, proteins, or other sample components. Alternatively, liquid desorption (LD) can be used, by placing the stir bar, typically, in a small vial (2 mL, or vial with insert) being desorption performed with apolar solvents (*e.g.* hexane). The thermal desorption system is used for optimum desorption and re-concentration before GC analysis. Thermal desorption temperatures between 150 $^{o}$C and 300 $^{o}$C are typically used. But, because more sorptive extraction phase is used, the desorption process is slower than for a SPME fibre. Longer desorption times (10 min) in combination with desorption flows between 10 mL/min and 100 mL/min are typically used. A programmed-temperature vaporizing (PTV) injector is operated as a cryotrap for cryogenic refocusing of the thermally desorbed analytes. Temperatures as low as −150 $^{o}$C are used along with liquid nitrogen cooling (David & Sandra, 2007).

SBSE have large surface area of stationary phase than SPME, leading to a higher phase ratio and hence a better recovery and higher sample capacity. Typically, the coated volume layer is 50 to 250 times larger. Consequently, the extraction efficiency for solutes that are partially water soluble, *i.e.* polar compounds, is much better (David & Sandra, 2007). Typically, the life-time of a single stir bar is 20 to more than 50 extractions, depending on the matrix.

SBSE presents a series of advantages over the rest of extraction techniques: is solvent-free (environmental friendly); could be completely automated; don't requires pre-treatment of samples (reduces analytical errors) and; presents greater sensitivity than SPME, reaching lower detection and quantitation limits. However, it presents two clear disadvantages compared with the other extraction techniques: PDMS is the only phase commercially available to date, limiting the extraction of polar substances and; a specific thermal desorption unit is required for optimize the process (Castro et al., 2008). The increase of the extraction yields for the recovery of polar compounds could be carry out by in-site derivatization. Recently, other phases under development were referred, namely those based on the sol-gel technology, restricted access materials and molecular imprinted polymers (Prieto et al., 2010).

The number of applications to enological products is lower than SPME. However, due to the lower limits of detection achieved with SBSE, the number of published works has largely increased recently. Works refer to analysis of volatile compounds, indistinctly, in wines (Coelho et al., 2009; Fang & Qian, 2006; Tredoux et al., 2008) or grapes (Caven-Quantrill & Buglass, 2006; Salinas et al., 2004). Applications to particular classes of wine volatile compounds include volatile phenols (Díez et al., 2004), terpenes, $C_{13}$-norsoprenoids and $C_6$-compounds (Zalacain et al., 2007), haloanisoles and halophenols (Zalacain et al., 2004) and related-oak volatiles (Marín et al., 2005). Luan et al. (2005 and 2006) used SBSE to study the metabolism of geraniol in grape berry and to carry out an enantioselective analysis of monoterpenes in different grape varieties during berry ripening. Weldegergis & Crouch (2008) refer the use of HSSE to analyse volatile compounds.

## 3. Analyses of volatile extracts

Once the volatile compounds were extracted, they should be analyzed by gas-chromatography (GC). Depending on the characteristics of the extracts (solvent, analytes of interest), the GC conditions must be select and optimized. The chromatographic system is composed by an injector, a column inside an oven and a detector. A carrier gas is needed to transport the volatile compounds through the column; depending on the detector, other gases may be required. Finally, an acquisition system collects the information that arrives to the detector.

Some wine volatile compounds could be analysed, however, without previous extraction. Indeed, acetaldehyde, ethyl acetate, methanol and higher alcohols (1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol) could be directly analysed by injecting a filtered wine spiked with a suitable internal standard (*e.g.* 4-nonanol), in the split mode; in that case, the column stationary phase should tolerate the reception of vaporized aqueous samples, *e.g.* CP-Wax 57 CB (100 % chemically-bonded polyethylene glycol).

The final goal of an analytical method is to determine quantitatively each compound of interest. Furthermore, the method must be robust, accurate and reproducible. To achieve these goals, the analytes should ideally appear at the end of the column (detector) as individual peaks to be easily identified and quantified.

### 3.1 Isolation of volatile compounds by gas chromatography

Sample introduction is made by injecting 1 μL to 5 μL, commonly, by a micro-syringe into the injection port of the injector. Two types of injectors are commonly used: split/splitless injector and programmed-temperature vaporizing injector (PTV). In the split mode, only a small fraction of the sample enters the chromatographic column by splitting the gas flow (*e.g.* 1 % to 6 %); the rest is vented through the split outlet. This mode is used for highly concentrated samples in order to avoid system overloading and when sensitivity is not an issue. In the splitless mode on the other hand, in order to increase the sensitivity, the split valve is closed for a short period of time (*e.g.* 30 s to 2 min) after injection, ensuring that the entire sample is transferred for analysis (Grob & Barry, 2004). In a PTV inlet analytes are trapped at reduced temperature which commonly ranges between -150 ºC and -50 ºC; therefore, it could reduce analyte discrimination during the injection step and promote better recovery of thermo-labile compounds. The PTV inlet could also operate both in split and splitless modes.

The capillary column is the core of a chromatographic separation. Separation occurs based on the physical and chemical properties of each analyte in the sample in relation to the stationary phase of the column. There are a wide range of capillary columns available nowadays, mainly differing in the type of their stationary phases and dimensions. The choice depends mainly on the type and number of analytes to be separated and the complexity of the sample. In a non-polar stationary phase, the separation is made according to boiling point. On the contrary, the separation of polar compounds should be achieved by a polar phase mainly due to selective partitioning. The most extensively used stationary phases are polyethylene glycol –PEG, also known as Wax– and polydimethylsiloxane–PDMS– (Ferreira & Cacho, 2009; Grob & Barry, 2004). Other polyethylene glycol based phases, differing slightly in polarity, are also adopted; for example, FFAP phases are mostly used to analyse volatile fatty acids and phenols. The length, internal diameter and film thickness ($d_f$) of the column are other three parameters to be chosen. A longer column will give better separation but leads to a longer analysis time. Narrow bore columns will improve separation efficiency and thicker films will provide better sample loading capacity. A typical column should be 30 m long, 0.25 mm internal diameter and 0.25 μm film thickness (30 m × 0.25 mm, $d_f$ = 0.25 mm); however, nowadays, for fast GC analysis, columns of 15 m to 30 m long and 0.10 mm to 0.15 mm internal diameter are commonly used. As the partition between carrier gas and the stationary phase is highly dependent on the temperature, the oven is usually programmed, one or more ramps, from a low temperature (*e.g.* 50 ºC) to maximum operation temperature of the column (*e.g.* 250 ºC); this procedure permit to obtain a good separation in the possible shortest time and to clean chromatographic column. Comprehensive two-dimensional gas chromatography (GC×GC) emerged recently as a powerful technique to analyse the composition of complex mixtures. GC×GC uses two columns of different characteristics, coupled in sequence through a suitable interface known as modulator, that allow peaks from the primary column to be

transferred onto the secondary column, so that an additional separation, and ideally complete resolution for all constituents, may be achieved (Weldegergis et al., 2011). The first dimension column is often non-polar and the second dimension column, shorter (1 m to 3 m), for fast analysis is polar.

The used carrier gases are nitrogen, hydrogen and helium. For a given GC system, best results, *i.e.* higher separation efficiency, are achieved with hydrogen. However, due to security factors, helium is commonly the choice. Modern equipment has electronic pneumatic controller (EPC) to maintain a constant flow during the analysis; in former systems a pressure controller is usually installed.

Detectors used commonly to analyze grape and wine volatile compounds could be classified broadly as universal or selective detectors. Flame ionization detector (FID) and mass spectrometer detector (MS) are the most used universal detectors. Atomic emission detector (AED) and electron capture detector (ECD) have been applied to halogenated compounds (Campillo et al., 2008; Schneider et al., 2003), flame photometric detector (PFD), pulsed flame photometric detector (PFPD) and sulphur chemiluminescence detector (SCD) are used in the analysis of sulphur compounds (Mestres et al., 2000). The mass spectrometer detector is the most important as it provides apart from the chromatogram an extra dimension of information, *i.e.* the mass spectra of the peak compound. Common MS routine analysis uses electron impact ionization (EI), while when searching structural information of an unknown compound, chemical ionization (CI) or even $MS^n$ should be also applied. Quadruple (qMS) and ion trap (ITMS) analysers are the most used, either in scan mode, where scanning of all possible fragment ions within the specific range (*e.g.* 35 *m/z* to 350 *m/z*) takes place, or in the selected ion monitoring (SIM) mode, where only pre-selected ions will be detected; in the later mode, MS became a selective detector with lower detection limits. Furthermore, when faster acquisition rate is required, a time-of-flight mass analyser (TOFMS) should be used. TOFMS is fully compatible with GC×GC, providing sufficient data density for an accurate definition of the narrow peaks (Weldegergis et al., 2011).

### 3.2 Identification of volatile compounds

The simple way to identify volatile compounds is comparing retention times of the interest peaks with those of pure standard compounds. However, when dealing with complex mixtures, sometimes presenting non-resolved peaks, this method is very fallible; even when spiking the extract with pure standard compounds, although being safer, the identification remain problematic. For all the detectors except MS detector, this is the only way to identify volatile compounds.

When a pure standard is not commercially available and its synthesis is not possible, identification may be carried by comparing retention indexes (*e.g.* linear retention indexes, Kovats retention indexes) of compounds of interest with those of published in literature; retention indexes are based on the values attributed, by definition, to a series of homologous compounds (*e.g.* alkanes; $C_8 = 800$, $C_9 = 900$) and its relation with its retention time.

On the other hand, using a MS detector the identification is much more trustworthy because the conjugation of the fragment fingerprint of a molecule and its retention time is practically infallible. Furthermore, the conjugation of mass spectra and retention indexes may permit the identification of some suspected peaks. Mass spectra libraries are commercially available

and can be also constructed by injected and collect the spectra, under the same conditions, of purchase/synthesized pure standards; this last library gives the best results.

### 3.3 Quantification of volatile compounds

The quantification of the identified peaks of volatile compounds must be made after calibration of the method. External standards, internal standard and standards addition are the three approaches. Calibration with external standards involves the construction a curve, by regression of the points obtained (concentration, peak area), after the injection of several standard solutions (*e.g.* 6 points). This approach presupposes that losses occurred in samples and standard solutions treatment is exactly the same. However, samples analysis usually involve some treatment before extraction and, when injected by a micro-syringe, losses could occur during the injection; in this case one or more internal standards are added to the sample at the beginning of the procedure, in a known amount. Nevertheless, because extraction procedure and detector don't respond similarly to all volatile compounds, a relative response factor should be determined anyway. The standard additions methodology may be adequate when matrix effects occur. This method is much more time-consuming than the other two approaches because it adopts the spiking of each sample with increasing amounts of the compound to be quantified.

If the interest of an analysis is to identify a great number of compounds and/or to compare samples, it should be sufficient the use of a unique internal standard without previous determination of the response factors. The analysis is only semi-quantitative. However, when quantitative determination is imperative, the relative response factor for each compound must be determined. A more reliable quantitative analysis is conducted using labelled isotopes of each compound of interest as internal standards (Kotseridis et al., 1999).

## 4. GC-MS application on the evaluation of grape and wine aroma in NW Iberian Peninsula

### 4.1 Grapes and wines characterization

Atlantic Viticulture (NW Iberian Peninsula) include Galicia (NW Spain) and North Portugal, situated mainly on Atlantic Ocean border, where are grown several common cultivars. Five Appellations of Origin (AO) are included in Galicia, *Rías Baixas*, *Ribeiro*, *Valdeorras*, *Ribeira Sacra* and *Monterrei* and white (*Albariño, Loureira, Treixadura, Godello, Caíño blanco*) and red cultivars (*Mencia, Espadeiro, Caíño tinto, Mouratón, Brancellao, Souson*) are grown in this geographic area. In another hand, the *Vinhos Verdes* Apellation of Origin is situated in the northwest of Portugal being divided into 9 sub-regions: *Amarante*, *Ave*, *Baião*, *Basto*, *Cávado*, *Lima*, *Monção & Melgaço*, *Paiva* and *Sousa*. The Atlantic Ocean and the relief markedly influence the climate. There are seven recommended white grape varieties (*Alvarinho, Arinto, Avesso, Azal, Batoca, Loureiro* and *Trajadura*) and eight red grape varieties (*Amaral, Borraçal, Brancelho, Espadeiro, Padeiro de Basto, Pedral, Rabo de Ovelha* and *Vinhão*) used to produce these wines.

*Albariño* in Galicia (NW Spain) and *Alvarinh*o in north Portugal is the most important white cultivar grown in Atlantic Viticulture, because this grape variety is only cultivated for the production of wines of recognized high quality. Contrasting to *Alvarinho* wines, malolactic fermentation is almost always applied to produce *Albariño*. Apart from *Alvarinho* and

*Albariño*, four other cited varieties are the same: *Espadeiro*; *Treixadura* and *Trajadura*; *Caíño tinto* and *Borraçal*; *Brancellao* and *Brancelho (*EU Project GenRes 081, 1997).

The Albariño variety, both as a grape and a wine, has been the objective of several studies (Carballeira et al., 2001; Dieguez et al. 2003; Fernández et al. 1999; Orriols & Camacho, 1991; Versini et al., 1994). Albariño from Galicia (NW Spain) was characterized by a high intensity of floral and fruity descriptors and free monoterpenes being responsible for these floral notes (Carballeira et al., 2001; Falqué et al., 2001). Ribéreau-Gayon et al. (2000) compared *Albariño* wine to *Riesling, Muscadelle* and *Sauvignon* wines and showed that *Albariño* wine was the richest in terpene compounds. Studies performed with this cultivar growed in Rias Baixas AO from Spain (Vilanova & Sieiro, 2006b) showed that the wines contained a higher concentration of terpenes in free form than in bound form. Only linalool and eugenol assumed to have the strongest odour impact on the aroma of *Albariño* wines. The *Albariño* wines were characterized by balsamic, fruity and sweet series when odour activity values (OAV) were calculated. Volatile compounds with fruity and floral odours showed the highest odour activity values, contributing in a great measure to the aroma of *Albariño* wines.

Oliveira et al. (2000) performed a comparative study on volatile composition of two aromatic cultivars from *Vinhos Verdes* AO, *Alvarinho* and *Loureiro*, characterized by freshness and floral and fruity flavours. These cultivars are employed for high quality monovarietal wine production because they are over all appreciated by their aromatic characteristics. *Loureiro*, as is already known, is an aromatic variety because of the levels of linalool in the free fraction (Oliveira et al., 2000). *Alvarinho* variety, which is poorer than *Loureiro* with respect to the free fraction, presents interesting levels of terpenic compounds in the bound fraction, as well as *Loureiro. Loureiro* and *Alvarinho* varieties have an important reserve of volatile compounds (Oliveira et al., 2000, 2004). This fact may become important in winemaking, since these compounds, particularly linalool, can be liberated from a glycoside moiety by specific enzymes and so contribute to the final wine flavour; other compounds such as monoterpenic oxides and diols, at the concentrations found in this study, may be rearranged at acidic pH to produce aromatic compounds. The ratio between (*Z*) and (*E*) isomers of 8-hydroxylinalool present in the glycosidically bound fraction seems to be important in differentiating *Alvarinho* and *Loureiro* varieties, with values near 6 for the first cultivar and about unity for the second one. The results of this work showed that it is possible to differentiate the recommended grape varieties for the *Vinhos Verdes* Region with regard to the quantification of monoterpenic compounds either in the free or in the glycosidically bound fraction. In another study, Oliveira et al. (2004) characterize five *Vinhos Verdes* grape varieties (*Alvarinho*, *Loureiro*, *Avesso*, *Amaral* and *Vinhão*) in terms of monoterpenic compounds present either in free form (17) or in glycosidically bound form (21). Nevertheless, apart from *Alvarinho* and *Loureiro*, the other three cultivars are poor respecting monoterpenic compounds.

Wines elaborated with *Loureiro* and *Alvarinho* cultivars from *Vinhos Verdes* Region showed similar composition on volatiles. *Loureiro* wines are globally richer than *Alvarinho* ones respecting monoterpenic compounds in both free and glycosidically bound forms. The varietal compounds which could influence particularly the aroma of these wines seem to be only linalool, HO-trienol, α-terpineol and β-damascenone. Terpenols seem to be more important to *Loureiro* wines and the $C_{13}$-norisoprenoids for *Alvarinho* ones. Respecting

fermentative compounds, *Alvarinho* wine is also particularly rich in fatty acids ethyl esters related to lipid metabolism and acetates of fusel alcohols, which can provide it a fruity character; *Loureiro* contains higher levels of esters of organic acids and 2-phenylethanol, conferring fruity and floral notes. Sensory analysis agreed with chemical analyses showing a pronounced tree and tropical fruit character for *Alvarinh*o wines while *Loureiro* wines present more intense citrus fruit notes (Oliveira et al., 2008a, 2008b). The authors indicated the possibility of discriminating *Loureiro* from *Alvarinho* wines by the ratio between (*E*) and (*Z*) isomers of 3-hexen-1-ol, in free form, and of 8-hydroxylinalool, in the glycosidically bound form.

Genisheva & Oliveira, 2009, compared the volatile composition of all white cultivars from *Vinhos Verdes* AO (*Arinto, Azal, Avesso, Batoca, Trajadura, Alvarinho* and *Loureiro*). In the free fraction, the *Loureiro* variety could be easily differentiated from the other six varieties by the important levels of linalool, above the odour perception threshold. This was in contrast to *Alvarinho, Avesso, Arinto, Azal* and *Trajadura*, where geraniol prevailed. The Batoca variety showed a very poor monoterpenic profile in the free form. *Arinto, Avesso, Azal* and *Trajadura* had a more equilibrated profile in terms of aroma compounds in both fractions. The *Arinto* variety showed a high potential of aroma compounds in the bound fraction. On the other hand, *Alvarinho* was the richest variety in respect to the glycosidically bound form, followed by *Loureiro*. In this fraction, linalool and 3,7-dimethylocta-1,5-dien-3,7-diol were the most abundant compounds in the *Loureiro* grape cultivar; additionally, the isomer (*E*)-8-hydroxylinalool was in a higher concentration than (*Z*)-8-hydroxylinalool, while for the rest of the varieties the (Z) isomer prevailed. The results of this study showed that it was possible to differentiate the seven recommended white varieties for the production of *Vinho Verde*, regarding free and bound monoterpene composition. Cultivars *Loureiro, Arinto* and *Alvarinho* were clearly distinct from the other studied varieties, while *Trajadura* showed an intermediate position.

Wines from minority two white cultivars (*Blanco lexítimo* and *Agudelo*) from Galicia also were characterized by Gas chromatography (Vilanova et al., 2009). These cultivars are grown in Betanzos, the most northern viticultural geographic area from Galicia (NW Spain). The results obtained suggest that ethyl octanoate (apple flavour), isoamyl acetate (banana), ethyl hexanoate (fruity) and β-damascenone (floral) were the most powerful odorants for the white wines *Blanco lexítimo* and *Agudelo* from Betanzos. *Blanco lexítimo* was the most aromatic wine dominated by citric, banana, apple and pineapple aroma. *Agudelo* wine which is a minor aromatic, compared to *Blanco lexítimo*, presents high levels of fruity aromas.

Studies about red grapes and wines from Atlantic viticulture also were performed. In recent years there has been a trend towards recovering the use of native Galician grape cultivars, whose presence had become reduced. These cultivars are well adapted to the area and transmit to their wines the characteristics of the climate and soil in which they are grown. Although currently it is produced only in small quantities, the red cultivar *Caíño* is one of the most appreciated of the *Rías Baixas* and *Ribeiro* Appellation of Origin areas from NW Spain. *Caíño tinto, Caíño longo* and *Caíño bravo* were analysed by gas chromatography (Vilanova et al., 2007a). The wines made from the different cultivars and the vintages were clearly different. *Caíño longo* wines had the highest concentrations of acetates and esters. The concentrations of ethyl esters and acetates in *Caíño bravo* wines were comparatively very low. From an oenological point of view, the *Caíño tinto* was the most interesting wine

because its composition was the most equilibrated. Non-terpenic compounds were the most abundant aroma substances in the considered varieties (Vilanova et al., 2008).

Wines produced from other minority *Vitis vinifera* red cultivars *Castañal* and *Serradelo* from Galicia (NW Spain) also were studied (Vilanova & Martinez 2007; Vilanova et al., 2009). From the 36 compounds identified in *Castañal* wine, 10 were determined as the most powerful odorants: β-ionone, 3-methyl-1-butanol, benzyl alcohol, 2-phenylethanol, ethyl acetate, isoamyl acetate, ethyl lactate, ethyl butyrate, ethyl hexanoate and ethyl octanoate. These data suggested *Castañal* wines as a fruity (blackberry) and floral (rose) product (Vilanova & Martinez, 2007). In another hand, ethyl octanoate and β-damascenone (fruity and floral aroma, respectively) were the most odorant for the red wine *Serradelo* form Betanzos (Vilanova et al., 2009). Ethyl octanoate and β-damascenone were the most odorant for the *Serradelo* red wine.

### 4.2 *Terroir* effect on grape and wine volatile composition

*Terroir* has been acknowledged as an important factor in grape and wine quality, particularly in European viticulture. The *terroir* concept was born in the Europe Appellations of Origin (AO) and was used for many purposes: to guarantee the authenticity of the products against frauds, to justify an economical advantage linked to a specific property, to synthesize an historical local experience, to strengthen the defence of a community of growers facing economical competition and to explain the characteristics of the wines.

Quite recently, around the 1980's, a scientific approach of *terroir* was developed by several teams and led to establish some relations between some elements of the natural environment and the grape. Today *terroir* concept can be defined as interactive ecosystem, including climate, soil and the vine (van Leeuwen et al., 2004).

The Atlantic Ocean and the relief markedly influence the climate. This region is in Viticultural Zone C I a) of the Winegrowing Regions of the European Communities. The produced wine is unique and its specific characteristics are mainly due to the climate and soil. Several studies have been conducted to know the grape and wine volatile composition in basis to the *terroir* in Atlantic viticulture (Oliveira et al., 2000; Vilanova et al., 2007b; Zamuz & Vilanova, 2006a, 2006b).

The white *Vinho Verde* is softly alcoholic, with a delicate, fresh and fruity bouquet. Oliveira et al., (2000) investigated the influence of the climate and the soil on the volatile compounds of grapes from two autochthonous white grape varieties, *Alvarinho* and *Loureiro*, in two sub-regions for each one of them (*Alvarinho* in *Monção* and *Lima*; *Loureiro* in *Lima* and *Cávado*). The results showed that the global characteristics of the grapes from the two varieties depend on the harvest factor (climate) rather than on the sub-region where the vine is planted. The characteristics of the soil have an even lesser influence on the variableness of the samples, although the *Alvarinho* variety picked in the *Lima* sub-region seems to have different characteristics.

*Albariño* must and wine from NW Spain was studied in three different areas from *Rías Baixas* AO (Vilanova et al., 2007b; Zamuz & Vilanova, 2006a). The Rías Baixas was originally made up of three subzones, *Val do Salnés*, *O Rosal* and *Condado do Tea*. The Atlantic climate, with

wet winters and sea mists, varies between the subzones. The coolest is *Val do Salnés*, and the hottest is southerly *Condado do Tea* and *O Rosal*, with occasional temperatures over 35 $^{o}$C and colder winters. Differences of climate and geography make the wines from the different origins individual in their own right. Non-terpenic compounds were the most abundant compounds in the free aroma fraction of the *Albariño* musts from the three geographic areas; of these, 2-phenylethanol (rose aroma) was the most important. The *Albariño* must from *O Rosal* should be the most aromatic since it had significantly higher volatile compounds content, with the bound compounds making up the largest group, quantitatively. The *Albariño* must from *Val do Salnés* had the lowest concentrations of volatile compounds and should therefore be the least aromatic; this could be related to its lower maturation index.

Respecting wines, *Albariño* from *O Rosal* was characterized by a high content of higher alcohols, while wines from *Val do Salnés* show the highest concentrations of free terpenes, acetates and ethyl esters. Wines of *Condado do Tea* show the highest concentrations of $C_{13}$-norisoprenids, principally due to α-ionone (Zamuz & Vilanova, 2006b). The results obtained in the study of *Albariño* wines showed that significant differences have been found among different geographic areas of *Rías Baixas* AO (northwestern Spain) in terms of the concentrations of most aromatic compounds.

Another comparative study was conducted on *Albariño* wine produced from musts from northern and southern Galicia (NW Spain) (Vilanova et al., 2007b). The influence of *terroir* on varietal and fermentative volatile compounds was studied. Data obtained from gas chromatography showed that differences were present in wine volatiles. The *Albariño* wines from northern Galicia showed the highest total concentration of volatiles analysed, dominated by higher contents in total free terpenes and acetates. Total higher alcohols and ethyl esters characterised the *Albariño* wine from the south. Among the terpenes found, geraniol was markedly abundant in the north, while nerol and linalool were most abundant in the south. Among the alcohols, 2-phenylethanol and benzyl alcohol showed the highest concentrations in the south and in the north, respectively. *Albariño* wines from the south were more heterogenic than those from the north. Differentiation of these wines was possible. This behaviour could be due to the predominance of terroir over the varietal character of the wines.

### 4.3 Vinification techniques influence on volatile composition of wines

Changes in volatiles during maturation in bottles of monovarietal *Vinhos Verdes* wines from *Loureiro* and *Alvarinho* grape varieties were followed by chemical and sensory analyses (Oliveira et al., 2008a). Young wines and wines matured for 8 and 20 months were studied. The volatiles were determined by gas chromatography-mass spectrometry (GC-MS) after extraction on XAD-2 resin. Straight chain fatty acid ethyl esters and acetates of fusel alcohols decreased quicker for *Loureiro* wine, while the increase in ethyl esters of branched fatty acids was similar for both varieties. Linalool, HO-trienol, α-terpineol and β-damascenone could be used to differentiate between each variety. However, linalool decreased to negligible values after 20 months of maturation. β-damascenone decreased but remained high enough to be useful for differentiating each variety. Sensory analysis indicated a decrease of tropical fruit and tree fruit characters with conservation time for *Alvarinho* wine, and the opposite for *Loureiro;* moreover, citrus fruit character decreased in both varieties.

### 4.4 Yeast influence on volatile composition of wines

The yeast responsible for alcoholic fermentation in winemaking is usually introduced into the must from the surface of the grapes, the surface of winery equipment, or from specifically prepared cultures. The fermentation process can occur either naturally, without inoculation, or by inoculating the must with selected starters. The use of locally selected yeast strains (usually belonging to the species *Saccharomyces cerevisiae*), with strain-specific metabolic characteristics can positively affect the final quality of the wine (Regodon et al., 1997; Romano et al., 2003). Several studies have clearly shown the effects of indigenous and inoculated yeast populations on the wine volatile composition (Mateo et al., 2001; Nurgel et al., 2003).

From Atlantic viticulture, a comparative study was made of the fermentation products of Spanish *Albariño* wines produced with spontaneous yeast flora and an indigenous selected *Saccharomyces cerevisiae* strain (Vilanova & Sieiro, 2006a). The gas chromatography data showed that the wines differed in their volatiles contents. The wine produced by spontaneous fermentation showed higher contents in higher alcohols, ethyl esters (except ethyl hexanoate and ethyl octanoate) and acetates. Alb16 selected yeast strain led to the production of extra ethyl hexanoate and hexyl acetate. The wines obtained by the spontaneous fermentation were more aromatic than those obtained with the Alb16 yeast strain.

Another study (Vilanova & Masneuf-Pomarède, 2005a, 2005b) was performed with *S. cerevisiae* cerevisiae yeast strains (ASln1, ASln2 and ASln20) isolated from musts obtained from *Albariño* grapes harvested in the *Rías Baixas* region (Spain). ASln20 produced the highest amounts of alcohol. Marked differences in the volatile composition of the fermented musts, determined by GC were dependent upon the yeast strain used. *S. cerevisiae* ASln1 and ASln20 produced the greatest quantities of esters, (ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate) which can give to wine a fruity aroma. These results suggest that the production level of these compounds is characteristic of the individual yeast strains, which highlights the importance of characterising yeast strains for industrial use.

## 5. Correlation between volatiles by GC and sensory properties of wines

To understand the chemical compounds in wine that shown sensory characteristics, is necessary some information regarding both, the volatile composition and the sensory properties. Gas chromatography is an important analysis technique of wine volatile components, although the aromatic impact of volatiles identified is evaluated, generally by determining perception thresholds.

The odour activity value (*OAV*) is a useful parameter to assess the relative importance of individual chemical components present in a wine. The aroma active compounds are volatiles whose concentration in wine is above their odour threshold ($OAV>1$). However, even when the *OAV* of a particular compound is less than 1, it still might contribute to the aroma of a wine as a consequence of some additive or synergic effect among compounds with similar aroma nature.

In another hand, sensory analysis invoices the detection and description of qualitative and quantitative sensory components of a product by trained panel of judges (Meilgaard et al.,

1999). Quantitative Descriptive Analysis (Stone & Sidel, 1998) is one of the most comprehensive and informative tools used in sensory analysis. This technique can provide complete sensory descriptions of a product as wine.

Relationships between volatile composition and sensory descriptors of wines have been explored by other researchers (Barbe et al., 2008; Francis & Newton, 2005). Several authors have suggested the use of multivariate strategies such as Partial Least Square (PLS) regression to predict sensory descriptors from chemical composition in wine (Koussissi & Paterson, 2007; Tenenhaus et al., 2005).

The study of correlation between volatile compounds by instrumental analysis and sensory properties of *Albariño* wine from NW Spain was performed by Vilanova et al. (2010). The results of the investigation showed the compounds that most contributed to the flavour of *Albariño* wines in instrumental analysis were those related to fruity (ethyl esters and acetates) and floral aromas (monoterpenes). Similar results were found in sensory analysis where the descriptors with the highest Geometric Mean were fruity and floral aromas too (citric, flowers, fruit, ripe fruit, apple and tropical). Therefore, this work demonstrates that some relationship between sensory data and volatile compounds exist to asses sensory properties in *Albariño* wines.

Other studies analysed the aroma of wines from several minority red cultivars from NW Spain by Gas Chromatography Mass Spectrometry (GC-MS) and sensory descriptive analysis (SDA) (Canosa et al. 2011; Vilanova et al., data not published). Sixteen volatile compounds and twelve sensory attributes showed significant differences among red wines *Brancellao, Mencía, Merenzao, Mouratón* and *Sousón*. Twenty out of fifty one quantified volatile compounds were present in some samples at concentrations higher than their corresponding odour thresholds (*OAV* >1), thus contributing to the final wine aroma. Principal component analysis (PCA) applied to volatile compounds showed three groups of cultivars: *Mencía-Brancellao, Mouratón-Merenzao* and *Sousón. Souson* and *Mouraton* wines were the most different from a sensory viewpoint, both cultivars being clearly linked to valuable traits like aroma and taste intensity and quality. Partial Least Square (PLS) regression applied to volatile compounds and aroma descriptors yielded a satisfactory model for the prediction of four important aroma descriptors in this set of wines - aroma quality, aroma intensity, herbaceous and red fruit - from instrumental analysis data. Free and bound compounds from *Pedral* and *Espadeiro* red cultivars (NW Spain) also were studied (Canosa et al., 2011). *Pedral* cultivar showed an important contribution of glycosidically bound compounds, especially $C_{13}$-norisoprenoids.

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8

# The Aroma of Rojal Red Wines from La Mancha Region – Determination of Key Odorants

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## 1. Introduction

Wine aroma is one of the most influential properties when it comes to consumer preference, and is mainly determined by the volatile compounds. The flavour of young wines results from a series of different biochemical and technological processes. Formation of volatile compounds begins in the grape, while during juice production, fermentation, maturation, ageing and storage the chemical composition continues to change. The amount and type of chemicals that influence wine flavour therefore depend on many factors including the origin of the grapes, grape varieties and ripeness, soil and climate, yeast used during fermentation and a variety of other winemaking practices (Kotseridis & Baumes, 2000; Rapp, 1998; Spranger et al., 2004).

Grape aroma compounds mainly appear either in their free form, directly contributing to wine aroma, or as non-volatile sugar-bound conjugates (monoglucosides or disaccharide glucosides), these being the predominant form in aromatic varieties (Günata et al., 1985). To release the aglycones that enrich wine aroma, bound forms must be subjected to acid or enzyme hydrolysis, normally using commercial preparations with β-glucosidase activity (Marais & Rapp, 1988; Carballeira et al., 2001). Over the past ten years, many aroma compounds in monovarietal wines have been identified and quantified. Particular attention has been devoted recently to the analytical characterization and the quality improvement of the varietal aroma of wines. Several studies have focused on the identification of volatile components of different varieties including the volatile components originating from the nonvolatile precursors (Gunata et al., 1985; Sánchez-Palomo et al., 2006, 2007; Ugliano et al., 2006; Ugliano & Moio, 2008; Rocha et al., 2010; García-Carpintero et al., 2011a, 2011b). These precursors have been reported as glycosides having the aroma compounds as their aglycons. The knowledge of the varietal volatile composition offers a means of evaluating the potential aroma of a variety and to improve the wine aroma quality.

To understand the chemical compounds in wine that showed sensory characteristics, it is necessary to obtain some information regarding both volatile composition and sensory properties (Francis & Newton, 2005). Gas chromatography is an important analysis technique for volatile and non-volatile components to the aroma of the wine. However, the wine volatile fraction is extremely complex, mainly because of the great number of

compounds which form it. To date, more than 1000 compounds have been identified, which are from different chemical classes, covering a wide range of polarities, solubility and volatilities. The volatile compounds responsible for the varietal aroma are present in only trace amounts, which means that to carry on their identification and quantification, an effective method of enrichment is required prior to their analysis by gas chromatography–mass spectrometry (GC–MS).

The search of adequate extraction techniques allowing the identification and quantification of wine volatile compounds has attracted the attention of many scientists. This has resulted in the availability of a wide range of analytical tools for the extraction of these compounds from wine. These methodologies are mainly based on the solubility of the compounds in organic solvents (liquid–liquid extraction: LLE, simultaneous distillation liquid extraction: SDE), on their volatility (static and dynamic headspace techniques), or based on their sorptive/adsorptive capacity on polymeric phases (solid phase extraction: SPE, solid phase microextraction: SPME, stir bar sorptive extraction: SBSE). In addition, volatile compounds can be extracted by methods based on combinations of some of these properties (headspace solid phase microextraction HS-SPME, solid phase dynamic extraction: SPDE).

One of the most commonly used method for the analysis of volatile compounds in wine is SPE. The possibility of using different sorbent phases and eluents makes SPE a very selective technique, and the fact that only minor amounts of organic solvents are used compared to LLE, is why SPE has been extensively used for the analysis of volatile aroma compounds (Ferreira et al, 1998; Dominguez et al., 2002; Lopez et al., 2002; Ibarz et al., 2006; Campo et al., 2007; Loscos et al., 2009) and off-flavours (Dominguez et al., 2002; Insa et al., 2005) in wines. Solid-phase extraction (SPE) is widely used in analytical laboratories for either sample extraction or sample clean up procedures. This technique based on adsorbent materials where analytes are bound to active sites on a surface, allows the determination of a wide range of volatile compounds, requires smaller quantities of solvents and shorter time of analyses but is relatively tedious. Many benefits of SPE methods have been commonly cited including its robustness, potential for automation, capacity for providing clean extracts, selective isolations and even a fractionation of the different sample components. For these reasons, SPE is a powerful pre-concentration technique which can be easily adapted for routine analysis and, in fact, many studies based on SPE procedures for monitoring different compounds in wine samples have been published in the last years (Vianna & Ebeler, 2001; Rodriguez-Bencomo et al., 2002; Sala et al., 2002). However, as the SPE systems have a low number of chromatographic plates (Vianna & Ebeler, 2001) the selectivity (measured as the ratio between the chromatographic retention factors of analytes and interferences) must be high in order to get good separations. SPE has been successfully used to study the evolution of aromatic compounds of grapes during ripening (Lopez et al., 2002) and to determine the potential aroma in several varieties of Spanish grapes (Loscos et al., 2009).

Over the last few decades the introduction and spread of world renowned varieties has caused a massive loss of indigenous grapevine varieties traditionally grown in various grape-growing regions. *Vitis vinifera cv*. Rojal a minority grape variety is cultivated in La Mancha region in little areas with special climatologic conditions (warm summers, cold winters and low rain) that could influence on its aroma composition cultivated in a little restringed area. Only the knowledge of the chemical composition and sensory properties of

this variety can give opportunities for the adaptation of the characteristics of this minority grape variety to the winemaking procedures ruled by the consumer's preferences. As far as we know, the aroma of this grape variety has not yet been characterized.

The aim of this study was to characterize the free and bound volatile aroma compounds of Rojal red wines from La Mancha region (Spain) during four consecutive vintages by GC-MS, and determine the key odorants of the aroma of these wines.

## 2. Material and methods

### 2.1 Wine samples

Red, *cv*. Rojal grapes were obtained from the vineyards of La Mancha in the central-southeastern region of Spain. They were harvested at their optimal stage of ripeness and in health conditions, over four consecutive vintages (2007-2010).

Wines were elaborated from two batches of grapes (500 kg each) were elaborated in 250 l-Stainless steel tanks with skin maceration until the alcoholic fermentation. Winemaking conditions, included the addition of 100 ppm of $SO_2$, as $K_2S_2O_7$, after stemming and crushing, inoculation with *Saccharomyces cerevisiae* selected yeasts (UCLM S325, Fould-Springer), and fermentation temperature maintained at 24ºC. Manual punching down was done twice a day. Separation of wines from solids was performed when relative density reached a constant value. Subsequently, the malolactic fermentation was induced by inoculation with *Oenococcus oeni* lactic acid bacteria (Lactobacter SP1; Laffort). This second fermentation terminated in 2–3 weeks, as confirmed by TLC (Thin Layer Chromatography); the wines were then racked. After one month, the wines were racked again, filtered through 1.2 µm membranes (Millipore, Bedford, MA, USA), bottled, and stored in room with a constant temperature between 16 and 18 ºC.

### 2.2 Reagents and standards

Dichloromethane and methanol were purchased from Merck (Darmstadt, Germany). Ammonium sulfate and anhydrous sodium sulfate were from Panreac (Barcelona, Spain). Pure water was obtained from a Milli-Q purification system (Millipore, U.S.). LiChrolut EN resins were purchased from Merck (Darmstadt, Germany). The chemical standards were supplied by Sigma (St. Louis, MO, USA), Aldrich (Gillingham, UK), Firmenich (Geneva, Switzerland), Panreac (Barce lona, Spain), Merck (Darmstadt, Germany), Fluka (Buchs, Switzer land), and Lancaster (Strasbourg, France). An alkane solution (C8–C28) in dichloromethane was employed to calculate the linear retention index (LRI) of each analyte.

### 2.3 Analysis of major volatiles

Major volatile compounds were analyzed by direct injection (Sánchez-Palomo et al., 2006) of a HP-5890 GC with a FID detector, using a CP-Wax-57 capillary column (50 m×0.25mm i.d.; 0.25 µm film thickness). The oven temperature program was: 40 °C (5 min)–4 °C/129 min–120 °C. Injector and detector temperature were 250 and 280 °C, respectively. One microliter (1 µl) was injected in split mode, split ratio 1:15. Carrier gas was He (0.7 ml/min).

### 2.4 Extraction of minor volatiles

The aroma compounds were separated by adsorption/desorption on preconditioned polypropylene-divinylbenzene cartridges (Sánchez-Palomo et al., 2006) (LiChrolut EN, Merck, 0.5 g of phase). One hundred milliliters of wine added of 40 µl of 4-nonanol, as an internal standard, was passed through the LiChrolut EN column at a flow rate of 1 ml/min. The column was rinsed with 50 ml of pure water to eliminate sugars and other low-molecular-weight polar compounds. The free fraction was eluted with 10 ml of dichloromethane. All dichloromethane extracts were cooled to−20 °C to separate the frozenwater from the organic phase by decantation, and then dried over anhydrous sodiumsulfate using nitrogen stream, the organic phase was concentrated to a final volume of 200 µl. The bound fraction was eluted with 25ml of ethyl acetate. Ethyl acetate extracts were evaporated to dryness under vacuum, and then re-dissolved with 1 ml of methanol.

### 2.5 Enzymatic hydrolysis of bound fraction

A 500 µl methanol extract was evaporated to dryness under nitrogen stream. The dried glycosidic extract was dissolved in a 100 µl citrate–phosphate buffer (0.2 M, pH 5). Enzymatic treatment with AR2000 (Gist Brocades) was completed at 40 °C for 18 h according to optimum conditions described previously (Sánchez-Palomo et al., 2006). The mixture was then extracted five times with 2 ml of pentane–dichloromethane (2:1 v/v). After adding 4-nonanol (1 g/l) as the internal standard the extract was concentrated to a final volume of 200 µl under nitrogen stream.

### 2.6 Gas Chromatography–Mass Spectrometry (GC–MS) analysis

An Agilent Gas Chromatograph model 6890N coupled to a Mass Selective Detector model 5973 inert equipped with a BP-21, Polyethylene glycol TPA treated, capillary column (60 m×0.25 mm i.d.; 0.25 µm film thickness) was used. Operating conditions were as follows. Oven temperature program was: 70 °C (5min)–1 °C/min–95 °C (10min)–2 °C/min–200 °C (40 min). Injector and transfer line temperatures were 250 °C and 280 °C, respectively. Mass detector conditions were: electron impact (EI) mode at 70 eV; source temperature: 178 °C; scanning rate: 1 scan/s; mass acquisition: 40–450 amu. One microlitre (1 µl) was injected in splitless mode. Carrier gas was helium (1 ml/min). Retention time, Wiley mass-spectral library, and pure volatile compounds were used for identification, confirmation and preparation of standard solutions of volatile compounds. The relative response areas for each of the volatile compounds to the internal standard were calculated and interpolated in the corresponding calibration graphs. For the calibration, standard solutions were prepared in 12% v/v ethanol with 5 g/l tartaric acid and the corresponding internal standard in the same concentration as in the samples. Calibration curves were drawn for each standard at eight different concentration levels. The measurements of all standards were performed in triplicate. When the authentic standard were not available the identification was based on the comparison with the spectral data of Wiley A library and the chromatographic dates of the literature, semi-quantitative analysis of these compounds were made assuming response factor equal to one.

### 2.7 Odor activity values

To evaluate the contribution of a chemical compound to the aroma of a wine the odor activity value (OAV) was determined. OAV is a measure of importance of a specific compound to the odor of a sample. It was calculated as the ratio between the concentration of an individual compound and the perception threshold found in literatures (Francis & Newton, 2005; Vilanova, et al., 2008).

### 2.8 Sensory analysis

Wines were evaluated in duplicate by a panel consisting in 10 experienced wine-testers (7 female and 3 male) ranging ages from 24 to 45 years. Assessment took place in a standard sensory-analysis chamber (ISO 8589, 1998) equipped with separate booths. Wines were sniffed. Three wines were presented in each session, in coded standard wine-testing glasses according to standard ISO 3591, 1997 and covered with a watch-glass to minimize the escape of volatile components. Testing temperature of wine was 18º C.

### 2.9 Statistical analysis

Analysis of variance (ANOVA) was performed using the general linear model procedure to determine significant differences in the concentration of volatile compounds of Rojal wines on different vintages. Student–Newman–Keuls test was conducted when the samples exhibited significance between them, with the level of significance set at $P<0.05$. Both ANOVA and Student–Newman–Keuls test were performed with SPSS 19.0 (2010) for Windows statistical package.

## 3. Results and discussion

### 3.1 Free volatile aroma composition of Rojal wines

The free volatile compounds of wines were extracted with dichloromethane. Representative wine aroma extracts for chemical and olfactory analysis were obtained using this solvent. Fig. 1 is the TIC of free volatile compounds of Rojal wines detected by SPE–GC–MS. Quantitative data of the volatile compounds found in free aroma fraction of the young red wines from Rojal grape variety are shown in Tables 1 and 2. The data are expressed as means (µg/l) of the GC–MS analyses of duplicate extractions and they correspond to the average of the analyzed wines. Improvement in the analytical method used to extract the volatile compounds from these wines has allowed us to identify and quantify 80 free volatile compounds in Rojal red wines including alcohols, esters, acids, terpenes, $C_{13}$ norisoprenoids, $C_6$ compounds and benzenic compounds. They have been positively identified and quantitatively determined.

#### 3.1.1 Varietal aroma compounds

**$C_6$ compounds.** All Rojal wines displayed higher concentration of $C_6$ alcohols, principally 1-hexanol. C6 compounds, which supply "vegetal" and "herbaceous" nuances to the wine, usually have a negative effect on wine quality when their concentration is above their odor threshold values (Ferreira et al., 1995). However, 1-hexanol and (Z)-3-hexen-1-ol were found

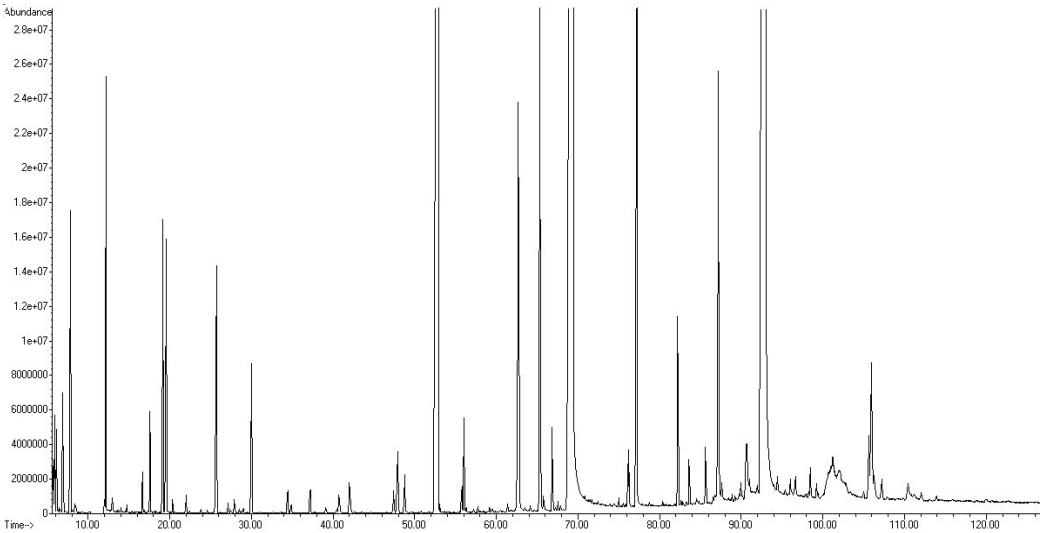


Fig. 1. TIC of free volatile compounds of Rojal wines detected by SPE–GC–MS.

at concentrations under their odor threshold values (8000 µg/l and 400 µg/l respectively) in the analyzed Rojal wines. Rojal wines from 2008 vintage showed the lower concentration of $C_6$ compounds, due to a lightly superior ripening stage than the rest of studied vintages. Among the compounds found, we give prominence to the ratio between *trans*- and *cis*-3-hexen-1-ol contents. As previously reported (Hatanaka, 1993), the composition of the $C_6$ compounds is strongly dependent on four enzymes, which catalyze the biosynthesis of these compounds, and among these four, lipoxygenase and hydroperoxide lyase are particularly important. Thus, the level and relationships between these compounds could be considered as characteristic of the *V. vinifera variety*. Specifically, for Rojal, *cis*-3-hexen-1-ol was higher than *trans* form in all of the samples analyzed from the four vintages considered; these results are in agreement with Boido et al., 2003 in wines from Tannat grape variety and by García-Carpintero et al., 2011a, in wines from Bobal grape variety and opposite result were found by García-Carpintero et al., 2011b in wines from Moravia Agria grape variety.

**Terpenes and $C_{13}$ norisoprenoids.** Terpene compounds are characteristic of aromatic varieties such as Muscat, have a low olfactory threshold and are generally associated with floral and citric aromas (Etiévant, 1991; Guth, 1997). Linalool, β-citronellol and *trans*-geraniol were detected in wines from four vintages.

The concentration of linalool, β-citronellol and *trans*-geraniol was higher in the 2007 vintage. However, their contribution to Rojal wine seems negligible when their odor thresholds are considered (15 µg/l linalool, 100 µg/l β-citronellol and µg/l, 30 µg/l *trans*-geraniol Guth,1997). The first study on volatile composition of Rojal wines over four vintages from La Mancha region shows that the aroma of this cultivar is not terpene dependent.

| | RI* | Aroma Compounds | Vintage | | | |
|---|---|---|---|---|---|---|
| | | | 2007 | 2008 | 2009 | 2010 |
| Fluka | 1282 | 1-Hexanol | 2187$^{c}$ (0.10) | 1413$^{a}$ (0.12) | 2013$^{b}$ (2.35) | 2023$^{b}$ (1.56) |
| Sigma-Aldrich | 1286 | (*E*)-3-Hexen-1-ol | 203$^{c}$ (4.50) | 173$^{b}$ (0.26) | 164$^{b}$ (2.14) | 135$^{a}$ (1.05) |
| Sigma-Aldrich | 1296 | (*Z*)-3-Hexen-1-ol | 208$^{b}$ (0.80) | 185$^{b}$ (0.22) | 198$^{b}$ (1.39) | 149$^{a}$ (0.58) |
| Sigma-Aldrich | 1300 | (*E*)-2-Hexen-1-ol | 15.7$^{a}$ (2.80) | 9.01$^{a}$ (0.18) | 11.9$^{a}$ (2.57) | 15.5$^{a}$ (0.86) |
| | | **Total $C_6$ compounds** | **2614** | **1780** | **2387** | **2323** |
| Fluka | 1529 | Linalool | 13.9$^{b}$ (2.80) | 11.8$^{b}$ (1.23) | 12.2$^{b}$ (2.34) | 9.11$^{a}$ (1.26) |
| Fluka | 1755 | β-Citronellol | 14.5$^{b}$ (4.20) | 8.93$^{a}$ (1.05) | 12.3$^{b}$ (2.24) | 13.9$^{b}$ (2.56) |
| Firmenich | 1801 | β-Damascenone | 0.48$^{c}$ (2.80) | n.d | 0.32$^{b}$ (0.87) | 0.21$^{a}$ (0.85) |
| Fluka | 1831 | *Trans* geraniol | 9.87$^{b}$ (2.00) | 8.87$^{a}$ (1.36) | 9.21$^{b}$ (1.23) | 9.47$^{b}$ (0.59) |
| T.I. | 2558 | 3-Hydroxy-β-damascone | 29.5$^{c}$ (2.30) | 7.52$^{a}$ (1.00) | 15.6$^{b}$ (2.33) | 19.3$^{b}$ (1.26) |
| T.I. | 2582 | 3-Oxo-α-ionol | 10.3$^{a}$ (1.23) | 25.6$^{c}$ (1.08) | 18.7$^{b}$ (3.14) | 18.5$^{b}$ (0.24) |
| T.I. | 2722 | 3-Hydroxy-7,8-dehydro-β-ionol | n.d | 1.90$^{c}$ (2.05) | 1.02$^{a}$ (1.87) | 1.45$^{b}$ (0.94) |
| | | **Total Terpene + $C_{13}$ norisoprenoids** | **77.6** | **65.6** | **69.4** | **71.9** |
| Sigma-Aldrich | 1503 | Benzaldehyde | 2.54$^{a}$ (2.80) | 2.81$^{c}$ (0.58) | 2.64$^{b}$ (2.36) | 2.88$^{c}$ (1.08) |
| T.I. | 1505 | 3(2H)-2-methyldihydro-thiophenone | 83.8$^{b}$ (5.30) | 81.3$^{a}$ (3.21) | 86.3$^{b}$ (1.87) | 91.7$^{c}$ (1.98) |
| Sigma-Aldrich | 1882 | Guaiacol | 41.7$^{a}$ (8.00) | 62.5$^{b,c}$ (1.28) | 58.3$^{b}$ (5.21) | 66.9$^{c}$ (0.88) |
| Sigma-Aldrich | 1895 | Benzyl Alcohol | 291$^{c}$ (1.40) | 239$^{a}$ (0.59) | 261$^{b}$ (4.26) | 287$^{c}$ (2.19) |
| T.I. | 1899 | 1,2-Benzothiazole | 1.39$^{a}$ (5.10) | 2.42$^{c}$ (1.21) | 1.99$^{b}$ (2.87) | 1.98$^{b}$ (1.69) |
| Sigma-Aldrich | 1971 | Phenol | 3.04$^{a}$ (3.30) | 11.5$^{c}$ (1.67) | 5.64$^{b}$ (1.49) | 9.58$^{c}$ (0.82) |
| Lancaster | 2055 | 4-Ethylguaiacol | 3.47$^{c}$ (2.70) | n.d. | 0.68$^{a}$ (1.24) | 1.97$^{b}$ (1.07) |
| Sigma-Aldrich | 2193 | Eugenol | 5.01$^{a}$ (2.80) | 6.18$^{b}$ (2.04) | 5.21$^{a}$ (1.14) | 6.51$^{b}$ (2.07) |
| Sigma-Aldrich | 2208 | 4-Ethyl phenol | 10.4$^{a}$ (1.90) | 23.3$^{c}$ (0.74) | 13.4$^{b}$ (1.23) | 20.7$^{c}$ (1.92) |
| T.I. | 2212 | 4-Hydroxy-2-methyl acethophenone | 1.56$^{a}$ (0.85) | 1.23$^{a}$ (1.59) | 1.89$^{b}$ (2.14) | 1.97$^{b}$ (1.48) |
| Sigma-Aldrich | 2219 | 4-Vinylguaiacol | 46.2$^{c}$ (2.10) | 15.3$^{a}$ (0.66) | 28.6$^{b}$ (3.29) | 12.4$^{a}$ (0.88) |
| Sigma-Aldrich | 2225 | Syringol | 156$^{b}$ (2.80) | 107$^{a}$ (0.85) | 172$^{b}$ (2.87) | 194$^{b}$ (1.07) |
| Lancaster | 2302 | Isoeugenol | 21.3$^{b}$ (1.20) | 26.7$^{b}$ (1.09) | 23.6$^{b}$ (1.82) | 17.2$^{a}$ (1.45) |

| | RI* | Aroma Compounds | Vintage | | | |
|---|---|---|---|---|---|---|
| | | | 2007 | 2008 | 2009 | 2010 |
| Sigma-Aldrich | 2378 | Benzoic acid | 178$^{b}$ (2.84) | 183$^{b}$ (2.04) | 167$^{b}$ (0.99) | 153$^{a}$ (0.08) |
| T.I. | 2501 | Benzeneacetic acid | 27.2$^{a}$ (1.10) | 46.2$^{c}$ (3.01) | 37.2$^{b}$ (0.87) | 33.6$^{b}$ (1.01) |
| Panreac | 2511 | Vanillin | 6.76$^{b}$ (0.50) | 4.55$^{a}$ (9.18) | 5.12$^{a}$ (2.47) | 5.81$^{a}$ (2.67) |
| Sigma-Aldrich | 2543 | Methyl vanillate | 15.9$^{a}$ (2.20) | 18.3$^{b}$ (1.69) | 17.8$^{b}$ (2.07) | 19.3$^{b}$ (0.27) |
| Lancaster | 2676 | Ethyl vanillate | 195$^{b}$ (8.10) | 98.2$^{a}$ (0.73) | 174$^{b}$ (1.08) | 182$^{b}$ (1.45) |
| Sigma-Aldrich | 2685 | Acetovanillone | 114$^{b}$ (7.30) | 79.2$^{a}$ (0.07) | 91.5$^{b}$ (1.65) | 75.8$^{a}$ (1.91) |
| Sigma-Aldrich | 2936 | Zingerone | 6.35$^{b}$ (2.69) | 2.88$^{a}$ (0.98) | 3.64$^{a}$ (2.48) | 15.9$^{c}$ (0.09) |
| Sigma-Aldrich | 2755 | 3,4-Dimethoxy phenol | 6.87$^{a}$ (0.20) | 7.56$^{a}$ (0.41) | 9.64$^{a}$ (1.05) | 12.0$^{b}$ (1.37) |
| Sigma-Aldrich | 3045 | Cinamic acid | 60.5$^{c}$ (0.29) | 60.8$^{c}$ (0.99) | 51.3$^{b}$ (5.32) | 44.6$^{a}$ (0.61) |
| T.I. | 3030 | Methyl vanillil eter | 15.4$^{a}$ (4.10) | 19.3$^{b}$ (3.09) | 18.1$^{b}$ (0.78) | 16.5$^{a}$ (2.39) |
| | | **Total Bencenic compounds** | **1292** | **1099** | **1237** | **1273** |

*Linear retention index on a DB Wax column
nd: not detected.
$^{a, b, c, d}$ According to the result of the Student–Newman–Keuls test, values to that no share a common superscript are significantly different ($p<0.05$).
T.I. Tentatively identified

Table 1. Mean concentration of free volatile compounds (µg/l) and relative standard deviations (n=2) of Rojal wines.

$C_{13}$-norisoprenoids are volatile compounds that could come from the direct degradation of carotenoid molecules such as β-carotene, lutein, neoxanthin and violaxanthin (Marais et al., 1992), and have an important role on the varietal character of wines because they have a very low odor threshold (Guth, 1997). These compounds were present in low concentration in all vintages. β-damascenone is normally considered a positive contributor to wine aroma (Escudero et al., 2007) and its odor threshold (0.05 µg/l) (Guth, 1997) was exceeded in 2007, 2009 and 2010 vintages. Floral and exotic fruit notes are attributed to β-damascenone, and due to its low odour threshold it can have a great sensorial impact on wines (Guth, 1997). However, other authors attribute its importance to a potentiating role of the fruit aromas of other compounds, rather than β-damascenone acting alone (Pineau et al., 2007).

**Benzene compounds.** Benzene compounds are an important group in varietal aroma, abundant in wines, including aromatic alcohols, aldehydes, volatile phenols and shikimic acid derivates. The volatile phenols in wines can come from grapes, both as free and bound aroma, or be generated during the alcoholic fermentation by chemical reactions such as phenolic acid degradation, or in the case of vinylphenols due to brettanomyces contamination (Suarez et al., 2007). Volatile phenols are considered characteristic components of wine aroma, although their influence on the final product may be positive or

negative depending on their concentrations. In our case volatile phenols did not attain levels sufficient to prompt off-flavours. Guaiacol, 4-ethylguaiacol and 4-vinylguaiacol were identified in wines. These compounds are principally formed during the fermentation process. Guaiacol is associated with medicinal flavours, the olfactory threshold of this compound 10 µg/l (Guth, 1997) was exceeded in all studied wines. Olfactory threshold of volatile phenols 4-ethylguaiacol, (110 µg/l), 4-vinylguaiacol (10000 µg/l) (Swiegers et al., 2005) were not exceeded in any case. Other remarkable benzenic compounds were the shikimic acid derivates, which point out by the elevated sensory impact. These compounds are formed by the aromatic aminoacid metabolic routes in plants or by yeasts, also can be extracting from wood (Swiegers et al., 2005). Benzaldehyde, benzyl alcohol and eugenol were other benzenic compounds identified in wines. Benzaldehyde and benzyl alcohol concentrations were not exceeded their olfactory threshold (350 and 10000 µg/l, respectively) (Etiévant, 1991), although these compounds could add a synergic effect to wine aroma with fruity and floral notes. The identification of eugenol in wines is related to sweet spice aroma, especially with clove aroma in wines, this compound exceeded in all wines studied their olfactory threshold (5 µg/l) (Guth, 1997).

### 3.1.2 Volatile compounds formed principally during the alcoholic fermentation

Although compounds from the grapes themselves are responsible for the varietal character of wines, the compounds formed during alcohol fermentation via yeast metabolism may have a positive or negative influence on wine sensory properties (Ferreira et al., 1995). Table 2 shows concentrations of volatile compounds formed principally during the alcoholic fermentation of Rojal red wines over four vintages expressed as mg/l as mean of two replicates. The major fermentation compounds such alcohols, ethyl esters, acetates and fatty acids were detected at similar total levels in both wines, some minor differences were observed.

**Aldehydes.** Acetaldehyde is the majority aldehyde in the wine. It is formed mainly by the metabolism of yeasts, and is associated with fruity aromas and notes to nuts or dried fruits. The amount of acetaldehyde found in the wines is closely related to enzymatic manning of the strain of yeast used but also, this concentration can be changed with the conditions of fermentation, especially with the amount of $SO_2$ added to the medium (Herraiz et al., 1989). In this study, the conditions of fermentation and the amount of $SO_2$ added to the musts were the same in all case, so that the observed differences can be attributed to the different composition of the initial must used in the elaboration that may be attributed to climatic variations.

**Alcohols:** Alcohols were one of the largest group of free volatile compounds in La Mancha Rojal wines. The most abundant compounds were the higher alcohols, in accordance with the literature (Baumes et al., 1986). These compounds can be recognized by their strong and pungent smell and taste and they are related to herbaceous notes. The total concentration of higher alcohols in Rojal wines was below 300 mg/l. This allowed them to contribute positively to the aroma of the Rojal wines, giving it complexity (Mateo et al., 2001; Selli et al., 2004). Among the aliphatic alcohol 3-methyl-1-butanol showed the highest concentration in the four vintages.

Methanol is derived from the demethylation of skin pectins. Since we have mentioned previously, the conditions of fermentation were the same for all wines, so in this case the concentration differences can be attributed to the decreased permeability of the skin of Rojal grapes.

| | RI* | Aroma Compounds | Vintage | | | |
|---|---|---|---|---|---|---|
| | | | 2007 | 2008 | 2009 | 2010 |
| Sigma-Aldrich | 800 | Acetaldehyde | 7.93$^{b}$ (3.60) | 3.08$^{a}$ (0.99) | 6.21$^{b}$ (1.26) | 10.6$^{c}$ (1.25) |
| | | **Total aldehydes** | **7.93** | **3.08** | **6.21** | **10.6** |
| Sigma-Aldrich | 879 | Metanol | 81.6$^{c}$ (2.10) | 57.6$^{b}$ (3.27) | 51.3$^{b}$ (3.28) | 40.1$^{a}$ (1.05) |
| Sigma-Aldrich | 1060 | 1-Propanol | 14.7$^{a}$ (2.60) | 20.4$^{b}$ (1.29) | 17.2$^{a}$ (1.08) | 15.3$^{a}$ (0.36) |
| Merck | 1214 | Isobutanol | 41.1$^{c}$ (2.30) | 31.5$^{a}$ (3.64) | 35.2$^{b}$ (1.68) | 30.9$^{a}$ (2.36) |
| Sigma-Aldrich | 1221 | 2-Methyl-1-butanol | 58.0$^{a}$ (5.30) | 60.2$^{b}$ (2.09) | 58.7$^{a}$ (0.87) | 57.6$^{a}$ (0.14) |
| Sigma-Aldrich | 1221 | 3-Methyl-1-butanol | 181$^{a}$ (1.00) | 199$^{d}$ (1.18) | 191$^{c}$ (3.08) | 185$^{b}$ (2.58) |
| Fluka | 1328 | 4-Methyl-1-pentanol | 0.09$^{a}$ (5.66) | 0.03$^{a}$ (6.17) | 0.05$^{a}$ (2.87) | 0.08$^{a}$ (3.69) |
| T.I. | 1337 | 2-Penten-1-ol (Z)** | 4.58$^{c}$ (1.28) | 4.91$^{c}$ (3.17) | 3.29$^{b}$ (0.84) | 2.68$^{a}$ (1.29) |
| Fluka | 1341 | 3-Methyl-1-pentanol | 0.10$^{a}$ (1.00) | 0.12$^{a}$ (5.65) | 0.11$^{a}$ (1.69) | 0.11$^{a}$ (4.69) |
| Fluka | 1472 | 1-Heptanol | 0.04$^{a}$ (3.97) | 0.02$^{a}$ (5.29) | 0.02$^{a}$ (0.34) | 0.03$^{a}$ (5.18) |
| Fluka | 1545 | 2,3-Butanediol (levo)** | 0.06$^{a}$ (5.01) | 0.08$^{a}$ (4.67) | 0.06$^{a}$ (1.91) | 0.07$^{a}$ (5.36) |
| Fluka | 1585 | 2,3-Butanediol (meso) | 0.02$^{a}$ (4.58) | 0.03$^{a}$ (7.08) | 0.02$^{a}$ (2.11) | 0.04$^{a}$ (3.47) |
| Sigma-Aldrich | 1725 | 3-Methylthio-1-propanol | 2.51$^{a}$ (0.58) | 2.68$^{a}$ (1.18) | 2.63$^{a}$ (0.17) | 2.57$^{a}$ (2.64) |
| Fluka | 1892 | Phenylethylalcohol | 25.1$^{a}$ (2.20) | 30.7$^{b}$ (0.28) | 28.4$^{b}$ (3.27) | 34.5$^{b}$ (2.36) |
| | | **Total alcohols** | **409** | **407** | **388** | **369** |
| Sigma-Aldrich | 834 | Ethyl acetate | 36.9$^{b}$ (2.60) | 35.6$^{b}$ (0.25) | 28.9$^{a}$ (1.29) | 24.5$^{a}$ (2.01) |
| Fluka | 1080 | Ethyl butanoate | 0.06$^{a}$ (5.90) | 0.07$^{a}$ (1.36) | 0.06$^{a}$ (2.17) | 0.08$^{a}$ (3.78) |
| Sigma-Aldrich | 1145 | Isoamyl acetate | 0.03$^{a}$ (2.90) | 0.05$^{a}$ (1.48) | 0.04$^{a}$ (2.64) | 0.05$^{a}$ (5.87) |
| Fluka | 1185 | Ethyl hexanoate | 0.43$^{b}$ (1.40) | 0.38$^{a}$ (2.34) | 0.41$^{b}$ (2.11) | 0.35$^{a}$ (2.45) |
| Sigma-Aldrich | 1294 | Hexyl acetate** | 1.99$^{a}$ (7.80) | 2.65$^{b}$ (1.92) | 2.08$^{a}$ (2.74) | 3.07$^{b}$ (0.70) |
| Sigma-Aldrich | 1326 | Ethyl lactate | 15.9$^{a}$ (1.80) | 16.7$^{a}$ (0.58) | 17.6$^{a}$ (2.98) | 22.7$^{b}$ (1.64) |
| T.I. | 1321 | 2-hydroxy 3-methylethyl butanoate** | 6.95$^{b}$ (2.34) | 5.64$^{b}$ (1.26) | 4.12$^{a}$ (1.54) | 3.24$^{a}$ (2.18) |
| Sigma-Aldrich | 1436 | Ethyl octanoate | 0.45$^{a}$ (11.2) | 0.37$^{a}$ (6.17) | 0.39$^{a}$ (1.67) | 0.41$^{a}$ (2.81) |
| T.I. | 1461 | 2-Hydroxy 2-methylpropyl butanoate** | 2.96$^{a}$ (1.23) | 5.48$^{b}$ (2.35) | 3.86$^{a}$ (1.78) | 4.25$^{b}$ (2.45) |
| T.I. | 1499 | 3-Hydroxy-ethyl butanoate | 0.46$^{a}$ (0.80) | 0.49$^{a}$ (4.07) | 0.47$^{a}$ (1.24) | 0.51$^{a}$ (4.11) |
| T.I. | 1522 | Ethyl, dI-2-hydroxycaproate** | 25.4$^{b}$ (2.64) | 16.3$^{a}$ (1.02) | 23.1$^{b}$ (2.42) | 38.4$^{c}$ (2.71) |

| | RI* | Aroma Compounds | Vintage | | | |
|---|---|---|---|---|---|---|
| | | | 2007 | 2008 | 2009 | 2010 |
| Sigma-Aldrich | 1605 | Diethyl malonate** | 0.14$^{a}$ (5.40) | 0.18$^{a}$ (3.01) | 2.23$^{b}$ (2.53) | 1.81$^{b}$ (1.65) |
| Fluka | 1655 | Ethyl decanoate | 0.08$^{a}$ (1.02) | 0.07$^{a}$ (4.57) | 0.07$^{a}$ (1.92) | 0.07$^{a}$ (3.08) |
| Fluka | 1702 | Diethyl succinate | 2.56$^{c}$ (2.64) | 4.14$^{d}$ (2.36) | 1.14$^{b}$ (1.05) | 0.87$^{a}$ (2.11) |
| Fluka | 1787 | Methyl salicylate** | 15.6$^{b}$ (3.24) | 11.8$^{a}$ (0.54) | 13.1$^{a}$ (1.25) | 18.7$^{b}$ (2.31) |
| T.I. | 1783 | 4-Hydroxy ethyl butanoate | 1.71$^{a}$ (1.50) | 1.67$^{a}$ (1.28) | 1.62$^{a}$ (0.84) | 1.57$^{a}$ (0.82) |
| Fluka | 1936 | 2-phenylethyl acetate | 0.03$^{a}$ (3.00) | 0.09$^{a}$ (2.04) | 0.05$^{a}$ (3.11) | 0.13$^{a}$ (1.85) |
| Sigma-Aldrich | 2070 | Diethyl malate | 1.13$^{b}$ (0.20) | 0.22$^{a}$ (4.08) | 0.84$^{b}$ (1.45) | 1.07$^{b}$ (0.18) |
| T.I. | 2331 | Ethyl monosuccinate | 3.30$^{a}$ (3.90) | 3.59$^{a}$ (2.48) | 3.52$^{a}$ (2.37) | 3.91$^{a}$ (3.77) |
| | | **Total esters** | **63.0** | **63.4** | **55.1** | **56.2** |
| Sigma-Aldrich | 1426 | Acetic acid | 0.03$^{a}$ (5.20) | 0.08$^{a}$ (4.25) | 0.05$^{a}$ (6.14) | 0.06$^{a}$ (1.12) |
| Sigma-Aldrich | 1546 | Propanoic acid** | 1.45$^{a}$ (11.3) | 1.81$^{b}$ (1.64) | 1.63$^{a}$ (2.31) | 1.89$^{b}$ (2.33) |
| Fluka | 1583 | Isobutanoic acid | 1.34$^{a}$ (4.50) | 1.49$^{b}$ (0.08) | 1.43$^{b}$ (1.08) | 1.52$^{b}$ (2.51) |
| Fluka | 1600 | Butanoic acid | 1.40$^{c}$ (2.80) | 1.25$^{b}$ (2.34) | 1.21$^{b}$ (1.09) | 0.92$^{a}$ (1.94) |
| T.I. | 1642 | 3-Methyl butanoic acid | 2.38$^{a}$ (1.00) | 2.17$^{a}$ (1.18) | 2.08$^{a}$ (2.31) | 1.87$^{a}$ (0.74) |
| Fluka | 1703 | Pentanoic acid | 0.01$^{a}$ (1.10) | n.d. | 0.01$^{a}$ (2.47) | 0.02$^{a}$ (1.23) |
| Fluka | 1816 | Hexanoic acid | 2.62$^{b}$ (1.50) | 1.85$^{a}$ (2.36) | 2.32$^{b}$ (2.36) | 1.58$^{a}$ (2.12) |
| Sigma-Aldrich | 1917 | Heptanoic acid** | 6.59$^{b}$ (1.20) | 2.69$^{a}$ (1.58) | 6.24$^{b}$ (2.14) | 11.4$^{c}$ (2.32) |
| T.I. | 1929 | *(E)*-2-Hexenoic acid** | 12.6$^{b}$ (6.40) | 8.81$^{a}$ (1.47) | 11.4$^{b}$ (0.85) | 13.6$^{b}$ (2.33) |
| Fluka | 2024 | Octanoic acid | 2.48$^{a}$ (2.40) | 2.31$^{a}$ (2.61) | 2.24$^{a}$ (2.10) | 1.98$^{a}$ (2.14) |
| Sigma-Aldrich | 2289 | Decanoic acid | 0.48$^{a}$ (2.80) | 0.53$^{a}$ (1.58) | 0.59$^{a}$ (0.39) | 0.62$^{a}$ (1.20) |
| Sigma-Aldrich | 2439 | Dodecanoic acid | 0.03$^{a}$ (6.00) | 0.04$^{a}$ (3.68) | 0.03$^{a}$ (3.21) | 0.03$^{a}$ (2.34) |
| | | **Total acids** | **10.8** | **9.72** | **9.96** | **8.60** |

n.d. not detected.
$^{a, b, c, d}$ According to the result of the Student–Newman–Keuls test, values to that no share a common superscript are significantly different ($p<0.05$).
** Units expressed as µg/l.
* Linear retention index on a DB Wax column
T.I. Tentatively identified.

Table 2. Mean concentrations (mg/l) and relative standard deviations (n=2) of volatile compounds formed during alcoholic fermentation of Rojal wine.

**Esters:** Table 2 shows the wine concentrations of the 19 esters identified. Most of them are ethyl esters of fatty acids produced during the alcoholic fermentation; broadly speaking, they played a positive role in the generation of the quality of the aroma of these wines, especially the fruity aromas (Etiévant, 1991). The wines from the four vintages contained between 55.1 and 63.4 mg/l of esters. High levels were observed for ethyl acetate, ethyl lactate, monoethyl succinate and diethyl succinate.

**Acids:** Fatty acids production is governed by the initial composition of the must and by fermentation conditions and have been described with fruity, cheese, fatty, and rancid notes (Rocha et al., 2004). The most abundant acids in Rojal wines were butyric acid, isobutyric acid, isovaleric acid, hexanoic acid, octanoic acid and decanoic acid. The contents of 6, 8, and 10-carbon atom fatty acids, were in agreement with those found by García-Carpintero et al., 2011a,b in wines made with others grape varieties and fermented in the same conditions.

### 3.2 Bound aroma compounds enzymatically released of the wines

The evaluation of glycosidic precursors in non-aromatic varieties has gain relevance during the last years (Sánchez-Palomo et al., 2007; Loscos et al., 2009; Pedroza et al., 2010; García-Carpintero, et al., 2011a, 2011b). The ability of the enzyme preparation used to release glycosidically-bound compounds from grapes, AR-2000, has been confirmed in numerous studies (Baek & Cadwallader, 1999; Sánchez-Palomo et al., 2006, 2007; García-Carpintero et al., 2011a, 2011b).

The results obtained by enzymatic hydrolysis are exactly the aglycones liberated by the aroma precursors (without further chemical transformations), while with acid hydrolysis other chemical transformations of the liberated compounds are possible. These transformations may be important since they are related with the evolution of the varietal aroma during wine storage (Rodriguez-Bencomo et al., 2011).

The volatile compounds released from the bound fraction by enzyme hydrolysis in Rojal wines are shown in Table 3. As can be observed, from the different chemical families considered in the analysis of the aglycones liberated by the aroma precursors, 60 compounds (three $C_6$-compounds, nine terpenes, five $C_{13}$-norisoprenoids, 22 benzenic compounds, four alcohols, five esters and 13 aliphatic acids) have been found in quantifiable amounts in studied wines.

In Rojal wines benzene and $C_{13}$-norisoprenoids compounds were the most abundant bound compounds, followed by terpene compounds. The $C_6$ compounds concentrations in all the wines studied were significant lower than the observed on free volatile aroma (Table 1). These results confirm the limited importance of $C_6$ compounds on the bound fraction, as occurs with other variety grapes (Cabaroglu et al., 2003; Sánchez-Palomo et al., 2006, 2007; García-Carpintero et al, 2011a, 2011b). In all wines studied the major component of this group of compounds was 1-hexanol.

It can be seen that the total concentration of terpenes + $C_{13}$ norisoprenoid compounds in the bound forms was always higher than that of the free forms in all studied wines, as would correspond to a quality variety (Diéguez et al., 2003). All of the terpene compounds present in wines were found in low concentrations as expected for a neutral grape variety and some of these compounds α-terpineol, trans-linalool oxide (furanoid), cis-linalool oxide (furanoid), cis-linalool oxide (pyranoid), nerol and geranic acid were not present in the free fraction of wines. The bound fraction of others, such as linalool, geraniol and β-citronellol, was more abundant than the free fraction. Geranic acid, geraniol and linalool were the major components of this group in bound fraction of La Mancha Rojal red wines.

| Source | RI* | Aroma Compounds | Vintage | | | |
|---|---|---|---|---|---|---|
| | | | 2007 | 2008 | 2009 | 2010 |
| Fluka | 1282 | 1-Hexanol | 95.5$^{c}$ (2.30) | 85.6$^{b}$ (0.07) | 80.1$^{b}$ (0.32) | 42.8$^{a}$ (0.57) |
| Sigma-Aldrich | 1296 | (*Z*)-3-Hexen-1-ol | 10.5$^{a}$ (7.80) | 10.5$^{a}$ (2.14) | 26.3$^{c}$ (1.28) | 15.1$^{b}$ (2.04) |
| Sigma-Aldrich | 1300 | (*E*)-2-Hexen-1-ol | 45.0$^{c}$ (10.4) | 36.8$^{b}$ (1.08) | 32.5$^{a}$ (1.34) | 38.1$^{b}$ (1.27) |
| | | Total $C_6$ compounds | 151 | 133 | 139 | 96.0 |
| T.I. | 1455 | *Cis* linalool oxyde furan | 6.03$^{b}$ (1.02) | 5.45$^{b}$ (0.25) | 2.38$^{a}$ (1.32) | 4.17$^{b}$ (2.64) |
| T.I. | 1483 | *Trans* linalool oxyde furan | 3.03$^{a}$ (11.7) | 2.89$^{a}$ (1.23) | 2.31$^{a}$ (1.42) | 3.01$^{a}$ (0.84) |
| Fluka | 1529 | Linalool | 17.3$^{b}$ (3.70) | 15.3$^{b}$ (0.15) | 16.5$^{b}$ (1.01) | 11.6$^{a}$ (2.15) |
| Fluka | 1607 | *a* –Terpineol | 5.14$^{b}$ (0.90) | 4.65$^{b}$ (1.64) | 3.68$^{a}$ (1.37) | 4.89$^{b}$ (1.36) |
| T.I. | 1716 | *Cis* linalool oxyde pyran | 5.28$^{b}$ (3.90) | 4.52$^{b}$ (1.09) | 5.08$^{b}$ (1.34) | 3.54$^{a}$ (2.03) |
| Fluka | 1755 | β-citronellol | 4.28$^{b}$ (9.30) | 4.14$^{b}$ (1.28) | 3.21$^{a}$ (2.31) | 3.96$^{a}$ (1.36) |
| Fluka | 1819 | Nerol | 7.77$^{c}$ (7.50) | 6.25$^{b}$ (2.01) | 5.14$^{a}$ (1.09) | 7.70$^{c}$ (1.54) |
| Fluka | 1831 | Geraniol | 30.3$^{b}$ (9.90) | 29.5$^{b}$ (2.48) | 20.6$^{a}$ (2.30) | 28.1$^{b}$ (1.77) |
| Sigma-Aldrich | 2289 | Geranic acid | 60.1$^{a}$ (1.70) | 68.4$^{b}$ (0.12) | 64.2$^{b}$ (1.09) | 67.2$^{b}$ (1.08) |
| | | Total Terpene compounds | 139 | 141 | 123 | 134 |
| Sigma-Aldrich | 1703 | 4-Oxo-isophorone | 3.27$^{b}$ (1.00) | 3.19$^{b}$ (1.58) | 2.21$^{a}$ (1.31) | 2.12$^{a}$ (1.85) |
| T.I. | 2558 | 3-Hydroxy-β-damascone | 401$^{a}$ (8.60) | 427$^{b}$ (0.64) | 441$^{b}$ (2.31) | 395$^{a}$ (2.05) |
| T.I. | 2582 | 3-Oxo-α-Ionol | 134$^{b}$ (4.10) | 128$^{b}$ (1.23) | 130$^{b}$ (1.98) | 106$^{a}$ (0.31) |
| T.I. | 2722 | 3-Hydroxy-7,8-dehydro-β-ionol | 202$^{b}$ (2.30) | 185$^{b}$ (1.74) | 153$^{a}$ (2.08) | 191$^{b}$ (1.08) |
| Sigma-Aldrich | 1873 | α-Ionone | 2.31$^{a}$ (11.7) | 2.85$^{b}$ (1.99) | 2.14$^{a}$ (3.01) | 3.20$^{b}$ (2.30) |
| | | Total $C_{13}$ norisoprenoids compounds | 743 | 746 | 728 | 697 |
| Sigma-Aldrich | 1503 | Benzaldehyde | 3.93$^{c}$ (1.60) | 3.58$^{c}$ (0.25) | 2.54$^{b}$ (2.36) | 1.84$^{a}$ (1.08) |
| Fluka | 1667 | Acetophenone | 3.21$^{a}$ (1.21) | 3.85$^{a}$ (1.35) | 4.84$^{b}$ (1.34) | 4.67$^{b}$ (2.18) |
| T.I. | 1750 | N-ethyl benzeamine | 3.87$^{a}$ (0.25) | 6.51$^{c}$ (1.47) | 4.65$^{b}$ (0.98) | 8.94$^{d}$ (0.59) |
| Sigma-Aldrich | 1882 | Guaiacol | 62.1$^{c}$ (4.50) | 58.4$^{b}$ (1.05) | 50.7$^{a}$ (0.48) | 60.3$^{c}$ (0.47) |
| Sigma-Aldrich | 1895 | Benzyl Alcohol | 712$^{b}$ (0.40) | 608$^{a}$ (2.36) | 897$^{d}$ (0.96) | 835$^{c}$ (0.85) |
| Fluka | 1892 | Phenylethylalcohol | 567$^{a}$ (0.70) | 905$^{d}$ (1.01) | 706$^{c}$ (1.64) | 684$^{b}$ (1.28) |
| T.I. | 1899 | 1,2-Benzothiazole | 6.84$^{a}$ (0.56) | 7.12$^{a}$ (0.66) | 10.2$^{b}$ (0.33) | 9.58$^{b}$ (0.99) |
| Sigma-Aldrich | 1971 | Phenol | 18.5$^{b}$ (6.90) | 15.9$^{a}$ (0.65) | 26.3$^{c}$ (0.65) | 24.6$^{c}$ (1.28) |
| T.I. | 2038 | Benzenepropanol | 5.32$^{c}$ (1.60) | 6.45$^{c}$ (1.25) | 2.81$^{a}$ (0.19) | 3.64$^{b}$ (0.15) |
| Sigma-Aldrich | 2193 | Eugenol | 23.4$^{a}$ (2.60) | 20.7$^{a}$ (2.65) | 29.4$^{b}$ (0.70) | 32.6$^{b}$ (1.88) |
| Sigma-Aldrich | 2219 | 4-Vinylguaiacol | 89.1$^{c}$ (3.60) | 73.6$^{b}$ (3.21) | 80.6$^{c}$ (3.65) | 67.8$^{a}$ (1.01) |

| Source | RI* | Aroma Compounds | Vintage | | | |
|---|---|---|---|---|---|---|
| | | | 2007 | 2008 | 2009 | 2010 |
| Sigma-Aldrich | 2225 | Syringol | 6.35$^{b}$ (3.60) | 4.15$^{a}$ (0.41) | 3.68$^{a}$ (1.85) | 5.94$^{b}$ (2.31) |
| Lancaster | 2302 | Isoeugenol | 13.5$^{b}$ (11.7) | 10.6$^{a}$ (0.99) | 13.2$^{b}$ (1.41) | 11.8$^{a}$ (1.07) |
| Sigma-Aldrich | 2378 | Benzoic acid | 96.2$^{c}$ (3.20) | 95.3$^{c}$ (1.24) | 78.4$^{a}$ (1.24) | 84.1$^{b}$ (0.22) |
| Sigma-Aldrich | 2424 | (*E*)-4-Allylsyringol | 16.4$^{a}$ (1.01) | 20.3$^{b}$ (2.65) | 15.4$^{a}$ (0.11) | 19.6$^{b}$ (0.17) |
| T.I. | 2501 | Benzeneacetic acid | 10.8$^{a}$ (2.30) | 15.4$^{b}$ (1.48) | 11.3$^{a}$ (0.74) | 17.6$^{b}$ (1.09) |
| Panreac | 2511 | Vanillin | 61.2$^{b}$ (2.20) | 59.6$^{b}$ (2.31) | 57.9$^{a}$ (1.32) | 54.8$^{a}$ (10.80) |
| Sigma-Aldrich | 2543 | Methyl vanillate | 205$^{c}$ (3.30) | 199$^{c}$ (1.24) | 153$^{a}$ (0.90) | 178$^{b}$ (2.18) |
| Lancaster | 2676 | Ethyl vanillate | 32.3$^{a}$ (1.01) | 44.7$^{c}$ (0.54) | 28.9$^{a}$ (1.25) | 39.4$^{b}$ (5.21) |
| Sigma-Aldrich | 2685 | Acetovainillone | 205$^{b}$ (1.70) | 146$^{a}$ (1.44) | 198$^{b}$ (0.81) | 153$^{a}$ (1.65) |
| Sigma-Aldrich | 2936 | Zingerone | 68.6$^{b}$ (3.30) | 62.4$^{a}$ (0.86) | 65.2$^{b}$ (2.10) | 61.6$^{a}$ (4.14) |
| Sigma-Aldrich | 2755 | 3,4-Dimethoxy phenol | 79.5$^{c}$ (4.70) | 72.3$^{b}$ (2.64) | 67.3$^{a}$ (1.89) | 71.3$^{b}$ (2.01) |
| | | Total Bencenic compounds | 2290 | 2439 | 2507 | 2430 |
| Sigma-Aldrich | 1221 | 3-Methyl-1-butanol | 55.3$^{b}$ (2.36) | 36.1$^{a}$ (1.05) | 64.3$^{c}$ (2.31) | 75.8$^{d}$ (1.62) |
| Sigma-Aldrich | 1273 | 3-Methyl-2-buten 1-ol | 3.62$^{a}$ (2.15) | 6.57$^{b}$ (1.65) | 5.21$^{b}$ (1.67) | 7.98$^{b}$ (1.25) |
| | | Total Alcohols | 58.9 | 42.7 | 69.5 | 83.8 |
| T.I. | 1355 | Octanoic acid methyl ester | 3.25$^{a}$ (0.96) | 2.65$^{a}$ (2.15) | 1.36$^{a}$ (2.00) | 4.28$^{b}$ (2.74) |
| Fluka | 1655 | Decanoic acid ethyl ester | 1.23$^{a}$ (0.54) | 0.67$^{a}$ (3.15) | 0.95$^{a}$ (5.78) | 1.14$^{a}$ (1.25) |
| T.I. | 1704 | Ethyl methyl succinate | 6.68$^{c}$ (1.24) | 11.3$^{d}$ (0.09) | 3.14$^{b}$ (1.45) | 1.05$^{a}$ (1.06) |
| Fluka | 1787 | Methyl Salicylate | 2.67$^{a}$ (2.85) | 2.31$^{a}$ (0.25) | 2.55$^{a}$ (1.91) | 2.53$^{a}$ (0.74) |
| T.I. | 1827 | Butanoic acid, 4-hydroxy-methyl ester | 7.68$^{b}$ (1.26) | 5.32$^{a}$ (1.97) | 11.2$^{c}$ (1.62) | 9.64$^{b}$ (1.63) |
| | | Total Esters | 21.5 | 22.3 | 19.2 | 18.6 |
| Sigma-Aldrich | 1426 | Acetic acid | 21.1$^{b}$ (6.60) | 18.5$^{b}$ (1.54) | 20.9$^{b}$ (2.07) | 15.6$^{a}$ (1.65) |
| Sigma-Aldrich | 1546 | Propanoic acid | 7.50$^{b}$ (4.20) | 3.98$^{a}$ (1.65) | 6.14$^{b}$ (1.25) | 3.37$^{a}$ (1.26) |
| Fluka | 1600 | Butanoic acid | 28.8$^{b}$ (6.70) | 21.4$^{a}$ (1.06) | 25.9$^{b}$ (0.32) | 24.0$^{b}$ (2.31) |
| Sigma-Aldrich | 1642 | Isovaleric acid | 30.5$^{b}$ (2.40) | 28.4$^{b}$ (2.65) | 24.6$^{a}$ (0.65) | 30.2$^{b}$ (1.34) |
| Fluka | 1703 | Pentanoic acid | 4.27$^{b}$ (9.60) | 3.39$^{b}$ (1.99) | 2.36$^{a}$ (1.99) | 4.18$^{b}$ (1.27) |
| Fluka | 1816 | Hexanoic acid | 154$^{c}$ (2.65) | 139$^{b}$ (1.65) | 152$^{c}$ (2.48) | 112$^{a}$ (2.14) |
| T.I. | 1857 | 2-Ethyl hexanoic acid | n.d. | n.d. | 1.25$^{a}$ (1.28) | 1.37$^{a}$ (1.48) |
| Sigma-Aldrich | 1917 | Heptanoic acid | 11.5$^{b}$ (4.20) | 9.64$^{b}$ (3.15) | 7.51$^{a}$ (1.09) | 11.4$^{b}$ (1.20) |

| Source | RI* | Aroma Compounds | Vintage | | | |
|---|---|---|---|---|---|---|
| | | | 2007 | 2008 | 2009 | 2010 |
| Fluka | 2024 | Octanoic acid | 381$^{d}$ (3.80) | 241$^{c}$ (1.65) | 134$^{a}$ (1.39) | 197$^{b}$ (0.64) |
| Sigma-Aldrich | 2108 | Nonanoic acid | 65.9$^{c}$ (0.80) | 61.3$^{c}$ (1.74) | 48.7$^{a}$ (2.62) | 56.1$^{b}$ (1.06) |
| Sigma-Aldrich | 2289 | Decanoic acid | 140$^{d}$ (7.30) | 121$^{c}$ (1.81) | 112$^{b}$ (1.85) | 69.4$^{a}$ (1.07) |
| Sigma-Aldrich | 2439 | Dodecanoic acid | 43.1$^{b}$ (11.7) | 45.0$^{b}$ (1.15) | 33.4$^{a}$ (1.99) | 49.0$^{b}$ (4.65) |
| Sigma-Aldrich | 2653 | Tetradecanoic acid | 6.87$^{a}$ (2.20) | 21.6$^{c}$ (0.97) | 20.9$^{c}$ (2.36) | 10.3$^{b}$ (2.61) |
| | | Total Acids | 895 | 714 | 590 | 584 |

*Linear retention index on a DB Wax column; nd: not detected.
$^{a, b, c, d}$ According to the result of the Student–Newman–Keuls test, values to that no share a common superscript are significantly different ($p<0.05$)
T.I. Tentatively identified

Table 3. Mean concentration (µg/l) and relative standard deviations (n=2) of bound volatile compounds released by enzymatic hydrolysis of Rojal wines.

Norisoprenoids detected in negligible amounts or not found in the free fraction of studied wines, were relatively abundant in the bound fraction. The $C_{13}$-norisoprenoid pattern was composed by 3-hydroxy-β-damascone, 3-oxo-α-ionol, 3-hydroxy-7,8-dihydro-β-ionol and, in smaller concentrations, α-ionone and 4-oxo-isophorone. By contrast, β-damascenone –detected in the free fraction in wines– was not detected in bound form as this compound is formed principally from the precursors 3-hydroxy-β-damascone and 3-hydroxy-7,8-dehydro-β-ionol; the highest concentration of both compounds found in fraction of aroma of Rojal wines could be related to that in the free fraction Rojal wines presented lower concentration of β-damascenone.

The bound fraction of benzenic compounds was major quantitative than the free fraction in Rojal wines. *(E)*-4-Allylsyringol was not present in the free fraction of wines but was detected in the bound fraction. Benzyl alcohol and 2-phenylethanol were the compounds in higher concentration in this fraction in Rojal wines. Guaiacol, phenol, eugenol, isoeugenol, vanillin, methyl vanillate and ethylvanillate present higher concentrations in bound fraction of aroma of Rojal wines. García-Carpintero et al., 2011b founded higher concentrations in the total benzenic compound of bound fraction in Moravia Agria wines than in our studied wines.

The alcohols qualitative composition in bound aroma was lower than in free aroma, attributable to these compounds are principally formed by the yeast metabolism. The concentration of aliphatic acids in bound aroma were noteworthy lower than in free aroma, due to the principal formation pathway of this compounds is by the yeast metabolism.

During winemaking, some of these bound aroma compounds give rise to odorant compounds that play a role in certain aroma characteristics of wine; similar results were observed by Hernandez-Orte et al., 2009 studying the ability of glycosidase activity of

several lactic acid bacteria (LAB) to change the volatile fraction of wine by releasing aroma compounds. These authors observed that the studied LAB strains were able to release terpene compounds, $C_{13}$-norisoprenoid compounds, volatiles phenols and vanillin derivates. According to the result, the bound fraction of Rojal wines can be considered a potential aroma source which reveals the enrichment in varietal compounds of the must as a result of the transfer of these compounds from the skin (Cabaroglu et al., 2003; Sánchez-Palomo et al., 2006, 2007; García-Carpintero et al., 2011a, 2011b).

### 3.3 Odour activity values

The aroma of Rojal red wines from La Mancha region has been studied by sensory analysis. Relevant aroma sensory descriptors given by the expert panel are summarized in Table 4.

| Aroma descriptors |
|---|
| Red fruit |
| Fresh |
| Clove |
| Pepper |
| Leather/Tobacco |
| Sweet |
| Fresh fruit |

Table 4. Sensory Aroma Descriptors given by the expert panel to the La Mancha Rojal wines.

The table 5 shows the odour descriptors and odour threshold of the aroma compounds in La Mancha Rojal wines obtained by the bibliographic references (Kotseridis & Baumes, 2000; Lopez et al., 2003). With over 50 aroma components of wide-ranging intensities and no single character impact compounds, it is difficult to predict the overall aroma impact of these wines from the sheer size of the data. To estimate overall wine aroma, the odour descriptors were grouped in different aromatic series and every compound is assigned to one or several aromatic series based on similar odour descriptor used.

| Compounds | Sensory description | Odorant series* | Odour Threshold (µg/L) |
|---|---|---|---|
| Acetaldehyde | pungent, ripe apple | 1,6 | 500[a] |
| Ethyl acetate | fruity, solvent | 1,6 | 7500[a] |
| Ethyl butyrate | fruity | 1 | 20[a] |
| Isoamyl acetate | banana | 1 | 30[c] |
| Methanol | chemical , medicinal | 6 | 668000[b] |
| 1-propanol | ripe fruit, alcohol | 1,6 | 830[b] |
| Isobutanol | oily, bitter, green | 3,6 | 40000[b] |
| 3-Methyl-1-butanol | burnt, alcohol | 4,6 | 30000[a] |
| 1-Butanol | medicinal, phenolic | 6 | 150000[b] |
| Ethyl caproate | green apple | 1 | 14[b] |
| 1-Pentanol | almond, syntetic, balsamic | 6 | 64000[b] |
| Hexyl acetate | green, floral | 2,3 | 1500[c] |
| Ethyl pyruvate | vegetable, caramel | 4,7 | 100000[b] |
| Ethyl lactate | acid, medicine | 6 | 154636[c] |

| Compounds | Sensory description | Odorant series* | Odour Threshold (µg/L) |
|---|---|---|---|
| 1-Hexanol | flower, green, cut grass | 2,3 | 8000[a] |
| 4-Methyl-1-pentanol | almond, toasted | 4 | 50000[c] |
| 3-Methyl-1-pentanol | vinous, herbaceous, cacao | 1,3,7 | 50000[c] |
| (Z)-3-Hexen-1-ol | green, cut grass | 3 | 400[a] |
| Ethyl caprilate | sweet, fruity | 1,4 | 5[b] |
| Acetic acid | sour, pungent, vinegar | 6 | 200000[a] |
| 1-Heptanol | oily | 6 | 2500[b] |
| 3-Hydroxy, ethyl butyrate | caramel, toasted | 4 | 20000[b] |
| Benzaldehyde | sweet, fruity | 1,4 | 350[c] |
| Propanoic acid | pungent, racid, soy | 6 | 8100[c] |
| 2, 3-Butanediol (levo) | fruity | 1 | 150000[b] |
| Linalool | floral | 2 | 15[a] |
| Isobutyric acid | rancid, butter, cheese | 6 | 2300b |
| 2,3-Butanediol (meso) | fruity | 1 | 150000[b] |
| γ-Butyrolactone | sweet, toast, caramel | 4 | 35[c] |
| Butyric acid | rancid, cheese, sweat | 6 | 173[b] |
| Ethyl caprate | sweet/fruity | 1,4 | 200[c] |
| Isovaleric acid | sweet, acid, rancid | 4,6 | 33[c] |
| Diethyl succinate | vinous | 7 | 200000[b] |
| 3-(Methylthio)-1-propanol | cooked vegetable | 7 | 1000[a] |
| 2-Phenylethyl acetate | floral | 2 | 250[a] |
| β-damascenone | sweet, fruity | 1,4 | 0,05[a] |
| Hexanoic acid | sweat | 6 | 420[b] |
| Geraniol | roses, geranium | 2 | 30[a] |
| 2-Methoxyphenol, | medicine, sweet, smoke | 4,6 | 10[c] |
| Benzyl Alcohol | sweet, fruity | 1,4 | 200000[b] |
| 2-Phenyethyl alcohol | floral, roses | 2 | 10000[a] |
| Diethyl malate | over-ripe, peach, cut grass | 1 | 760000[b] |
| Octanoic acid | sweat, cheese | 6 | 500[c] |
| Eugenol | spices, clove, honey | 4,5 | 6[c] |
| 4-Vinylguaiacol | spices/curry | 5 | 40[a] |
| Decanoic acid | rancid fat | 6 | 1000[b] |
| Ethyl cinnamate | fruity, honey, cinnamon | 1,4,5 | 1,1[a] |
| Isoeugenol | clove | 5 | 6[b] |
| Ethyl monosuccinate | caramel, coffee | 4 | 1000000[c] |
| Benzoic acid | chemical | 6 | 1000[b] |
| Vainillin | vanillin | 5 | 60[b] |
| Methyl vanillate | honey, vanillin | 4,5 | 3000[b] |
| Ethyl vanillate | sweet, honey, vanillin | 4,5 | 990[b] |
| Acetovanillone | sweet spices | 5 | 1000[b] |

*1 = fruity; 2 = floral; 3 = green, fresh; 4 = sweet; 5 = spicy; 6 = fatty; 7 = others.
[a] Guth 1997; [b] Etiévant, 1991 ; [c] Ferreira et al., 2000

Table 5. Odour descriptors, odorant series and odour threshold (µg/L) of the aroma compounds in monovarietal and co-winemaking wines.

Table 6 lists the OAV for all vintages studied and average OAVs values for the 31 aroma compounds with OAV>0.1 studied in Rojal wines during four consecutive years the odorant series of these compounds. The method based in the OAV has been used in the latter years in studies on wine aroma, such as in the discrimination of wines obtained from different grapes varieties (Guth, 1997), in works on accelerated ageing wines (Muñoz et al., 2007), in works on wines subjected to biological ageing (Moyano et al., 2002; Zea et al., 2007) and in studies of characterization of impact compounds of monovarietal wines (Sánchez-Palomo et al., 2010; García-Carpintero et al., 2011a, 2011b).

| Compounds | Vintage | | | |
|---|---|---|---|---|
| | 2007 | 2008 | 2009 | 2010 |
| Ethyl caprilate | 90.0 | 74.0 | 78.0 | 82.0 |
| Isovaleric acid | 72.1 | 65.8 | 63.0 | 56.7 |
| Ethyl caproate | 30.7 | 27.1 | 29.3 | 25.0 |
| 1-propanol | 17.7 | 24.6 | 20.7 | 18.4 |
| Acetaldehyde | 35.8 | 6.2 | 12.4 | 21.2 |
| Butyric acid | 8.09 | 7.23 | 6.99 | 5.32 |
| 3-Methyl-1-butanol | 6.03 | 6.63 | 6.37 | 6.17 |
| Guaiacol | 4.17 | 6.25 | 5.83 | 6.69 |
| Hexanoic acid | 6.24 | 4.40 | 5.52 | 3.76 |
| beta-damascenone | 9.60 | 0.00 | 6.40 | 4.20 |
| Octanoic acid | 4.96 | 4.62 | 4.48 | 3.96 |
| Ethyl acetate | 4.92 | 4.75 | 3.85 | 3.27 |
| Isoeugenol | 3.55 | 4.45 | 3.93 | 2.87 |
| Ethyl butyrate | 3.00 | 3.50 | 3.00 | 4.00 |
| 2-phenyethyl alcohol | 2.51 | 3.07 | 2.84 | 3.45 |
| 3-Methylthio-1-propanol | 2.51 | 2.68 | 2.63 | 2.57 |
| Isoamyl acetate | 1.00 | 1.67 | 1.33 | 1.67 |
| Eugenol | 0.84 | 1.03 | 0.87 | 1.09 |
| Isobutanol | 1.03 | 0.79 | 0.88 | 0.77 |
| Linalool | 0.93 | 0.79 | 0.81 | 0.61 |
| 4-vinylguaiacol | 1.16 | 0.38 | 0.72 | 0.31 |
| Isobutyric acid | 0.58 | 0.65 | 0.62 | 0.66 |
| Decanoic acid | 0.48 | 0.53 | 0.59 | 0.62 |
| (Z)-3-Hexen-1-ol | 0.52 | 0.46 | 0.50 | 0.37 |
| Ethyl caprate | 0.40 | 0.35 | 0.35 | 0.35 |
| Geraniol | 0.33 | 0.30 | 0.31 | 0.32 |
| 1-Hexanol | 0.27 | 0.18 | 0.25 | 0.25 |
| 2-Phenylethyl acetate | 0.12 | 0.36 | 0.20 | 0.52 |
| Propanoic acid | 0.18 | 0.22 | 0.20 | 0.23 |
| Benzoic acid | 0.18 | 0.18 | 0.17 | 0.15 |
| Ethyl vanillate | 0.20 | 0.10 | 0.18 | 0.18 |
| Ethyl lactate | 0.10 | 0.11 | 0.11 | 0.15 |
| Methanol | 0.12 | 0.09 | 0.08 | 0.06 |
| Vainillin | 0.11 | 0.08 | 0.09 | 0.10 |
| Acetovanillone | 0.11 | 0.08 | 0.09 | 0.08 |

Table 6. Odor activity values of free aroma compounds in Rojal wines.

The aromatic series used in this work group volatile compounds with similar odour descriptors: fruity, floral, green/fresh, sweet, spice, fatty and other odours taking into account their use in previous papers (Sánchez-Palomo et al., 2010; Gómez García-Carpintero el al., 2011a, 2011b). Because of the high complexity of olfactive perceptions, some aroma compounds were included in two or more odorant series according to the finding of some authors (Zea et al., 2007; Charles et al., 2000).

The total intensities for every aromatic series were calculated as sum of the OAV of each one of the compounds assigned to this series and the results were graphed in Figure 2. This procedure makes it possible to relate quantitative information obtained by chemical analysis, to sensory perception, providing a single aroma profile based on an objective. It has recently been used some authors (Peinado et al., 2004, 2006; López de Lerma & Peinado 2011; García-Carpintero el al., 2011a, 2011b).

Intensity patterns in the category suggest that the major aroma characteristic of these wines would consist of fruity, sweet and fatty. Fruity was one of the aromatic series with major intensity (Figure 2). This series is formed principally by 7 esters, 1 alcohol and 1 $C_{13}$-norisoprenoid compound (beta-damascenone), identified and quantified by GC-MS. According to the results showed in Figure 2 can be observed that the aromatic series 4 (Sweet) showed the greatest intensity.

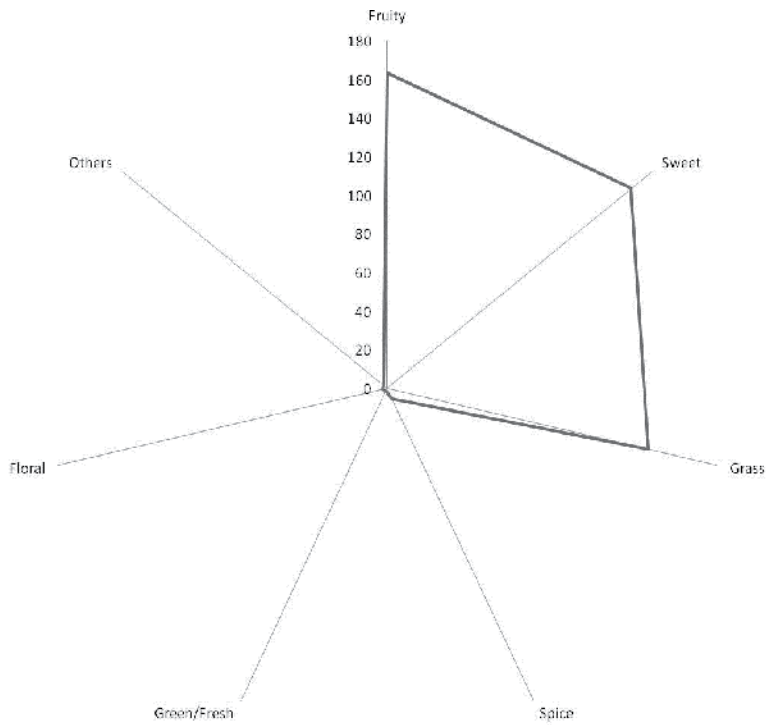


Fig. 2. Aromatic series in La Mancha Rojal wines ($\Sigma OAV_{medium}$ over four vintages).

The aromatic series 6 (pungent, chemical, fatty, dry) was also major aroma categories in the current study. These attributes were not detected in the sensory flavour profile studies of wines. The aromatic series 3 (green, fresh) was one of the minor aroma categories,

nevertheless this attribute was ones of the most characteristics in the sensory profile of Rojal wines. This can be due to that the values of total intensity in the different aromatic series were obtained as sum of the individual OAVs of each one of the components without bearing in mind the rest of present compounds in the matrix of wine. Nevertheless when combined, synergy, suppression and matrix effects may alter the intensity of the descriptors, masking the descriptors of some aromatic series (series 4 and 6) and increasing the intensity of others odour descriptors (series 2 and 3). These results are in agreement with the results obtained in red wines made from Merlot and Cabernet Sauvignon grape varieties (Gürbüz et al., 2006), in wines made from Moravia Agria grape variety (García-Carpintero et al., 2011b) and in wines made from Bobal grape variety (García-Carpintero et al., 2011a).

## 4. Conclusion

This work provide a better knowledge of the aroma composition of Rojal wines elaborated with grapes cultivated in La Mancha region, also this study presents results from the first experiment performed on the free and bound aroma compounds from this minority grape variety from Castilla La Mancha region. Rojal wines present a complex chemical profile with a high richness in their aromatic composition. The free aroma of La Mancha Rojal wines is characterized by large amounts of $C_6$ and benzene compounds. The most abundant glycosilated fraction was the benzene compounds followed by $C_{13}$-norisoprenoids compounds. By other hand, the sensory aroma profile of Rojal wines was characterized by red fruit, fresh, clove, leather, tobacco, sweet and fresh fruit aroma descriptors. This study showed that this grape variety present a great aroma potential providing a viable alternative to traditional grape varieties cultivated in La Mancha region, increasing the offer to the consumer, which favors the differentiation of La Mancha wines on the national and international market.

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# 9

# Application of an Automated Headspace Solid Phase Micro-Extraction for the GC-MS Detection and Quantification of Reductive Sulfur Compounds in Wines

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## 1. Introduction

Aromatic substances are among the key determinants of food and beverage quality, owing to their interactions with the senses of smell and taste. These can then determine consumer acceptance or rejection of a product. The aroma of foods is influenced by different compounds, among which sulfur containing compounds are an important group due to their abundance and aromatic impact. Likewise, wine contains various sulfur containing structures which have a major sensorial impact and play a significant role in wine aroma and flavour. Among these, certain sulfur containing volatiles can contribute to favourable sensory impacts, while others can have detrimental effects on wine quality. The latter refers to the off-odours that have been a major concern for the wine industry.

The analysis of reductive sulfur compounds in wine is a challenging task. This is due to three main problems, namely the complexity of the wine matrix, the low concentrations of the reductive sulfur compounds, and their highly reactive nature. In order to obtain good sensitivity, specificity and reliability, reductive sulfur compounds are usually analysed by gas chromatography coupled either with sulfur-specific detectors such as the flame photometric detector, the pulsed flame photometric detector, the sulfur chemiluminescent detector, or with a non-sulfur specific detector such as a mass spectrometry detector or an atomic emission detector. This chapter reports on an automated Headspace Solid Phase Micro-Extraction procedure followed by Gas Chromatography-Mass Spectrometry used for the detection and quantification of the common highly volatile (light) and less volatile (heavy) sulfur compounds in wine. These are defined as having boiling points below and above 90 °C, respectively, which is the boiling point of 3-(methylthio)-1-propanol. The methodology is based upon a publication by Fedrizzi et al. (2007), with additional considerations given to the choice of the SPME fiber for optimum extraction efficiency, the addition of magnesium sulfate to increase extraction yield, and other parameters such as extraction time and the choice of temperature to improve the Solid Phase Micro-Extraction methodology (Pawliszyn, 1997).

Table 1 below presents some information about the perception thresholds, odour descriptions and the concentrations of the reductive sulfur compounds commonly reported in the wine literature (Spedding & Raut, 1982; Mestres et al., 2002; Fang & Qian, 2005; Ribéreau-Gayon et al., 2006b; Landaud et al., 2008). Fourteen reductive sulfur compounds, out of sixteen compounds presented in this table, are the analytes of interest in the current study.

| Sulfur compounds | Perception threshold in wines (µg/L) | Odour description | Concentration range in wines (µg/L) |
|---|---|---|---|
| **Highly volatile** | | | |
| Hydrogen sulfide | 0.001 - 150 | Rotten eggs | nd - 370 |
| Methanethiol | 0.3** | Cooked cabbage | nd - 16 |
| Ethanethiol | 1.1 | Onion, rubber, putrefaction | nd - 50 |
| Dimethyl sulfide | 10 - 160 | Cabbage, asparagus, corn, molasses | nd - 910 |
| Carbon disulfide | > 38 | Cabbage, rubber | nd - 18 |
| Dimethyl trisulfide | 0.1* | Cabbage, onion, cooked vegetables | nd - 111 |
| **Less volatile** | | | |
| Diethyl sulfide | 0.93 - 18 | Garlic | nd - 10 |
| Dimethyl disulfide | 20 - 45 | Cooked cabbage, asparagus, onion | 0 - 22 |
| Diethyl disulfide | 4.3 - 40 | Garlic, onion, burnt rubber | nd - 85 |
| 2-Mercaptoethanol | 130 | Barnyard-like (*böxer*), poultry | nd - 400 |
| Methylthioacetate | 300* | Sulfury, rotten vegetables | nd - 115 |
| S-Ethylthioacetate | 40* | Sulfury | nd - 180 |
| 2-(Methylthio)-1-ethanol | 250** | Cauliflower, French bean | 88 - 139 |
| 3-(Methylthio)-1-propanol | 1200 | Cooked cabbage, cauliflower | 145 - 5655 |
| 4-(Methylthio)-1-butanol | 100 | Earthy, chive-garlic, onion | nd - 181 |
| Benzothiazole | 50 - 350 | Rubber | 0 - 30 |

Table 1. Common reductive sulfur compounds in wines

## 2. Material and methods

### 2.1 Chemicals and reagents

Fourteen reductive sulfur compounds of interest, ranging from highly volatile compounds such as thiols and disulfides to less volatile compounds, S-thioesters and thioether alcohols, were included. Of note is that another highly volatile compound, methanethiol, was also successfully detected and quantified without the use of a cryotrap, together with further high and low volatile sulfur compounds, commonly reported in the wine literature.

The fourteen reductive sulfur compounds were methanethiol (MeSH) (CAS No. 74-93-1), ethanethiol (EtSH) (75-08-1), dimethyl sulfide (DMS) (75-18-3), diethyl sulfide (DES) (352-93-

2), S-methyl thioacetate (MTA) (1534-08-3), S-ethyl thioacetate (ETA) (625-60-5), dimethyl disulfide (DMDS) (624-92-0), diethyl disulfide (DEDS) (110-81-6), carbon disulfide (CS2) (75-15-0), dimethyl trisulfide (DMTS) (3658-80-8), 2-mercaptoethanol (ME) (60-24-2), 2-(methylthio)-1-ethanol (MTE) (5271-38-5), 3-(methythio)-1-propanol (MTP) (505-10-2) and 4-(methylthio)-1-butanol (MTB) (20582-89-3). $^{2}H_{6}$-Dimethyl sulfide (926-09-0), isopropyl disulfide (4523-89-8) and 3-(methylthio)-1-hexanol (51755-66-9) were used as internal standards. The commercial standards were purchased from either Sigma-Aldrich (Auckland, New Zealand) or Alfa Aesa (Ward Hill, MA, USA). A methanethiol gas cylinder was supplied by Matheson Coleman and Bell (East Rutherford, NJ, USA). The standards were used to identify peaks in the chromatograms and to construct calibration curves for quantification purposes.

### 2.2 Sample extraction conditions

A pre-concentration step is required before chromatographic analysis of reductive sulfur compounds, due to their trace concentrations in wine. Solid Phase Micro-Extraction, a technique introduced in 1980s, has been increasingly used as an alternative to traditional pre-concentration methods, for the extraction of volatile compounds. It is a 'solvent-less' technique that employs a polymer-coated fiber immersed into a liquid sample or the gas headspace to extract and concentrate analytes from the matrix onto the fiber. The method allows the use of smaller sample volumes. Through the use of an automated Headspace Solid Phase Micro-Extraction (HS-SPME) procedure to extract the sulfur analytes onto the fiber, high through-put of samples is possible with minimal variations during sample preparation. The Gas Chromatography - Mass Spectrometry (GC-MS) method development for the analysis of reductive sulfur containing compounds was based on a publication of Fedrizzi et al. (2007).

#### 2.2.1 Selection of fiber coating

Different fibers are commercially available for use with SPME. Volatile analytes are retained more effectively on a thicker fiber coating and can be transferred into the GC injection port without losses. On the other hand, a thin coating is used to ensure rapid release of higher boiling point compounds during thermal desorption (Otles, 2009).

The commercially available SPME fibers can generally be classified into two groups. The first group involves a pure liquid polymer coating, such as polydimethylsiloxane (PDMS) and polyacrylate. The second group are mixed films containing solid particles and *liquid polymers*, such as Carboxen-*polydimethylsiloxane* (CAR-PDMS) and divinylbenzene-*polydimethylsiloxane* (DVB-PDMS) (Otles, 2009). Carboxen acts as a carbon molecular sieve[1] and is often used with PDMS for low molecular weight polar analytes, while DVB-PDMS is more suited to semi-polar analytes (Otles, 2009).

In the method reported by Fedrizzi et al. (2007), fibers with six different coatings were examined for their extraction efficiency with regard to reductive sulfur containing compounds. Although other 'light' sulfur compounds were included in the current study, the Divinylbenzene/Carboxen-Polydimethylsiloxane (DVB/CAR-PDMS; 50/30µm x 2 cm) fiber (Product No. 57298U, Supelco, Bellefonte, PA, USA) provided the best results, and was

[1] To trap very small molecular sized compounds (C2-C5)

employed to extract the analytes of interest. The fiber is a mixed film coated with Divinylbenzene/Carboxen on *polydimethylsiloxane*, bonded to a flexible fused silica that offers a less breakable fiber. The mixed film coating allows the extraction of the analytes by absorption with the liquid polymer and by adsorption with the porous solid particles.

### 2.2.2 Extraction and agitation

Incubation of the samples prior to extraction was carried out in order to give similar conditions before the fiber was exposed to the headspace for the extraction of the analytes.

Agitation using magnetic stirrer bars was employed during both incubation and extraction steps using a Gerstel Agitator/Stirrer, in which the agitation speed and temperature were automatically controlled using MAESTRO Software (Version 1.2.0) (Gerstel, Mülheim an der Ruhr, Germany). Variations in agitation conditions during incubation and extraction were therefore eliminated.

The extraction temperature and time were optimised, as part of the current method development, to give the best extraction effectiveness for the reductive sulfur compounds of interest. The experimental protocols and results are presented in Section 3.1.

### 2.3 Chromatographic conditions

Analysis of the reductive sulfur compounds was carried out on an Agilent Technologies 7890 GC system coupled with a 5975C inert XL MSD (Agilent Technologies, Santa Clara, CA, USA). Separation was performed on a tandem column composed of a 30 m x 0.320 mm x 0.25 µm HP-1MS[2] and a 30 m x 0.320 mm x 0.25 µm HP-Innowax[3] fused silica capillary column (Agilent, J&W Scientific, New Zealand). The transfer line temperature was set at 250 °C. Helium was used as the carrier gas at an initial flow rate of 1.8 mL/min, held for 5 min, then lowered to 1.5 mL/min for the rest of the run.

Different conditions, including injection temperatures and oven temperature programs, have been trialled to obtain good peak separation. The final conditions involved an injection port temperature of 250 °C. The oven temperature was initially set at 42 °C for 5 min, then ramped at 1.5 °C/min to 60 °C and at 4 °C/min to 150 °C, held for 5 min. After that, it was ramped at 40 °C/min to 230 °C and remained at this temperature for 10 min, until a final ramp at 70 °C/min back to 42 °C towards the end of the run.

### 2.4 Peak identification

The ions used for the identification and quantification of each compound were chosen according to the literature and NIST library. The resulting retention times of the analysed compounds and of the internal standards are presented in Table 2.

Fig.1 displays the peak separations of the internal standards and the investigated reductive sulfur compounds, obtained from an injection of a standard solution into the GC-MS system, as described in Section 2.3.

[2] Non-polar column with stationary phase made of 100 % dimethylpolysiloxane
[3] Highly polar column with stationary phase made of polyethylene glycol

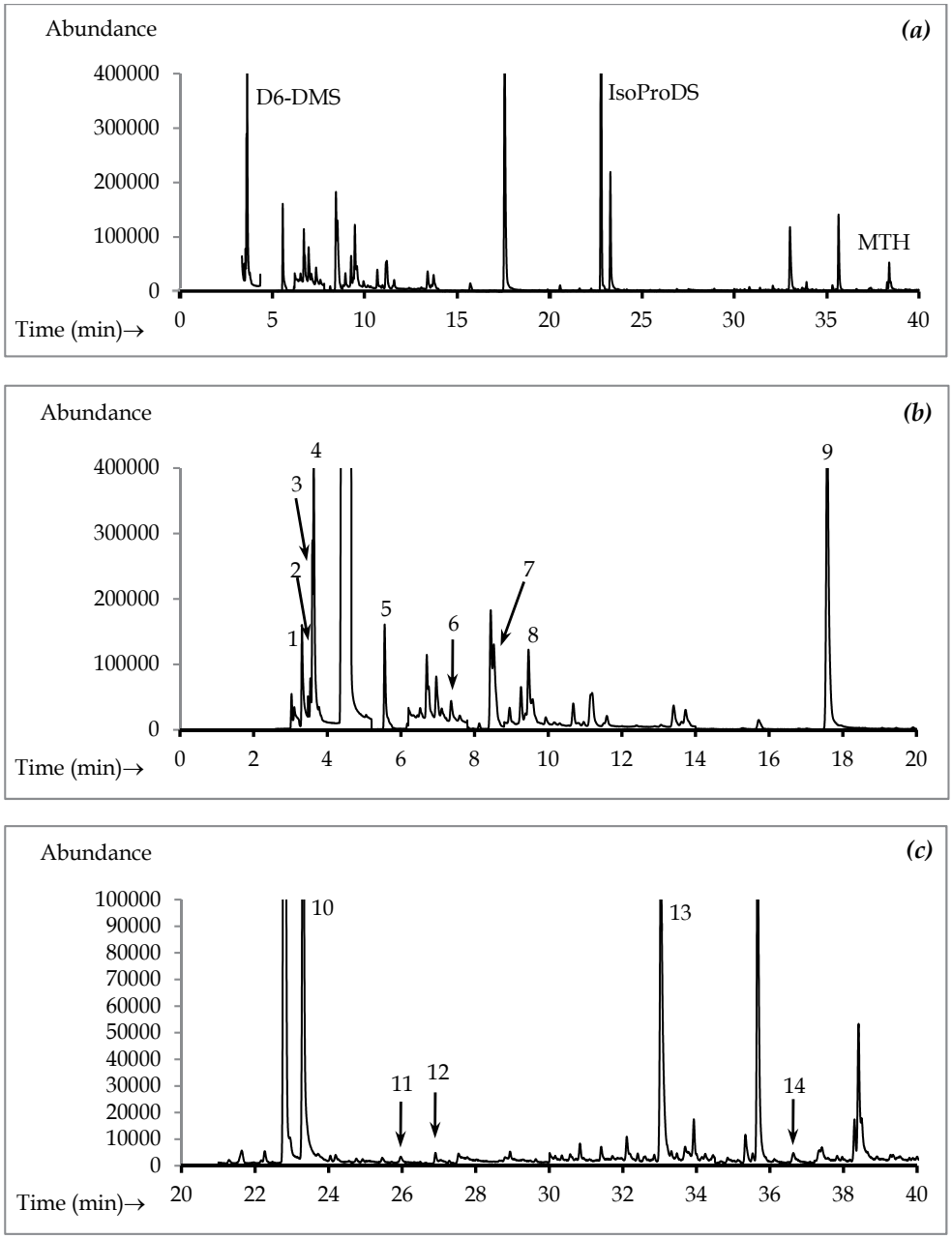


Fig. 1. Total ion chromatograms of a standard solution showing peaks for the internal standards (a): D6-DMS, m/z 68; IsoProDS, m/z 150; MTH m/z 148; and of the analytes (b & c): 1- MeSH, m/z 47; 2- EtSH, m/z 62; 3- DMS, m/z 62; 4- CS2, m/z 78; 5- DES, m/z 75; 6- MTA, m/z 90; 7- DMDS, m/z 79; 8- ETA, m/z 104; 9- DEDS, m/z 122; 10- DMTS, m/z 126; 11- ME, m/z 47; 12-MTE, m/z 92; 13- MTP, m/z 106; 14- MTB, m/z 120. The chromatographic conditions are described in Section 2.3 and the abbreviations used for the compounds can be seen in Table 2.

| Chemicals and reagents | Abbreviations | Quantifier ion (Qualifier ions) | Retention time (min) |
|---|---|---|---|
| **Analytes** | | | |
| *Methanethiol* | MeSH | 47 (45, 48) | 3.3 |
| *Ethanethiol* | EtSH | 62 (34, 47) | 3.53 |
| *Dimethyl sulfide* | DMS | 62 (47, 61) | 3.61 |
| *Carbon disulfide* | CS2 | 78 (44, 76) | 3.62 |
| *Diethyl sulfide* | DES | 75 (61,62,90) | 5.54 |
| *Methyl thioacetate* | MTA | 90 (43, 47, 75) | 7.22 |
| *Dimethyl disulfide* | DMDS | 79 (61, 64, 94) | 8.51 |
| *S-Ethyl thioacetate* | ETA | 104 (43, 60, 62) | 9.45 |
| *Diethyl disulfide* | DEDS | 122 (66, 94) | 17.56 |
| *Dimethyl trisulfide* | DMTS | 126 (64, 79) | 23.29 |
| *2-Mercaptoethanol* | ME | 47 (60, 78) | 25.96 |
| *2-(Methylthio)-1-ethanol* | MTE | 92 (47, 61) | 26.82 |
| *3-(Methylthio)-1-propanol* | MTP | 106 (57, 58, 61) | 33.02 |
| *4-(Methylthio)-1-butanol* | MTB | 120 (61, 87, 102) | 36.62 |
| **Internal standards** | | | |
| *$^2H_6$-Dimethyl sulfide* | D6-DMS | 68 (66,50) | 3.58 |
| *Isopropyl disulfide* | IsoProDS | 150 (108, 66) | 22.78 |
| *3-(Methylthio)-1-hexanol* | MTH | 148 (61,75) | 38.41 |

Table 2. Retention times and ions used for the identification and quantification of the reductive sulfur compounds

### 2.5 Calibration

#### 2.5.1 Preparation of deodourised wine for calibration

A simple method was employed to prepare a base wine for constructing a calibration curve for the quantification of the reductive sulfur compounds. Deodourisation of the base wine was carried out in order to remove the volatile sulfur compounds as much as possible, along with other volatile compounds, while retaining further components of the wine to obtain a matrix effect similar to that of a wine sample.

A research red wine (Cabernet Sauvignon) was used to construct the calibration curves for the reductive sulfur compounds of interest. The wine was deodourised twice using a Büchi Rotavapor R (BÜCHI Labortechnik AG, Flawil, Switzerland) sourced from Watson Victor Ltd., Australia & New Zealand. An aliquot of the wine (200 mL) was put in an evaporation flask placed in a water bath and the temperature was maintained at 30 °C. After the first evaporation, the wine was reconstituted with absolute ethanol and ultrapure water (Barnstead® NANOpure DIamond™ Water Purification System) and was evaporated for a second time. The wine was then reconstituted with absolute ethanol and ultrapure water to have 13.5 % v/v ethanol in the final reconstituted wine.

#### 2.5.2 Preparation of a global stock mixture of reductive sulfur compounds

Stock solutions of all thirteen reductive sulfur compounds, as mentioned in Section 2.1, except methanethiol, were separately prepared by introducing 50 µL of a commercial

standard by a SGE 100 µL gas tight syringe (*Part No*. 005250 100R-GT, Phenomenex NZ Ltd., New Zealand) into an amber screw top 20 mL vial (*Part No.* 5188-6537) (Agilent Technologies) containing 10 µL absolute ethanol and pre-flushed with an inert gas, closed with a certified ultraclean 18 mm screwcap with septum (*Part No*. 5188-2759, Agilent Technologies). Weight differences together with purities were then used to calculate the final concentrations. These stock solutions were stored at -80 °C.

Methanethiol was prepared by bubbling methanethiol gas through 10 mL ethanol in a collecting vial, which was placed in a dry ice container. The methanethiol stock solution was stored at -20 °C. All stock solutions had concentrations in the range between 1 and 15 g/L.

In order to construct the calibration curves, a global stock mixture in absolute ethanol of all the reductive sulfur compounds of interest was prepared from the individual sulfur stock solutions already made up from commercial standards. The volume of the individual stock solutions needed was calculated so that the final concentrations of reductive sulfur compounds in the standard solutions fell in the concentration ranges for wines reported in the literature.

The global stock mixture of reductive sulfur compounds was prepared using a SGE gas tight syringe, by adding the required amounts of individual stock sulfur compounds into a 20 mL amber vial containing the required volume of absolute ethanol. The vial had been previously purged with argon gas and closed with a certified ultraclean 18 mm screwcap with septum.

### 2.5.3 Preparation of the internal standard mixture

Individual stock solutions of internal standards were prepared in similar way as the reductive sulfur stock solutions. A mixture of internal standards was then prepared from the individual solutions, with the concentration of $^2H_6$-dimethyl sulfide at 5 mg/L, isopropyl disulfide at 0.4 mg/L and 3-(methylthio)-1-hexanol at 10 mg/L. Their addition led to concentrations in the wine samples of 25 µg/L, 2 µg/L and 50 µg/L, respectively. The internal standard mixture was stored at -20 °C for daily usage.

$^2H_6$-Dimethyl sulfide was used to quantify methanethiol, ethanethiol, dimethyl sulfide, diethyl sulfide, methyl thioacetate, S-ethyl thioacetate. Isopropyl disulfide was used for dimethyl disulfide, diethyldisulfide, carbon disulfide, dimethyl trisulfide. Other compounds including 2-mercaptoethanol, 2-(methylthio)-1-ethanol, 3-(methythio)-1-propanol and 4-(methylthio)-1-butanol were quantified using 3-(methylthio)-1-hexanol as the internal standard.

### 2.5.4 Preparation of standard solutions for calibration

Standard solutions used to build the calibration curves were prepared by adding the required volume of the global stock mixture into a 20 mL amber vial containing the reconstituted deodourised red wine (Section 2.5.1).

The vial had been purged with argon gas and closed with a certified ultraclean 18 mm screwcap with septum before introducing the global stock mixture of sulfur compounds through a SGE gas tight syringe to have a total sample volume of 10 mL. An aliquot of 50 µL internal standard solution (Section 2.5.3) was then placed in the vial before HS-SPME extraction and GC-MS analysis.

### 2.6 Method validation

A two-step approach was used for the determination of method detection and quantification limits for the sulfur analytes, as described in Lee & Aizawa (2003). The two step approach takes into consideration several factors that affect the analyte signal, including instrumental noise, variability in instrumental sensitivity, and variability in method efficiency, matrix effects and interference, and is simple to follow. Other methods, such as the Hubaux-Vos approach for the calculation of the detection limit can also be used, as reported in Fedrizzi et al. (2007). However, this later approach is complicated, time consuming and does not take either the variability in method efficiency or the matrix effects into consideration (Lee & Aizawa, 2003). A brief discussion on how to conduct the method validation using the two steps approach is mentioned in this section.

Each analytical instrument has a limitation in the amount of an analyte that can be detected. In addition, with complex matrices, interfering components cannot be completely eliminated, so their effects must be taken into account when determining the limit of detection (LOD) for an analyte-matrix combination. The LOD, for most modern analytical methods, can therefore be divided into two components, instrumental detection limit (IDL)[4] and method detection limit (MDL)[5]. A similar notion can be used for the limit of quantification (LOQ).

The approach described in Lee & Aizawa (2003) consists of two steps for the determination of the LOD and LOQ. These firstly involve determination of the Instrumental Detection Limit (IDL) and Instrumental Quantification Limit (IQL), and using these values to estimate the Method Detection Limit (MDL) and Method Quantification Limit (MQL), following calculation of the LOD and LOQ for the extraction/analysis method.

Step 1. Determination of IDL and IQL following the Root Mean Square Error method

The Root Mean Square Error (RMSE) method, recommended by the US Environmental Protection Agency, involves generation of a calibration curve and calculating the RMSE. The steps involved are as follows:

1. Generate a 4-5 point calibration curve with standards having concentrations within an order of magnitude[6] of the estimated detection limit. The detection limit may be estimated as a concentration that would produce a signal three times the peak-to-peak noise. The calibration curve should be generated by plotting the detector's response against concentration.
2. Perform a regression analysis on the calibration curve and calculate values for the slope (m) and intercept (i) for a number of standards (n).
3. The calibration curve generated by plotting detector response (x) versus concentration (c) is:

$$x = m.c + i \quad (1)$$

[4] IDL is the smallest amount of an analyte that can be reliably detected or differentiated from the background of an instrument.

[5] MDL is the smallest amount of an analyte that can be reliably detected or differentiated from the background for a particular matrix by a specific method. It should be applied to extraction and analysis methods developed for the analysis of specific analytes in a matrix.

[6] The concentrations of these standards are within a factor of 10 of the estimated detection limits.

4. Based on the values of slope (m) and intercept (i), calculate the predicted response ($x_P$) for each of the standards.
5. Calculate the error (E) associated with each measurement $|x_P-x|$.
6. Calculate the square of the errors for each standard and then calculate the sum of the square of the errors ($\sum E^2$) associated for the number of points (n).
7. After that, the RMSE is calculated as follows:

$$RMSE = [\sum E^2/n-2]½ \quad (2)$$

8. The predicted instrumental detection limit (IDL, $c_L$) is calculated as follows:

$$c_L=3.RMSE/m \quad (3)$$

9. The predicted instrumental quantification limit (IQL, $c_Q$) is calculated using:

$$c_Q=10.RMSE/m \quad (4)$$

The detection and quantification limits determined here ($c_L$ and $c_Q$) do not take the matrix interferences into account, because RMSE was determined from calibration standards. The value $c_Q$ is used in the next step to spike the blank to compute the LOD and LOQ of the method, which incorporates instrumental variations. Consequently, both matrix/analytes and the extraction/analysis are taken into account for the determination of LOD and LOQ.

Step 2. The $t_{99}\sigma_{LLMV}$ method to calculate the values of LOD and LOQ

1. Fortify the 'blank' with the analytes of interest (7 replicates) such that the concentration of the analytes in the matrix equals the estimated LOQ (eLOQ) as determined in the aforementioned step (the $c_Q$ value).
2. Extract and analyse these samples following the method used for sample analysis.
3. Determine the amount of each analyte in the fortified samples.
4. Calculate the standard deviation of these measurements ($\sigma_{eLOQ}$[7]).
5. Determine the 'one-tailed t-statistic' for n-1 observations at the 99 % confidence level ($t_{99(n-1)}$). The $t_{99(n-1)}$ for 7 replicates (6 degrees of freedom) is 3.413.
6. The method detection limit or limit of detection (LOD) and method qualification limit or limit of quantification (LOQ) for the matrix/analytes combination and the extraction/analysis procedure is computed as:

$$LOD = t_{99(n-1)}.\sigma_{ELOQ} = 3.413\sigma_{eLOQ} \quad (5)$$

$$LOQ = 3.LOD \quad (6)$$

## 3. Results and discussion

### 3.1 Extraction time and extraction temperature

At room temperature, the concentration of semi-volatiles in the gaseous phase is small. The mass transfer rates are thus substantially lower, resulting in a longer extraction time using a coated fiber compared to direct extraction (Pawliszyn, 1997). One of the options to shorten

[7] According to the definition the lowest level of method validation (LLMV), the standard deviation of the concentration of the analyte in these fortified samples ($\sigma_{eLOQ}$) is the $\sigma_{LLMV}$, which explains the name of the method (the $t_{99}\sigma_{LLMV}$ method).

the extraction time is to increase the extraction temperature. An increased extraction temperature leads to greater diffusion coefficients and decreased distribution constants. An elevated temperature, therefore, can effectively assist in the dissociation of analytes from the matrix and their movement into the headspace. This leads to a faster equilibration time for a more rapid extraction (Pawliszyn, 1997). Temperature, therefore, is a very important parameter to optimise.

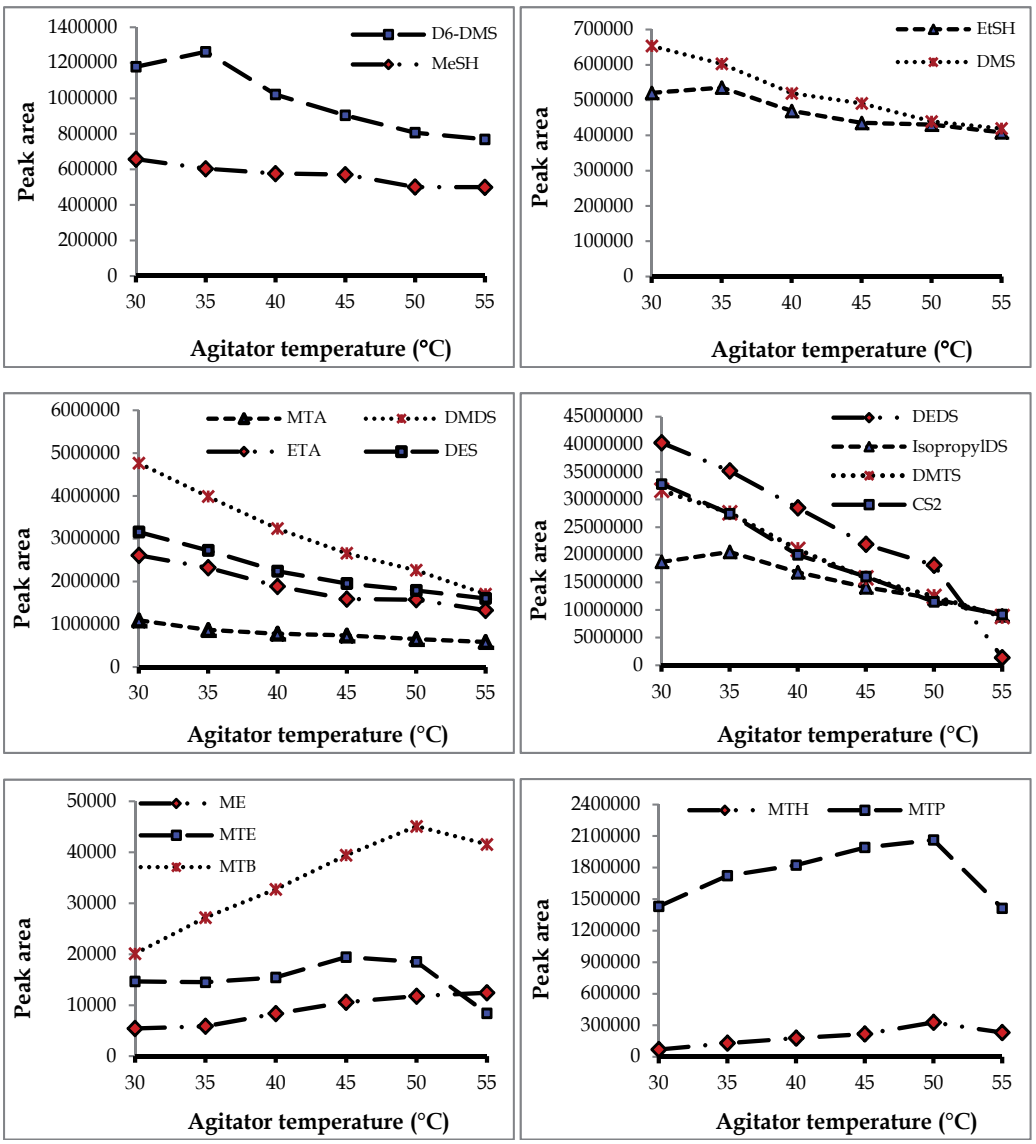


Fig. 2. Effect of extraction temperature on the chromatographic profile of the reductive sulfur compounds

Fig.2 presents the chromatographic profile, based on peak areas, of the reductive sulfur compounds, extracted at five different temperatures from 30 °C to 55 °C. As can be seen from these graphs, increasing the temperature led to a decrease in the amounts of the lighter sulfur compounds extracted onto the fiber. On the other hand, increasing the temperature to 50 °C resulted in increased peak areas for the heavier compounds, including ME, MTE, MTB, MTP and MTH. In order to get larger amounts of heavier compounds onto the fiber, without too much decrease in the quantity of the lighter reductive sulfur compounds, 45 °C was chosen as a compromise temperature.

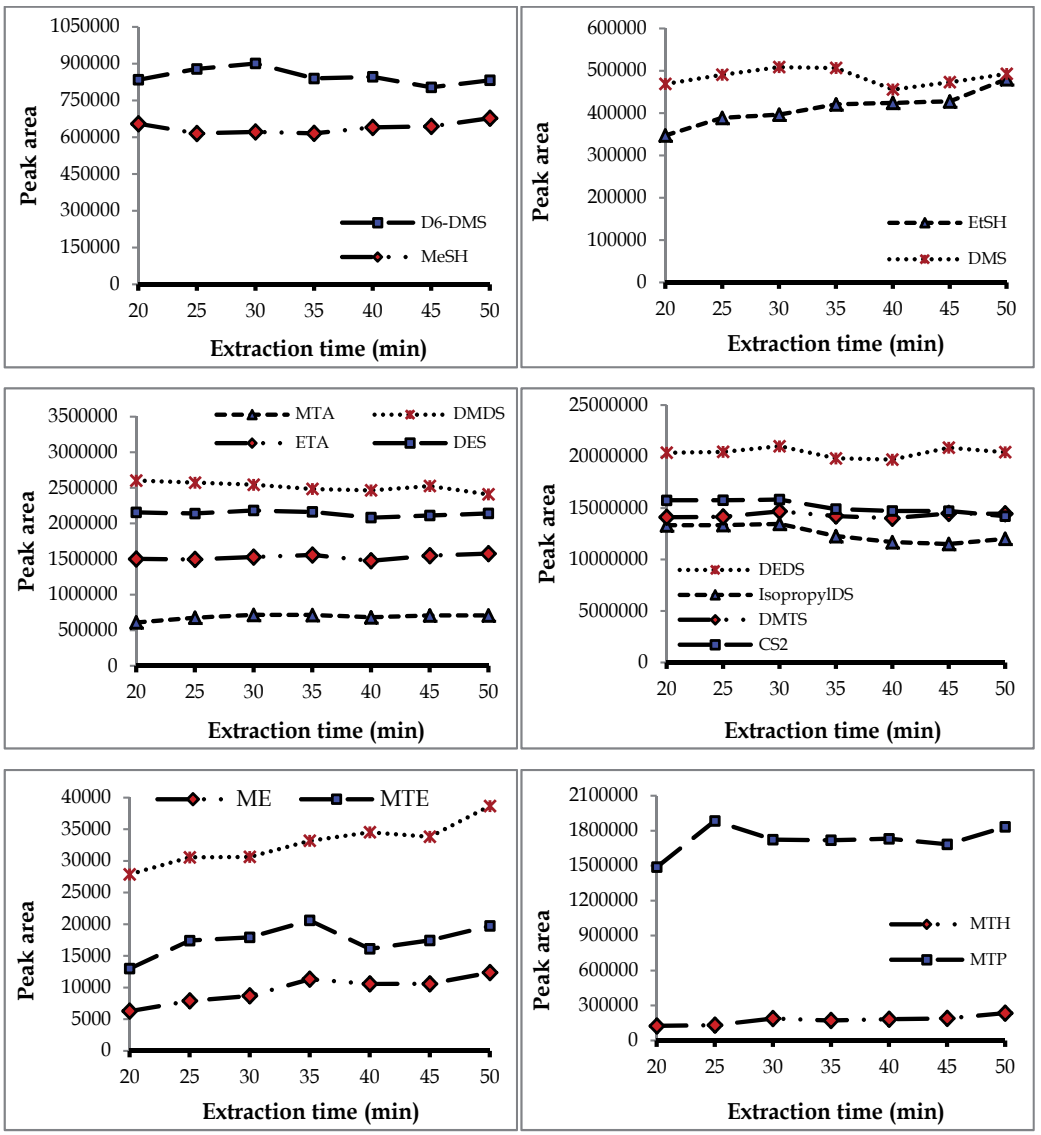


Fig. 3. Effect of extraction time on the chromatographic profile of the reductive sulfur compounds (continued)

The optimisation of the extraction time was also based on similar theory to the effect of increasing extraction temperature. Similar results were found when samples were extracted for different periods of time, from 20 to 50 min (Fig.3). The effect of extraction time on the chromatographic profile of the sulfur compounds was much less than the effect of temperature. Prolonging the extraction time to 35 min gradually increased the peak areas of most compounds. Increasing the extraction time from 30 to 35 min did not increase the peak areas of the heavy sulfur compounds very much, but led to a decline in peak areas for some of the lighter compounds such as D6-DMS, CS2, and also DMDS and DEDS. Therefore, an extraction time of 30 min was selected, with a practical consideration being the desire to keep the extraction time reasonably short.

The optimised HS-SPME extraction conditions and other parameters finally selected for use in sample preparation prior to GC-MS analysis are given in Table 3.

| **Parameters** | **Conditions** |
|---|---|
| **Fiber coating** | CAR-PDMS-DVB;<br>50/30 μm x 2 cm<br>(Product No. 57928-U, Supelco) |
| **Sample volume** | 10 mL |
| **Salt addition** | 1M $MgSO_4.7H_2O$<br>(2.5 g in 10 mL sample) |
| **Agitation speed** | 350 rpm<br>(10 sec on, 3 sec off) |
| **Incubation**<br>*Incubation time*<br>*Incubation temperature* | <br>5 min<br>45 °C |
| **Extraction**<br>*Extraction time*<br>*Extraction temperature* | <br>30 min<br>45 °C |

Table 3. Selected sample preparation conditions for HS-SPME extraction of the reductive sulfur compounds

### 3.2 Calibration, detection and quantification limits

Table 4 summarises the parameters obtained from the calibration graphs for all of the reductive sulfur analytes, along with the method detection and quantification limits for each compound. Linear regression analysis revealed that very good linearities ($R^2 > 0.992$) were obtained in the calibration graphs for all of the reductive sulfur compounds. The method provided very good detection limits, which were well below the sensory thresholds (See Table 1) of the analysed sulfur compounds.

| Compounds | Concentration range in the calibration (µg/L) | Slope | Intercept | $R^2$ | eLOQ (µg/L) | LOD (µg/L) | LOQ (µg/L) |
|---|---|---|---|---|---|---|---|
| **MeSH** | 0.40-16.1 | 6.33 | -0.80 | 0.993 | 0.37 | 0.16 | 0.49 |
| **EtSH** | 0.34-13.7 | 11.87 | -1.02 | 0.994 | 0.28 | 0.12 | 0.36 |
| **DMS** | 0.80-32.1 | 26.09 | 0.23 | 0.993 | 0.14 | 0.14 | 0.42 |
| **CS2** | 0.44-17.7 | 110.7 | 0.32 | 0.994 | 0.14 | 0.08 | 0.23 |
| **DES** | 0.40-16.0 | 4.12 | -0.14 | 0.995 | 0.25 | 0.03 | 0.10 |
| **MTA** | 0.85-34.0 | 32.94 | 0.08 | 0.998 | 0.39 | 0.15 | 0.44 |
| **DMDS** | 0.24-9.4 | 41.00 | 0.01 | 0.992 | 0.10 | 0.13 | 0.38 |
| **ETA** | 0.86-34.5 | 9.01 | 0.27 | 0.996 | 0.31 | 0.03 | 0.09 |
| **DEDS** | 0.39-15.5 | 4.61 | 0.21 | 0.996 | 0.12 | 0.06 | 0.19 |
| **DMTS** | 0.45-17.9 | 8.69 | 0.83 | 0.995 | 0.40 | 0.07 | 0.20 |
| **ME** | 11.9-237.1 | 2075 | 4.01 | 0.994 | 7.0 | 8.5 | 25.6 |
| **MTE** | 3.79-75.7 | 315.3 | 1.15 | 0.994 | 1.20 | 4.4 | 13.1 |
| **MTP** | 78.3-3130.1 | 172.4 | -10.98 | 0.996 | 29.5 | 8.4 | 25.2 |
| **MTB** | 3.41-136.6 | 280.9 | 0.79 | 0.995 | 2.71 | 7.8 | 23.3 |

Table 4. Parameters from the calibration graphs, along with method detection and quantification limits

### 3.3 Recovery and repeatability

Two red wines (Mission Estate 2009 Cabernet Merlot, Corbans 2009 Merlot) and a white wine (Oyster Bay 2008 Sauvignon blanc), after being deodorised and reconstituted with absolute ethanol and ultrapure water (See Section 2.5.1), were spiked with known amounts of the sulfur compounds. The non-spiked and the spiked reconstituted wines were then analysed in triplicate using the SPME extraction conditions given in Table 2 and the GC-MS separation and analysis conditions provided in Section 2.3. The concentrations were calculated by interpolation using the corresponding calibration curves. The recovery (R) (%) was estimated as:

$$R = [(C_s - C_0)*100]/C_a \quad (7)$$

where

$C_s$ - Calculated concentration in the spiked wine
$C_o$ - Calculated concentration in the non-spiked wine
$C_a$ - Concentration added

The repeatability of the method was also evaluated by calculating the relative standard deviation (% RSD) ($n$ = 3) for each compound using equation (8).

$$\%\ RSD = (STD*100)/Mean \quad (8)$$

where

*% RSD* - Relative standard deviation (%) of the mean

*STD* - Standard deviation of the mean
*Mean* - Mean value

Table 5 presents the calculated recoveries and the % RSD figures, which showed the good precision of the method for all of the analysed sulfur compounds. The recovery values were close to 100 % and the % RSDs were less than 10 % for all of the compounds.

| Compounds | Red wine 1 | Red wine 2 | White wine |
|---|---|---|---|
| **MeSH** | 108.6 (5.0) | 107.7 (2.6) | 101.3 (0.7) |
| **EtSH** | 94.7 (3.1) | 96.6 (6.8) | 104.8 (2.8) |
| **DMS** | 97.2 (1.3) | 95.5 (2.9) | 90.9 (4.8) |
| **CS2** | 98.9 (4.0) | 104.8 (5.1) | 105.9 (3.4) |
| **DES** | 109.1 (3.5) | 106.1 (0.39) | 99.8 (2.1) |
| **MTA** | 109.5 (4.8) | 110.0 (2.5) | 94.0 (0.10) |
| **DMDS** | 96.3 (6.8) | 97.4 (7.5) | 94.6 (10.1) |
| **ETA** | 99.6 (2.7) | 109.7 (4.4) | 91.7 (6.1) |
| **DEDS** | 100.3 (5.0) | 105.9 (5.3) | 92.3 (7.1) |
| **DMTS** | 105.5 (4.0) | 94.5 (1.8) | 90.4 (7.5) |
| **ME** | 92.1 (8.8) | 100.3 (13.8) | 106.3 (0.10) |
| **MTE** | 94.9 (4.4) | 85.9 (6.2) | 96.5 (1.3) |
| **MTP** | 88.5 (6.7) | 106.8 (6.0) | 98.8 (1.9) |
| **MTB** | 84.9 (4.3) | 94.4 (9.3) | 95.7 (0.85) |

Table 5. Recovery values (%), and in brackets the repeatability (RSD, in %)

## 4. Application

The method reported in this chapter was then employed to investigate the reductive sulfur compounds in some New Zealand red and white wines. It was particularly applied for the quantification of reductive sulfur compounds during red wine micro-oxygenation, an oxygen management tool that aims to improve wine quality (Nguyen *et al.*, 2010).

### 4.1 Reductive sulfur compound in some New Zealand red and white wines

The HS-SPME coupled with GC-MS method was employed to analyse some New Zealand commercial wines, including five white wines and five red wines, produced from different vintages from 2004 to 2008. Some older wines were included to look for the presence of as many reductive sulfur compounds as possible using the method developed, as high concentrations of some of these compounds are expected in older wines. The results obtained are displayed in Table 6. As can be seen from this table, ten out of the fourteen sulfur compounds that can be analysed using the HS-SPME coupled with GC-MS method, were quantified in these wines. Some compounds were present at fairly high levels, compared to their perception thresholds. The concentration of methanethiol (MeSH) was found to be higher than its perception threshold (0.3 μg/L in alcoholic solution) in all of the analysed wines, while the concentrations of dimethyl sulfide were also relatively high

compared to the reported threshold values, which range from 10 - 160 μg/L. It was found that only small quantities of the other compounds, including disulfides, thioacetates and thioether alcohols, were present in the examined wines, with the exception of 3-methylthio-1-propanol. The levels of these compounds were below their respective sensory thresholds, as can be seen from Table 6. DMTS was only found in the red wines examined, and at concentrations well above its perception threshold (0.1 μg/L). Interestingly, the concentrations of 3-methylthio-1-propanol were generally higher in the red wines than in the white wines, which followed the same trend reported by Fang & Qian (2005). Informal sensory evaluation by a group of Wine Science Post Graduate students (University of Auckland, New Zealand) noticed that most of the white wines examined, except for one of the Chardonnay wines (Chd1), exhibited quite strong reductive characters, especially those with higher concentrations of DMS. On the other hand, reductive odours were only moderately noticeable in the Cabernet Sauvignon (CSav1) and the Merlot wines analysed. This was the case even though higher concentrations of the reductive sulfur compounds were found in the red wines, and the concentrations of some compounds, such as 3-methylthio-1-propanol were higher than those of the white wines. This trend suggests that the complex red wine matrix could also play a role in the perception of the reductive notes. In a study on Spanish red wines, it was shown that the non-volatile components in the wines such as reducing sugars, alcohol and some phenolics, can have an impact on aroma sensory properties and thus on wine quality (Sáenz-Navajas et al., 2010).

| Compounds | MeSH | DMS | MTA | CS2 | DMDS | DMTS | ME | MTE | MTP | MTB |
|---|---|---|---|---|---|---|---|---|---|---|
| **Threshold (μg/L)** | *0.3* | *10-160* | *300* | *> 38* | *20 - 45* | *0.1* | *130* | *300* | *1200* | *100* |
| **2008 SB1** | 3.17 ± 0.12 | 19.1 ± 0.9 | 12.76 ± 1.44 | 1.62 ± 0.06 | 1.19 ± 0.35 | nd | nd | 23.2 ± 0.7 | 282 ± 36 | nd |
| **2005 SB2** | 4.40 ± 0.30 | 32.3 ± 0.6 | 12.34 ± 0.82 | 0.59 ± 0.07 | 1.06 ± 0.15 | nd | nd | nd | 237 ± 11 | nd |
| **2008 Chd1** | nd | 9.1 ± 0.2 | nd | 0.94 ± 0.06 | nd | nd | nd | 26.0 ± 2.6 | 624 ± 50 | nd |
| **2003 Chd2** | 2.20 ± 1.09 | 14.77 ± 0.01 | 5.42 ± 0.08 | 1.61 ± 0.01 | nd | nd | nd | 18.5 ± 1.4 | 230 ± 9 | nd |
| **2006 Rslg** | 3.24 ± 0.02 | 39.3 ± 0.7 | 4.94 ± 1.06 | 0.71 ± 0.01 | 2.17 ± 0.14 | nd | nd | nd | 374 ± 33 | nd |
| **2006 PN1** | 2.23 ± 0.19 | 36.3 ± 0.5 | 5.29 ± 0.29 | 1.48 ± 0.09 | nd | nd | nd | 26.7 ± 1.0 | 1064 ± 61 | 15.2 ± 4.0 |
| **2006 PN2** | 4.13 ± 0.14 | 10.2 ± 0.05 | 8.16 ± 2.56 | 3.44 ± 0.14 | 5.71 ± 0.61 | 0.92 ± 0.06 | nd | 37.0 ± 3.2 | 625 ± 54 | nd |
| **2006 CSav1** | 1.42 ± 0.01 | 54.4 ± 1.6 | 5.54 ± 0.61 | 7.18 ± 0.30 | nd | 0.87 ± 0.01 | 65.0 ± 9.5 | 27.8 ± 1.2 | 1119 ± 27 | 19.4 ± 4.0 |
| **2004 CSav2** | 1.76 ± 0.15 | 11.5 ± 1.2 | 9.53 ± 0.36 | 2.67 ± 0.03 | 2.35 ± 0.30 | 0.90 ± 0.04 | 88.4 ± 5.2 | 32.4 ± 3.4 | 2350 ± 133 | nd |
| **2004 Mer** | 4.85 ± 0.16 | 27.0 ± 0.4 | 5.97 ± 0.51 | 1.32 ± 0.03 | 2.28 ± 0.55 | 0.88 ± 0.03 | 43.6 ± 1.0 | 38.3 ± 2.5 | 1213 ± 8 | nd |

Table 6. Concentrations of reductive sulfur compounds (μg/L) ± standard deviations of the means ($n$ = 3) in ten commercial New Zealand white and red wines (SB = Sauvignon blanc, Chd = Chardonnay, Rslg = Riesling, PN = Pinot noir, Csav = Cabernet Sauvignon, Mer = Merlot).

### 4.2 Changes in the concentrations of reductive sulfur compounds in a red wine undergoing micro-oxygenation

The GC-MS method was then employed in a study on the effects of micro-oxygenation[8] (MOX) on the concentrations of reductive sulfur compounds in a red wine, commercially made from *Vitis vinifera* var. Cabernet Sauvignon grapes grown at Esk Valley, Hawkes Bay, New Zealand.

The grapes were harvested at 22.0 °Brix on the 13th April, 2008, crushed, destemmed and inoculated with Bio Springer BCS103 yeast (Bio Springer, Maisons-Alfort, France) at Corbans Winery (Hawkes Bay, New Zealand). The must was left to ferment on skins for 17 days before being drained off, centrifuged and pasteurised. The wine was then pumped onto a combination of French oak staves and untoasted oak chips at a rate of 2 g/L. The wine was then kept at 12 °C for 2 days to stabilise before oxygen was delivered in a fully replicated MOX trial, conducted using twelve 300 L tanks. Malolactic fermentation (MLF) occurred spontaneously at day 42 after MOX had been applied and was only completed towards the end of the trial. Oxygen dosing was carried out using a PARSEC SAEn 4000 Micro-oxygenation Unit supplied by Kauri New Zealand Ltd. (Wellington, New Zealand). Nine 300 L stainless steel tanks were used for the control (no oxygen supplied), the low oxygen (5 mg/L/month) and high oxygen (20 mg/L/month) rate treatments, while the other 3 tanks used for the third treatment were made of high density polyethylene, supplied by Flextank International (Abbotsford, Australia).

Seven reductive sulfur compounds, out of fourteen that could be analysed using the GC-MS procedure, were found in the Cabernet Sauvignon wine, and their concentrations were monitored at the beginning and the end of the trial. The highly volatile sulfur compounds (boiling point less than 90 °C) found to be present were methanethiol (MeSH) and dimethyl sulfide (DMS). The wine also contained five low volatile compounds (boiling point above 90 °C), including dimethyl disulfide (DMDS), methyl thioacetate (MTA), S-ethyl thioacetate (ETA), 2-(methylthio)-1-ethanol (MTE) and 3-(methylthio)-1-propanol (MTP) (methionol).

#### 4.2.1 Methanethiol, dimethyl sulfide and dimethyl disulfide

Methanethiol (MeSH) is one of the simplest sulfur compounds and plays a decisive role in reduction defects related to wine aromas (Ribéreau-Gayon et al., 2006b). The perception threshold of methanethiol in a hydroalcoholic solution is just 0.3 μg/L (ppb) and the compound can generate an odour reminiscent of cooked cabbage (Mestres et al., 2000).

In the current study, the concentration of MeSH in all of the wines was low and the wines were not overly reductive, as evaluated by local winemakers, although the concentrations were well above the perception threshold for MeSH. Our results indicated that at the end of the trial period, MOX did show some impact on the concentration of MeSH in the wine that received a higher oxygen dosage (20 mg/L/month) and in the wine stored in Flextanks. In

[8] MOX is a technique that was developed in the Madiran region of southern France in the 1980s and was commercially released in 1996 (Cano-López et al., 2006). Its principle is the continuous delivery of a small metered amount of oxygen into a wine by means of micro-bubbling using a porous micro-diffuser.

these two cases, the concentration of MeSH was significantly lower than in the control wine and in the wine treated with the lower oxygen rate (5 mg/L/month) (Fig.4).

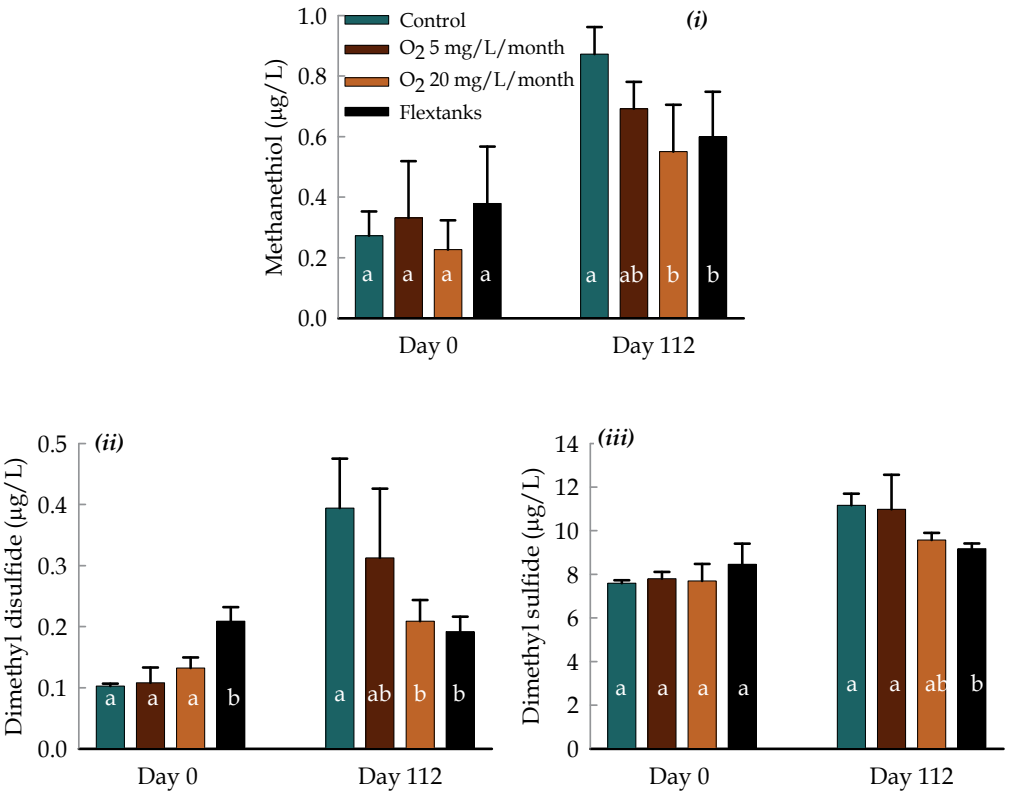


Fig. 4. Effect of MOX applied after alcoholic fermentation on (i) methanethiol, (ii) dimethyl disulfide and (iii) dimethyl sulfide in a Cabernet Sauvignon wine. Error bars represent the standard deviations of the mean ($n$ = 3). Columns with different letters denote values which significantly differ ($P < 0.05$, LSD and Tukey Post Hoc Tests) at the same observation date.

Our findings are in agreement with the study by McCord (2003) who reported a significant decrease in the concentration of MeSH and EtSH in a wine that underwent MOX. No biological activity was recorded in the wine used in the McCord study. However, spontaneous MLF did occur in the control and all treated wines during this MOX trial. Although the formation of methionine-derived reductive sulfur compounds from the metabolism of the lactic acid bacteria (LAB) *Oenococcus oeni* during MLF is still poorly understood, it was shown in a recent study that MLF may result in the formation of MeSH and DMDS (Vallet et al., 2008). In addition, thiols such as MeSH are nucleophilic compounds very susceptible to oxidation, and can readily react with different electrophilic species such as *o*-quinones and the carbocation form of procyanidin molecules, formed from the oxidation of wine polyphenols (Majcenovic et al., 2002; Ribéreau-Gayon et al., 2006b).

MeSH can also be oxidised to form DMDS and dimethyl trisulfide (DMTS) (Rauhut, 1993). The concentration of DMDS, which has a perception threshold of 20 - 45 μg/L in wine with cooked-cabbage and onion like odours (Mestres et al., 2000), however, was significantly lower ($P < 0.05$) in the wine treated with 20 mg $O_2$/L/month and in the Flextank wines (Fig.4), and DMTS was not detected in any of wines.

MOX did not affect the concentration of DMS, but the Flextank wine had a lower DMS concentration than the control (Fig.4). In McCord's study (2003), on the effect of toasted oak products with and without MOX on the ageing of a Cabernet Sauvignon wine, the concentration of DMS was found to decrease in all treatments with added toasted oak, while no such decrease was found in the micro-oxygenated counterparts. DMS can produce off-odours described as cooked cabbage and shrimp-like at concentrations above its perception threshold (10 - 160 μg/L in wine), whereas at low levels it produces odours reminiscent of asparagus, corn and molasses (Mestres et al., 2000; Jackson, 2008). On the contrary, DMS is thought to have a positive influence on the bouquet of some bottled wines when present at low levels (Spedding & Raut, 1982). The formation of DMS in wine has not yet been fully elucidated. Some researchers consider that cysteine, cystine and glutathione can be precursors of DMS, because yeasts cannot produce S-methyl methionine (SMM) (Landaud et al., 2008). On the other hand, it has been suggested that yeast may produce SMM, which subsequently can be enzymatically hydrolysed to release DMS and homoserine (Rauhut, 1993). The metabolism of LAB, e.g. *Oenococcus oeni*, during MLF, also produces diverse sulfur containing volatiles through methionine catabolism (Pripis-Nicolau et al., 2004). Although trace amounts of DMS were found in the basal medium inoculated with LAB strains, its formation did not correlate with methionine metabolism (Pripis-Nicolau et al., 2004). During wine ageing, the concentration of DMS can increase significantly, indicating that a chemical pathway could also be involved in its production (Landaud et al., 2008). Reduction of dimethyl sulfoxide (DMSO) (odourless), present at a concentration up to 1230 μg/L in a survey of New Zealand wines, has been proposed as a DMS precursor during wine aging (de Mora et al., 1993).

On a further note, it was found in a model wine ageing study that DMSO is only a minor precursor of DMS, yet SMM appeared to be a good source of DMS during wine ageing (Segurel et al., 2005). Loscos et al. (2008), for the first time, isolated SMM from grapes and established that it is a major DMS precursor in the grape. In the current MOX study, MLF started spontaneously after day 42, so either chemical or biochemical pathways could be sources of DMS. This could explain the increase in concentration values at day 112 compared to day 0 in all of the wines. This increase, however, was lower in the high oxygen treated wine and significantly lower in the Flextank wine, suggesting a limited effect of oxygen on the enzymatic biosynthesis of DMS from the precursor SMM during the course of the trial. In this case, the LAB that predominated in the research wine during spontaneous MLF might have been a facultative anaerobic species, such as *Oenococcus oeni* and *Lactobacillus*, which are better adapted to the absence of oxygen (Ribéreau-Gayon et al., 2006a), and thus enhanced the enzymatic hydrolysis of SMM in the control and the low oxygen treated wine.

### 4.2.2 S-thioesters

The S-methyl and S-ethyl esters of ethanethioic acid, methyl and ethyl thioacetates (MTA and ETA) were also found and monitored during the MOX trial. These compounds were identified and determined for the first time in beer and wine by Leppanen et al. (1980).

Despite their high sensory thresholds, the thioacetates are a potential source of off-odours, because they can be hydrolysed to give free thiols at low pH (Leppanen et al., 1980), although they can produce rotten vegetable smells on their own (Landaud et al., 2008). The formation of these esters could be due to wine yeast metabolism involving the highly volatile MeSH via acetyl coenzyme A (acetyl-CoA) (Landaud et al., 2008). In the present study, alcoholic fermentation had finished before oxygen was applied, so hydrolysis should have led to declines in the concentrations of MTA and ETA. In fact, the concentrations of both MTA and ETA in all wines did not show any difference among the treatments and with the control (Fig.5). This indicates that oxygen did not influence the hydrolysis process during the trial period, even though the ETA concentrations were different in the wines to start with. At the same time spontaneous MLF may have contributed to an increase in ETA concentrations in the control and the low oxygen treated wines, leading to similar levels of this compound in all of the wines at the end of the trial. During MLF, the metabolism of LAB can also transform sugars, organic acids or amino acids into aromatic compounds, including MeSH. The availability of MeSH, therefore, could lead to the formation of MTA through an enzymatic reaction between MeSH and acetyl-CoA. This mechanism has also been found to occur with yeast metabolism in beer (Landaud et al., 2008). However, whether such an enzyme catalysed reaction occurs in the metabolism of wine LAB has not yet been established.

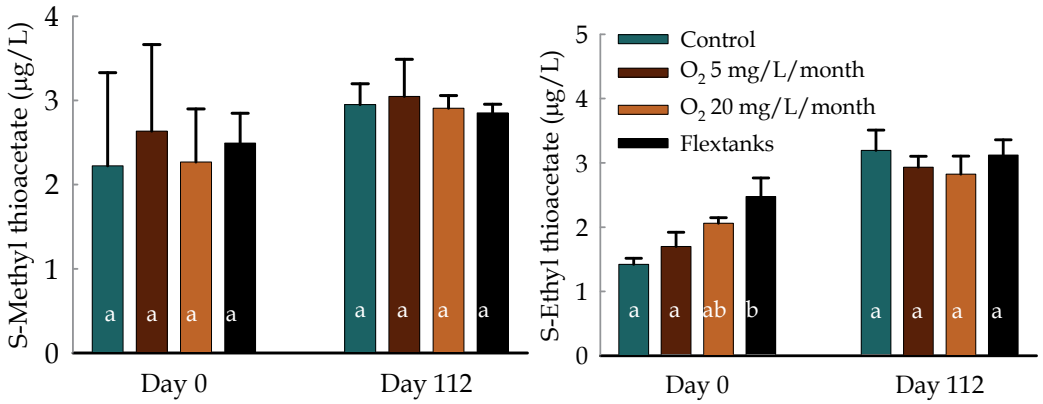


Fig. 5. Effect of MOX applied after AF on the S-thioesters in a Cabernet Sauvignon wine. Error bars represent the standard deviations of the mean ($n$ = 3). Columns with different letters denote values which significantly differ ($P < 0.05$, LSD and Tukey Post Hoc Tests) at the same observation date.

### 4.2.3 Thioether alcohols

Two other low volatile sulfur compounds included in this study were 2-methylthio-1-ethanol (MTE) and 3-methylthio-1-propanol or methionol (MTP). MTP is considered to be the most important heavy sulfur compound involved with reduction defects brought about by yeast activity (Ribéreau-Gayon et al., 2006b). MTP can generate odours reminiscent of cauliflower and cabbage at a concentration above its perception threshold (1200 µg/L). MTE

has a perception threshold of 250 µg/L in hydroalcoholic solution and has a French bean-like odour (Mestres et al., 2000). The concentration of MTP in wines with reduction notes, due to the heavy sulfur compounds that develop during alcoholic fermentation, is typically found at levels above its perception threshold. The concentration of MTE in wines with reductive characters may be very close to its perception threshold (Ribéreau-Gayon et al., 2006b).

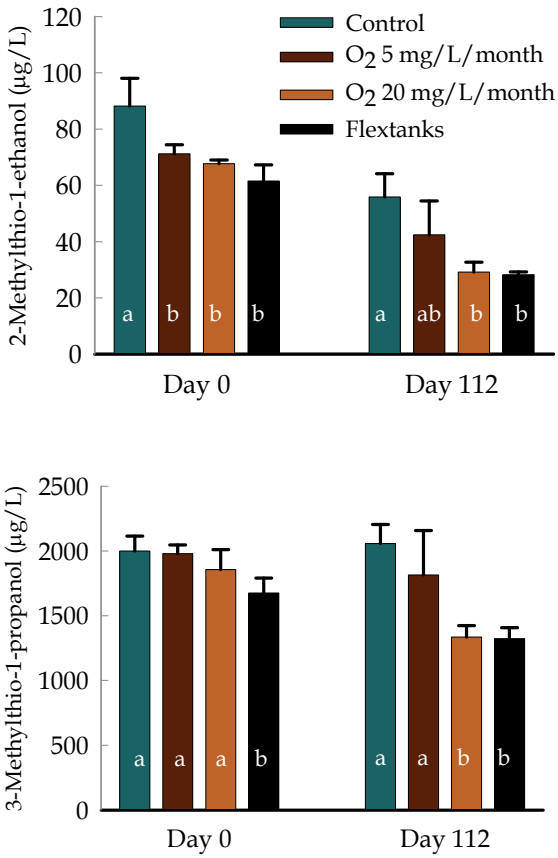


Fig. 6. Effect of MOX applied after AF on 2-methylthio-1-ethanol and 3-methylthio-1-propanol. Error bars represent the standard deviations of the mean ($n$ = 3). Columns with different letters denote values which significantly differ ($P < 0.05$, LSD and Tukey Post Hoc Tests) at the same observation date.

As can be seen from Fig.6, the concentration of MTE tended to decrease over time, with a greater drop in the high oxygen treated and Flextank wines at the end of the trial. However, possibly because of some variations caused during pumping the wine from the bulk storage tanks to the 300 L tanks, the MTE concentrations were different at day 0; so the question whether oxygen could have affected this compound during the trial is therefore undecided. Literature on the formation and evolution of MTE during winemaking is not well

documented. On the other hand, the metabolic formation of MTP in wine, involving the deamination of methionine produced by yeast, decarboxylation, then reduction of methional to methionol (MTP) via the Ehrlich pathway, is well known. Because the alcoholic fermentation had finished, no additional MTP formation was expected during the course of the trial. Instead, the higher oxygen treated wine and the Flextank wine showed a significant drop in the concentration of MTP by the end of the trial. The loss of MTP could be due to direct oxidation of MTP to form methional. Some authors have demonstrated a decrease of methionol in the presence of oxygen along with methional formation (Escudero et al., 2000). However, methional may also be formed from methionine via the Strecker mechanism in the presence of a dicarbonyl compound (e.g. methylglyoxal). This pathway was shown to be the main source of methional in white wines treated with oxygen (Silva Ferreira et al., 2002). Silva Ferreira et al. (2003) in a further study did not find that methional was present in a Port wine treated with oxygen, and suggested that the decrease in MTP would lead to the formation of other compounds as yet unidentified.

## 5. Conclusion

The method developed allows the concurrent quantification of fourteen high and low volatile reductive sulfur compounds commonly found in wines. Importantly, both methanethiol and ethanethiol were analysed without the use of a cryotrap, with good recovery and repeatability. The concentrations of reductive sulfur compounds in finished white and red wines revealed that although more reductive sulfur compounds were found in the examined red wines, the reductive odours were only moderately percieved. By contrast, strong reductive characters were exhibited in most of the white wines, suggesting that the complex red wine matrix could play a role in the perception of the reductive notes. The findings from the micro-oxygenation (MOX) trial also provide quantitative data to address the lack of scientific information regarding one of the proposed beneficial effects of MOX, namely to diminish the concentration of unwanted reductive sulfur compounds in red wines. MOX was applied to a Cabernet Sauvignon wine after the alcoholic fermentation in this study, and the results showed that oxygen could lower the concentration of MeSH. This may occur through an interaction with oxidised polyphenol quinones, but without an associated increase in the concentration of the disulfide DMDS, expected through direct oxidation of MeSH. More research is needed in this area to confirm the reaction pathways for the oxidation of methanethiol, and the potential formation of polyphenol adducts. The concentrations of other reductive sulfur compounds, except the thioesters, were also affected by the presence of oxygen. These losses have the potential to impact in a favorable manner on the removal of unwanted reductive odours from red wines. This issue also needs to be examined in future studies combining sensory analysis with the chemical analysis of reductive sulfur compounds.

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# Part 3

# Toxicology

# 10

# Determination of Volatile Substances in Forensic Samples by Static Headspace Gas Chromatography

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## 1. Introduction

The technique of static headspace gas chromatography has great acceptance in the forensic field, especially for the determination of ethanol in biological samples (Macchia et al., 1995; Tagliaro et al., 1992), so most forensic laboratories in the world have this equipment and perform this analysis on a routine basis, but in many of these laboratories, equipment is exclusively employed to determine ethanol, even when this technique can be used to determine many other substances of toxicological interest, volatile substances, without major changes to the equipment (Seto, 1994), thus we can conclude that these laboratories do not exploit all the possibilities of the technique.

The determination of volatile substances is one of the most important tests in forensic toxicology, (Broussard, 2003). Volatile substances can be defined as those organic compounds whose vapor pressures are greater than or equal to 0.1 mm Hg at 20 ° C; the Environmental Proteccion Agency (EPA) defines as volatile substances any compounds of carbon, excluding carbon monoxide, carbon dioxide, carbonic acid, metallic carbides, and ammonium carbonate which participates in atmospheric photochemical reactions. These substances may be involved in forensic cases as toxic agents and because of their use as drugs of abuse; in that context, the most important volatile substance is ethanol, however, there are other volatile substances of forensic interest, such as organic solvents, anesthetics, alkyl nitrites, etc. (table 1) (Moffat et al., 2004).

The determination of volatile substances in forensic samples has been carried out through titrations, spectrophotometric methods and chromatographic methods, as well (Seto, 1994). Titrations and spectrophotometric methods are not specific and usually lack sufficient sensitivity, besides not being able to analyze simultaneously all the volatile substances. In contrast, gas chromatography is qualitative (by the use of retention time) and quantitative (by the use of signal strength), so it is able to analyze simultaneously several volatile substances with the adequate sensitivity and specificity necessary in forensic environments,

therefore, this technique is used to make such determinations. Historically, three types of methods have been used for the preparation of the samples: solvent extraction, direct injection and headspace volume injection; as of late, forensic laboratories prefer the latter over the other two techniques.

| Solvents | Aromatic Hydrocarbons | Alkyl Nitrites | Anesthetics |
|---|---|---|---|
| Ethyl Alcohol | Toluene | Amyl Nitrite | Ethyl Ether |
| Methyl Alcohol | Benzene | Isobutyl Nitrite | Chloroform |
| Acetone | Xylenes | | Fluorocarbons |

Table 1. Examples of volatile substances of forensic interest.

The headspace injection technique has great advantages over other methodologies, namely providing a clean injection, resulting in lower spending of gas chromatograph consumables; it is simple, minimizes the possibility of artifacts during the analysis, diminishes the possibility of contamination and accurately quantifies analytes.

This paper will review theoretical aspects of the static headspace technique, as well as complementary additions to it (Kolb, 1999; Slack et al., 2003), the most significant substances from the standpoint of forensic toxicology that can be analyzed by this technique as ethanol (Kugelberg & Jones, 2007; Macchia et al., 1994; Tagliaro et al., 1992), congeners of alcoholic beverages (Iffland & Jones, 2003), inhalants (Angerer & Horsch, 1992; Seto, 1994), anesthetics (Pihlainen & Ojanperä, 1998), carbon monoxide (Boumba & Vougiouklakis, 2005; Vreman et al., 1984) and cyanide (Calafat & Stanfill, 2002; Felby, 2009); in each case, the analytical conditions and modifications necessary for the equipment to perform each analysis will be exposed, advantages and disadvantages of this technique over other existing procedures will be discussed and also, issues relating to development of methods for this technique and their validation.

Finally, newest developments in the area will be mentioned, and how they have replaced headspace injection in some applications.

## 2. Static headspace

If the components of interest in a solid or liquid sample are volatile, a good way to analyze them is to examine the concentration of these analytes in the gas phase above the matrix (headspace) when in a closed container, either by taking a sample directly from the gas phase or trapping and concentrating the gas prior to analysis. This type of extraction techniques are known as headspace analysis (Smith, 2003); the analysis and subsequent separation of volatile substances is normally carried out by the technique of gas chromatography, which is a mature technology, reliable and supported by a large body of work. The sample can be in contact and in equilibrium with the extractant gas (static or equilibrium headspace), or volatile compounds can be extracted by a steady stream of inert gas (dynamic headspace).

The different headspace sampling techniques can be classified into one-step procedures, such as static headspace, where an aliquot of the vapor phase is transferred in a closed container directly to the gas chromatograph, and two-step procedures, where the volatile analytes are transferred from the matrix of the headspace to a "trap" where they are released

by the action of heat or by a stream of carrier gas, and transferred to the gas chromatograph; dynamic headspace and solid phase microextraction (SPME) fall into this category. Regardless of the kind of headspace used, the sample is always a more or less diluted gas (Kolb, 1999). The technique you choose depends on several factors, such as the type of sample to be tested, if a quantitative or qualitative analysis is desired, the required sensitivity, automation and budget.

Static headspace extraction, also known as equilibrium headspace extraction, is one of the techniques used for qualitative and quantitative analysis of volatile substances in the forensic field, in this technique the sample is placed in a closed vial, the volatile analytes disseminate into the headspace of the vial (figure 1), once equilibrium is reached between the analyte concentration in the headspace and the analyte concentration in the sample, a portion of the headspace is taken and injected into the gas chromatograph; this can be done manually or with an autosampler, this process will be usually carried out at a pressure and temperature above ambient conditions (Slack et al., 2003).

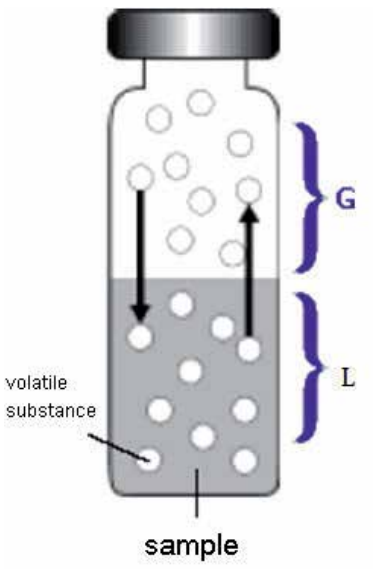


Fig. 1. Headspace extraction fundament. G: Gas, L: liquid

This technique is simple, relatively inexpensive, minimizes the formation of artifacts and can accurately quantify volatile substances with low solubility in water, its only disadvantage being its lower sensitivity with respect to dynamic headspace techniques, although this disadvantage is unimportant in most of its applications to forensic toxicology.

### 2.1 Instrumentation for static headspace extraction

The equipment for static headspace extraction consists of a container, where equilibrium takes place, a device that heats the container at a constant temperature and an injection device, which transfers a portion of the headspace gas to the gas chromatograph. The container is a glass vial of between 5 ml and 25 ml capacity, which is sealed with a septum coated with polytetrafluoroethylene (PTFE) and an aluminum cap, using a crimp. Injection

can be manual and automatic, however, the first of these techniques have poor reproducibility and there may be contamination between runs, so it is advisable to perform the technique using an autosampler (Seto, 1994).

Current autosamplers work with two different techniques. The first one employs sample loops; in order to fill these loops with headspace gas, the closed vial is pressurized to a pressure level above that present in the vial; the pressurized headspace is then temporarily connected to the sample loop, causing the headspace gas to expand through the sample loop and into the atmosphere; once the sample loop is filled, a valve is changed and its contents are transferred to the chromatographic column. This type of sampling has been commonly used for gas sampling in other areas, such as the petrol industry.

Instead of filling a sample loop, the headspace gas can be expanded directly to the chromatographic column, this type of sampling is called pressure-balance sampling; in this technique, the carrier gas enters the gas chromatograph through a V solenoid valve and is divided before the column. Some of the gas is directed to the column and some to the sampling syringe of the headspace injector. When the syringe enters the vial with the sample, the gas is pressurized; the gas transfer from the headspace to the chromatographic column takes place when the V solenoid valve is closed for a short time, suspending thus the flow of carrier gas; then the headspace gas expands, this time directly to the column, without any loss of headspace gas, as it happens with the previously mentioned procedure. Headspace gas replaces carrier gas during the sampling time, and the volume of headspace gas transferred to the column depends on the sampling time, therefore it is possible to calculate it with accuracy; this volume can be changed by simply changing the sampling time, unlike the previous technique, where the volume of injected gas depends on the sample loop and can only be changed by manually installing another sample loop of a different volume capacity (Kolb, 1999).

### 2.2 Sample preparation in static headspace extraction

One of the main advantages of static headspace extraction is how easy the sample preparation is; in the case of qualitative analysis, it suffices to place the sample in a vial and seal it with a PTFE septum and an aluminum lid; however, for quantitative analysis, it is necessary to understand and optimize the effects of the matrix, in order to obtain good sensitivity and, above all, accuracy.

#### 2.2.1 Solid samples

For solid samples, it may be necessary to change their physical state. This is achieved by grinding the solid to a powder as fine as possible, or dissolving or dispersing the solid into a liquid. In the first case, the contact volume of the solid is increased in order to establish a better partition between the volatile substance and the headspace gas; however, it is preferred to dissolve the solids in a liquid, because equilibrium is achieved faster this way, and is also more reproducible; on top of that, liquid samples are easier to work with (Slack, et al., 2003).

#### 2.2.2 Liquid samples

Liquid samples are usually simply poured into a vial and sealed immediately after, in order to prevent any evaporation losses (Slack, et al., 2003).

### 2.3 Optimization of static headspace extraction

Several factors must be optimized in a static headspace extraction in order to obtain a method with the desired extraction sensitivity, reproducibility and efficiency. These factors include the volume of the used vial, the temperature and pressure levels, and how the sample is to be prepared.

#### 2.3.1 Sample

The main factors controlling the sensitivity of the static headspace extraction are the analyte partition coefficient (K), which is the ratio between the analyte concentrations in the liquid phase with the concentration in the gas phase:

$$K=C^L/C^G \quad (1)$$

And the ratio of phases (β), according to the formula:

$$A=C^G=C°/K+\beta \quad (2)$$

Where A is the area of the chromatographic peak obtained for the analyte, $C^G$ is the concentration of analyte in the headspace, C° is the concentration of analyte in the liquid sample, K is the partition coefficient, and β is the ratio volume of the phases. The partition coefficient depends on the extraction temperature, while β is determined by the relative volume between the two phases; In static headspace extraction the sensitivity depends on the solubility of the analyte in the matrix; for analytes with a high partition coefficient, the most important parameter is the extraction temperature, since most of the analyte is in the liquid phase and it can only be passed into the headspace by heating the vial; on the other hand, for analytes with low partition coefficients, they are already present in the headspace even without any heating, so in this case, the most important parameter is the volume relation between the phases. That is, increasing the extraction temperature is only effective in polar volatile analytes, while the sensitivity of non-polar analytes remains essentially unchanged by the increase of the extraction temperature (Slack, et al., 2003).

Quite the opposite, changing the volume of the ratio phases has minimal effect on the sensitivity of polar analytes (high partition coefficients), but affects dramatically the sensitivity of non-polar analytes (low partition coefficients). Another way to increase the sensitivity of the method is by adding a salt, such as sodium chloride, to the sample.

Finally, if an analytical method focused on robustness rather than sensitivity is required, the relation between the partition coefficient and the polarity of the analytes can be used to achieve it; in order to do so, a matrix with high affinity for the analytes is used, so losses due to sample handling or second analyses are prevented. If the values of K are not known, one can simply analyze in the chosen matrix the substance to be determined, as well as determining the area against the sample volume (Slack, et al., 2003).

#### 2.3.2 Chromatographic conditions

Headspace extraction is solely a method of sampling and there are, in principle, no limitations for chromatographic columns and any type can be used, thus it can be selected according the demands of sensitivity resolution and analysis time of a particular analytical

problem. It has been recommended for headpace analysis (Kolb, 1999), to use in the first instance a capillary column of 0.32 mm of internal diameter (I.D.) and 30 m of length. However, if high resolution is required, a longer column with smaller I.D., for example a 50mX0.25 mm I.D. capillary column provides a better separation at the expense of longer analysis time; Nevertheless, in the case of an automated headspace sampler a long analysis time is less unfavorable, because the sample are analyzed anyway unattended overnight. If analysis time is the main parameter, very short capillary columns with I.D.´s of 100 μm or 50 μm can be used. (Kolb, 1999).

Film thickness is an important parameter in gas chromatography because it provides a higher sample capacity for compounds in high concentrations to avoid peak splitting or broadening by overload and consequently poor resolution, However, this is not a problem in headspace analysis, since headspace sample is a diluted gas sample and the amount of analytes is small enough to avoid peak broadening. Film thickness is chosen in headspace analysis to provide good resolution in an adequate time, unless, cryogenic trapping is used, where thick films are preferred.

Column capacity in Headspace gas chromatography has a different meaning and concerns the gas volume which can be introduced in the column without significant band broadening. To avoid any confusions the term headspace capacity has been used (Kolb, 1999). Resuming, peak broadening is determined by injection time, carrier gas flow and the internal diameter of the chromatographic column. Consequently, if high resolution is not required, and a mass spectrometer detector (MSD) is not used, capillary columns with an I.D. of 0.53 mm are preferred, to inject the biggest amount of gas from the headspace and to achieve the best sensitivity.

### 2.3.3 Enrichment techniques in static headspace extraction

When analytes are under the limit of detection (LOD) of the technique is necessary to use enrichment techniques. In headspace analysis, for this purpose the target analytes must be separated from the headspace gas either by absorption into a liquid or by adsorption onto a solid adsorbent and also by condensation in a cold trap. (Kolb, 1999). Solvent free techniques are particularly desirable in case of trace analysis to avoid problems with solvent impurities. Consequently, cryogenic trapping is the preferred choice to improved detection limits in static headspace analysis

Cold traps are used for two reasons: enrichment purposes and solute band concentration. There are two types of cryogenic trapping: by cryogenic condensation and cryogenic focusing.

In cryogenic condensation the volatile compounds are trapped simply by condensation in traps which usually contain no stationary phase or when the stationary phase has lost is properties as a chromatographic phase, Volatile substances are eluted from the trap by heating and depending on how the cold traps are heated, the analytes can be eluted in a very narrow band that thus, analytes are focused.

In cryogenic focusing, volatile compunds are trapped in the liquid phase of a chromatographic column at a low temperature which, however, preserve its chromatographic properties, In other words cryogenic focusing is based in the same

principles that thermal focusing, commonly used in gas chromatography and the difference in the nomenclature should only indicate the difference in the applied temperature, with cryogenic focusing carried below and thermal focusing above ambient temperature (Kolb, 1999).

Generally, cryogenic focusing is preferred over cryogenic condensation, for several reasons, the first technique needs higher temperatures, consequently its operation is simpler and its more easy to be automated, the rapid heating used in cryogenic condensation may cause decomposition of labile compounds. Moreover, cryogenic condensation has some inherent problems such as breakthrough by aerosol formation., Consequently, trapping may be incomplete by droplet formation, causing peak splitting or distorted peaks, last but not least, in cryogenic focusing, analytes are trapped inside the capillary column, thus, It's a more effective procedure and more simple to achieve than cryogenic condensation; for all these reasons, at the present moment cryogenic focused is favored over cryogenic condensation (Kolb, 1999).

Summarizing, the initial profile of a gas sample depends on sample volume and the inner diameter of the capillary column, film thickness has no effect on the band width causes no focusing effect under isothermal conditions. Only temperature programming helps to elute an originally broad band profile as a sharp peak. However, this approach for highly volatile substances needs low initial temperatures, which leads finally to cryogenic trapping

### 2.4 Quantitative techniques in static headspace extraction

The four most common approaches to quantitative static headspace gas chromatography calibration are external standard, internal standard, standard addition and multiple headspace extraction (MHE). The choice of technique depends on the type of sample being analyzed (Slack et al., 2003).

#### 2.4.1 External standard calibration

External standard calibration in static headspace gas chromatography is best for analytes in liquid samples where the analytes are soluble in the matrix and the matrix has no effect on the analyte response. In these type of calibration is important to match the standard and sample matrix as closely as possible and to demonstrate equivalence in the response between the standards and the samples. The main difficulty with external standard calibration is that is does not compensate for any variability due to the gas chromatograph injection or due to variation in the analyte matrix.

#### 2.4.2 Internal standard calibration

Internal standard calibration allows compensating for any variation due to matrix effects and gas chromatography injection. Prior to the extraction, a known additional analyte is added to each sample and standard. This compound is the internal standard.

The most important part in internal standard calibration is choosing an appropriate internal standard for any method. This compound must be available in extremely pure form and must never appear in the samples of interest, at least at the analyte concentration expected; it cannot interfere in either the extraction or the chromatography of the analytes. Finally, it

must be structurally similar to the analytes, so that it undergoes similar extraction and chromatography, otherwise the compensation will be lost.

### 2.4.3 Standard addition calibration

In standard addition calibration, an additional known quantity of the analyte is added directly to the sample, following an initial analysis. In standard addition, the sample is divided into several equal portions, then add increasing levels of standard. In other words, the calibration curve is prepared with the sample thus; all the points of the curve have the same composition, in this way matrix effects are eliminated. This type of calibration is not often used in gas chromatography static headspace.

### 2.4.4 Multiple headspace extraction

Multiple headspace extraction (MHE) determines the total peak area for an analyte in an exhaustive headspace extraction, so the analyst can calculate the total amount of analyte in the sample.

The main advantage of MHE is that matrix effects are eliminated because it determines the total amount of analyte in the sample; this is achieved by consecutive analysis on the same sample, in the same vial; in each analysis, the amount of analyte in the headspace will decrease until all the analyte is completely extracted.

Currently, it is not necessary to completely extract the analyte; usually, only three or four analyses are performed, and then an analysis by linear regression of the obtained data, in order to mathematically determine the total amount of analyte in the sample (Slack, et al., 2003).

## 3. Applications in forensic toxicology

In the next sections, the mean applications of static headspace gas chromatography will be described, with an emphasis in methods that could be performed without extensive modification of the equipment commonly present in the forensic toxicology laboratories, in any case, analytical considerations will be discussed, from sampling, materials and reactants needed, analysis, to interpretation of results, method validation and the importance of these test in the legal media, will be reviewed.

### 3.1 Ethanol

The determination of ethanol is one of the most important analysis in forensic toxicology, either in samples from corpses, or in drivers suspected of driving under the influence; in any case, precise and reliable ethanol determination is necessary. The technique of choice for the analysis of ethanol in biological samples is gas chromatography with a flame ionization detector (FID), using techniques of direct injection or headspace extraction (Kugelberg & Jones, 2007). Gas chromatography allows both a qualitative analysis by retention time, and a quantitative analysis using the area under the curve of the chromatographic peak, which gives it an advantage over older techniques (Seto, 1994).

Currently, the headspace extraction technique is preferred due to the minimal contamination produced to the injector and column of the gas chromatograph; this

technique for the determination of ethanol has been refined over time, to the extent that it is now possible to perform these tests quickly and accurately (Kugelberg & Jones, 2007; Musshoff, 2002).

To apply static headspace gas chromatography to ethanol determination, it is necessary to eliminate or minimize the effects of the matrix; to do so, several factors must be optimized, among which the most important is the calibration of the equipment. In the precise case of gas chromatography, internal standard calibration is recommended: in this type of calibration, a substance (internal standard) in the same concentration is added to all the samples and all the points of the calibration curve, the internal standard is a substance chemically similar to the substance to be analyzed but that does not exist in the sample, so that, in all chromatograms this substance must be identified with a similar intensity; any significant change in this signal will thus indicate errors in the process, and due to the fact that all the obtained signals are normalized according with internal signal standard, these errors can be corrected; in the particular case of the ethanol determination, substances such as n-propanol, t-butanol, i-propanol, i-butanol, etc. have been used as internal standards. It is recommended to use tertiary alcohols because in certain circumstances small amounts of n-propanol are produced during the putrefaction (Kugelberg & Jones, 2007; Musshoff, 2002; Seto, 1994). The determination of ethanol in the Forensic Medical Service of Mexico City is performed by static headspace gas chromatography with isobutanol as internal standard (table 2) with good results (figure 2).

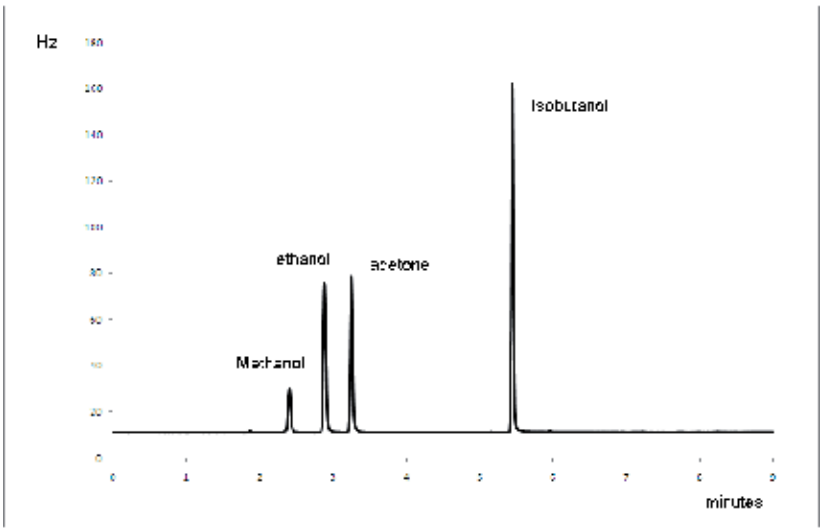


Fig. 2. Chromatogram of volatile substances determination with isobutanol 75 mg/dl as internal standard. Column DB-624, 30.0 m X 0.32 mm, Internal diameter (I.D.), film thickness 1.8 μm, carrier, nitrogen at 1.7 ml/min, Oven initial temperature 60°C for 4 minutes, ramp of 30°C/minute to 120°C final time 3 minutes, headspace temperature 60°C for 10 minutes. Ethanol 150 mg/dl, methanol, 75 mg/dl and acetone 30 mg/dl.

Another factor to consider is the temperature at which the exchange between the liquid and gaseous phase will be carried out and the time it will take; in this case one can choose between favoring the sensitivity of the method (using high temperatures) against its

| Specimen (ml) | Additives[1] | Temperature headspace (°C) | Time headspace (Min) | Injection headspace | Column | Temperature Oven (°C) | Carrier gas (ml/ min) | Reference |
|---|---|---|---|---|---|---|---|---|
| Blood (0.02) | n-propanol, (I.S.) Nitrite, fluoride | 60 | 3 | Manual | Porapack Q | 150 | Nitrogen, 30 | Wilkinson, et al,. 1975 |
| Blood (0.15) | n-propanol, (I.S.) dithionite, $(NH_4)_2SO_4$ | 60 | 30 | Automatic | Carbowax 20m on carbopack B, 1.8m X 2mm I.D. | 65 | Nitrogen , 30 | Christmore, et al., 1984 |
| Blood (0.2) | n-propanol, (I.S.) NaCl, | 20-40 | 30 | Automatic | Carbowax 1500 on carbopack C, 2mX 2mm I.D. | 125 | Nitrogen , 20 | Penton, 1985 |
| Several | n-propanol (I.S.) NaCl | 25 | 45 | Manual | Porapak S 2mX 2mm I.D. | 165 | Nitrogen , 45 | Watts & McDonald, 1987 |
| Blood (0.5) | n-propanol (I.S.) | 55 | 12 | Automatic | DB-1 & DB-WAX, 30 m | 45 | Helium, 75 | Brown & Long, 1988 |
| Blood (0.1) | n-propanol (I.S.) | 40 | 18 | Automatic | (1) Carbowax 1500 on carbopack C, (2) Carbowax 20M on Carbopack B, (3) Carbowax 20M on chromosorb B, 2mX 3mm I.D. all | 100 or 120 | Nitrogen, 20 | Jones & Schuberth, 1989 |
| Blood (1.0) | n-propanol (I.S.) | 30 | 45 | Manual | Porapack Q 2m | 160 | Ni, 54 | Senkowski & Thompson, 1990 |
| Several (0.1) | No | 75 | 30 | Automatic | HPFFAP, 5O mX0.2mm I.D. | 70 | Helium, 200kpa | Macchia, et al., 1995 |
| Blood (1.0) | Isobutanol (I.S.) | 55 | 15 | Automatic | Rtx-BAC2, 30mX 0.53 mm I.D. | -60-240 | Helium, 3.0 | Watanabe-Suzuki, et al., 1999 |
| Blood, vitreous fluid (0.2) | n-propanol, t-butanol (I.S.), NaCl | 65 | 8 | Automatic | Rtx-BAC1, RTX-BAC2, 30mX 0.32 mm I.D. | 50 | | Honey, et al., 2005 |
| Several | Isobutanol (I.S.),, nitrites | 60 | 10 | Automatic | DB-624, 30 mX 0.32 mm I.D. | 60-120 | Nitrogen, 1.7 | Suarez, et al., 2009 |

[1] I.S. Internal standard

Table 2. Headspace gas chromatography procedures for ethanol determination.

reproducibility, or the other way around, that is, to favor the reproducibility by applying low temperatures, even at room temperature. The reduction in reproducibility of the method is related to oxidation reactions suffered by ethanol in blood at temperatures above 40° C. To avoid such phenomenon, it is recommended to add sodium dithionite to the internal standard solution or applying low headspace temperatures (Christmore, et al., 1984; Musshoff, 2002; Seto, 1994).

Another factor that may decrease the reproducibility of the method is the difference between the amount of soluble proteins and ions present in different specimens of blood, as it alters the partition blood-air of alcohols; for example, ethanol and various volatile substances will not evaporate just the same in a sample of highly diluted blood, coming from a person who suffered a bleeding, than in a regular blood sample. To solve that problem, it is recommended to dilute the samples (usually with the solution of internal standard) in a relation of at least 1 to 5, but preferably of 1 to 10 (Kugelberg & Jones, 2007; Watts & McDonald, 1987). Finally, if it necessary to increase the sensitivity of the analysis, it can be done by adding a salt like sodium chloride, sodium nitrite, etc. (Christmore, et al., 1984), or by cryofocusing techniques (Watanabe-Suzuki, et al., 1999).

There are several columns for alcohol separation available on the market, that can be used for the chromatographic separation, but if it is necessary to determine non-polar volatile substances, it is advisable to choose a column that allows separating all the analytes of interest in a reasonable time; subsequent analyses in two or three chromatographic systems can be performed in order to confirm the obtained results, each one with different retention times for ethanol and internal standard; in some cases, it is even possible to perform a confirmation test with a different internal standard (Brown & Long, 1988; Jones & Schuberth, 1989), finally, FID is the chromatographic detector more commonly used for this kind of analyses. The chromatographic conditions most commonly used for the analysis of ethanol in biological samples are summarized in table 2.

The accuracy and reproducibility of the analysis performed in such a way is high, resulting in inter-laboratory coefficients of variation (CV) of 3% to 5%, and intra-laboratory CV of less than 1%, both with adequate sensitivity (LOD around 1 mg/dl) and high specificity (Jones & Schuberth, 1989; Jones, et al., 1992; Penton, 1985).

### 3.2 Congeners

Alcoholic beverages contain trace amounts of a wide variety of chemical substances, which are known collectively as congeners. The congener profile of a particular drink depends on the raw materials used in the fermentation process, such as the source of carbohydrates, whether derived from fruits, grape juice, grape mash, malted grain or barley (McAnalley, 2004). Others congeners might be introduced or removed during the distillation process, ageing and final storage of the beverage in special wooden cask; all these congeners helps to impart the special smell and taste of the final product (Iffland & Jones, 2003).

The qualitative and quantitative analysis of congeners have found several applications in forensic toxicology, the presence of those substances in abnormally high concentrations in blood could suggest deliberate criminal intent presumably by addition of these solvents to conventional alcoholic beverages or a failed distillation process. Otherwise, the person might have consumed accidentally denatured alcohol, which is not uncommon in alcoholics

(Jones, et al., 1989). In this particular case the analysis could be done with analytical conditions similar to the common used in ethanol analysis in blood.

Another application of alcoholic beverages congeners is as alcoholism markers (Musshof, 2002); the most associated volatile substances with the abuse of alcoholic beverages are methanol (Brinkmann, et al., 2000; Roine, et al., 1989; Suarez, et al., 2009) and the sum of acetone and isopropanol (Iffland, et al., 1988). Metabolism of methanol via liver alcohol dehydrogenase (ADH) is competitively inhibited by ethanol levels exceeding 20 mg/dl. Consequently, excessive and prolonged drinking results in high blood methanol levels, so methanol can be detected in blood, breath and urine for long after ethanol has returned to its endogenous concentration (Iffland & Jones, 2003; Majchrowicz & Mendelson, 1971; Musshof, 2002). On the basis of these findings methanol levels exceeding 1 mg/dl have been suggested as indicator of alcoholism, while 0.1 mg/dl can be considered a physiological level. On the other hand, join concentrations of acetone and isopropanol higher than 0.9 mg/dl are indicative of heavy drinking, normal levels of isopropanol are less of 0.01 mg/dl and 0.1-0.3 mg/dl for acetone (Iffland, et al., 1988; Iffland & Jones, 2003); this is the result of their reciprocal formation through the alcohol deshydrogenase system.

However, the specificity of isopropanol and acetone as alcoholism markers is low compared to methanol because both substances can be formed in some metabolic disorders or after strenuous exercise (Iffland & Jones, 2003; Musshof, 2002). Finally, has been suggested that these substances can be related to the severity of hangover (Bendtsen, et al., 1998; Calder, 1997; Pronko, et al., 1997).

The sensitivity of conventional headspace gas chromatography is sufficient for blood alcohol determinations down to 5 mg/dl, but for the detection of congeners as alcoholism markers LOD had to be improved to reach at least the physiological level of these substances several strategies could be used to achieve these, like adding a salt to enhanced the vaporization of the congeners (Bendtsen, et al., 1998; Suarez, et al., 2009) and the cryofocusing technique.

Another interesting forensic application of congener analysis is to evaluate claims of drinking after driving, This kind of defense arises frequently when the drunk driving suspect is not apprehended immediately after an vehicular incident, especially after hit and run incidents, in this situation, the congener profile can be used to identify the consumption of a particular kind of alcoholic beverage from the apprehended driver, comparing the profile present in the blood and urine of the driver with the known congener profile of the alcoholic beverage allegedly consumed after driving (Iffland & Jones, 2003).

The scientific basis of forensic congener analysis depends on the fact that drinks containing different amounts of various congeners produce different low molecular alcohols in blood and urine. Consequently, the qualitative and quantitative analysis of congeners in blood and urine can furnish useful information about the kind of alcoholic drink consumed and also the time of intake relative to the time of blood sampling (Iffland & Jones, 2003).

The main congeners of forensic interest are methanol, n-propanol, isobutanol, 2- butanol, and its metabolite methyl ethyl ketone and n- butanol, while isopropanol and acetone are endogenous substances always detected in blood and urine during a congener analysis by headspace gas chromatography. However, these substances are not ingredients of alcoholic beverages (Iffland & Jones, 2003).

Interpreting the results of congener analysis require to know the congener profile of the drinks consumed before and after driving as well as information about the disposition and fate of these substances in the body and the extent of any metabolic interaction with ethanol.

Congener analysis is a modification of the conventional headspace gas chromatography analysis for measuring blood ethanol concentration: However, the sensitivity of the method must be increased to allow measuring much lower concentrations (Iffland & Jones, 2003) (Table 3).

| Congener | Blood concentrations (mg/dl) |
|---|---|
| Methanol | 0.05-5 |
| n-propanol | 0.005- 0.3 |
| Isobutanol, n- butanol, 2-butanol and methyl ethyl ketone | 0.002-0.2 |

Table 3. Congener concentrations in blood.

Besides the conditions of the gas chromatographic analysis, the detections limits depend on the particular congener, its molecular weight, the amount of organic matrix in the biological material and the vapor pressure of the water in the sample.

Congener analysis require to enhanced the sensibility, usually by the addition of a salt, usually anhydrous sodium sulfate, various ways of sample pretreatment are used like ultrasonic disintegration prior ultrafiltration, low temperature vacuum distillation, ultracentrifugation, multiple headspace extraction or microdestillation. Headspace gas chromatography is performed with an internal standard calibration, usually with t-butanol as the internal standard and if necessary with a liquid nitrogen freeze trap (cryofocusing) to concentrate the sample, LOD usually achieved are about 0.01 mg/dl for methanol and about 0.001-0.002 mg/dl for n-propanol or isobutanol, Both FID and MSD have been used for the gas chromatography detection. An example of headspace gas chromatography conditions is shown (Iffland & Jones, 2003) (table 4).

| Additives[1] | Temperature headspace (°C) | Time headspace (Min) | Injection headspace | Column | Temperature Oven (°C) |
|---|---|---|---|---|---|
| t-butanol (I.S.), anhydrous sodium sulfate | 70 | 12 | Automatic | Carbowax 20M on Carbopack B, 2mX 2mm I.D. | 60-100 |

[1] I.S. Internal standard

Table 4. Headpace chromatographic conditions for congener analysis.

Finally, the successful application of congener analysis in casework requires considerable experience not only regarding laboratory analysis but also controlled drinking experiments. Studies of this kind furnish the information needed about the pharmacokinetics of specific congener, their interactions with ethanol metabolism and urine/blood relationships. Consequently, much basic research is necessary before embarking in actual casework, this expertise, at the present moment is available at only a few institutes of legal medicine on Germany.

### 3.3 Inhalants

Inhalants are a diverse group of volatile substances that may be inhaled accidentally or intentionally. Inhalants can be solids and liquids as well as gases. Their common feature is volatility, the property of being or being able to be converted to a form, susceptible to inhalation. Compounds having this property include aliphatic hydrocarbons (butane, hexane, propane, etc.); aromatic hydrocarbons (benzene, toluene, etc.); mixed hydrocarbons (gasoline, lighter fluid, etc.); halogenated hydrocarbons (chloroform, dichloromethane, etc.); chlorofluorcarbons (Freon 11, Freon 12, etc.) and oxygen containing compounds (acetone, nitrous oxide, etc.). Gaseous anesthetics will be studied in detail in the next section (Broussard, 2003).

Inhalants are present in many commercial products (solvents, glue, typewriter correction fluid, gasoline, lighter fluid, refrigerants, propellants for aerosol, etc.), consequently, inhalants are easily available and cheap, these circumstances, have contributed to its use as a drug of abuse, above all, in adolescents. Its use has risen in the last years (Hansen & Rose, 1995), despite legislation that limit its accessibility and to make their use by adolescent illegal. Worldwide, inhalants are one of the most dangerous classes of abused substances and one that is responsible of more deaths annually, that other drugs of abuse (Broussard, 2003).

The best sample for inhalants detection is blood, but urine can be used to detect metabolites, proper collection involves the use of glass tubes with minimal headspace remaining after collection. Volatile organic compounds by definition are highly evaporative, and analytes can be easily lost while samples or standards are being manipulated or stored; on the other hand, because, many of these substances are commonly found in laboratories, it's reasonable that contamination might occur during sample collection or analysis (Ashley, et al., 1996). Specimens should be stored between -5 to 4°C, it's recommended to add sodium fluoride as conservative, under these circumstances samples can be stored for up to forty days (Ashley, et al., 1996). Because of the tolerance that these substances produced, there is not a correlation between the blood inhalants concentrations and the clinical features of toxicity for any of these compounds. Table 5 lists concentration of inhalants found, usually, in postmortem cases (Baselt, 2004).

| Inhalant | Fatal Concentrations in blood in mg/dl (Baselt, 2004) | Basal concentrations in blood in ng/dl (Ashley, et al. 1996). |
|---|---|---|
| Benzene | Average, 3.6 | 13.0 |
| Toluene | Average, 2.2 | 52.0 |
| Propane | 1.1 and 110 | ---------- |
| Chloroform | Average, 6.4 | ---------- |
| Cresols | Average, 13.3 | ---------- |
| Dichloromethane | Average, 29.5 | ---------- |
| Diethyl ether | Range 9-375 | ---------- |
| Ethyl chloride | 42.3 | ---------- |
| Nitrous oxide | Average, 10.0 | ---------- |
| Tetrachloroethylene | Range 0.45-11.5 | 19.0 |
| Trichloroethane | Average, 12.6 | 34.0 |

Table 5. Toxic and background concentrations of some inhalants in blood

| Inhalant | Additives[1] | Temperature headspace (°C) | Time headspace (Min) | Injection headspace | Column | Temperature Oven (°C) | Carrier gas (ml/ min) | Detection | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Freon 11 | $CH_2CL_2$ (I.S.) | 40 | 30 | Manual | HP-5, 12m X0.2 mm I.D. | 30 | Helium, (1 psi) | MSD | Groppi, et al, 1994 |
| Several | Propanol (I.S.), NaCl | 60 | 3 | Automatic | (1) THEED on Carbopack B (2) and Carbowax 20 M on Carbopack B, 2m both | 67 or 73 | Nitrogen, 30 | FID | Logan, et al., 1994 |
| $CH_2Cl_2$, $CHCl_3$ | NaCl, Sec-butanol (I.S.) | 55 | 20 | Manual | DB-5, 30 mX 0.25 mm I.D. | 70–180 | Helium, (4 psi) | MSD | Kim, et al, 1996 |
| Difluro ethane | Propanol (I.S.) | 37 | 15 | Manual | RTX-BAC1 30 mX 0.32 mm I.D. | 65 | Helium, 2 | FID | Broussard, et al, 1997 |
| Several | NaCl | 80 | 26 | Automatic | DB-1, 30mX 0.25mmI.D. | 40-250 | Helium, 18 | MSD | Schuberth. 1997 |
| $C_2Cl_4$ | $C_2HCl_3$ | 60 | 10 | Manual | DB-5, 15 mX 0.25mm I.D. mm Carbowax on carbopack 2m x 0.32 mm I.D. | 75 or 110 | Helium, Argon 10% methane, 30 | MSD, ECD | Isenschmid, et al, 1998 |
| Ethyl chloride | Propanol (I.S.), NaCl | 37 | 5 | Manual | Innowax, 15mX0.25 mm I.D. | 50 | Helium, 1.05 | FID | Broussard, et al, 2000 |
| Toluene | citrate, iso-butanol (I.S.) | 60 | 20 | Automatic | Innowax, 30mX0.25 mm I.D. | 60-140 | Helium, 1.0 | FID | Kim, et al, 2000 |
| > 50 volatiles | t-butanol (I.S.), nitrite, fluoride | 70 | 30 | Automatic | DB1 and DB-WAX (Both 30mX0.25 mm I.D.), DB-624, 30mX0.53 mm I.D | 40-150 or 45-90 | Helium | FID, MSD | Sharp, 2001 |
| $CH_2Cl_2$ | No | 60 | 20 | Automatic | Carbowax 20 M on Carbopack B 2mX 2mm I.D. | 90 | Helium, 30 | FID | Zarrabeitia, et al, 2001 |
| Alkanes | No | 80 | 20 | Automatic | CB-624, 30mX0.25 mm I.D | 35-150 | Helium, (10 psi) | MSD | Gaulier, et al. 2003 |
| $CHCl_3$ | Trichloro ethane (I.S.) | 60 | | Automatic | RTX-BAC1 30m. | 60-150 | ---------- | FID | Singer & Jones, 2006 |
| MAPP | | 40 | 15 | Automatic | RTX-BAC1 30 mX 0.53 mm I.D. | 40 | ---------- | FID | Avella & Lehrer, 2004 |
| $CHCl_3$ | Butanol (I.S.) | 90 | 12 | Automatic | Poraplot Q, 25 mX 0.25 mm I.D. | 50-230 | Helium, 1.0 | MSD | Gaillard, et al. 2006 |

[1] I.S. Internal standard

Table 6. Headspace gas chromatography procedures for inhalants determination.

The analysis of inhalants has been performed for the diagnosis of solvent abuse and monitoring of industrial exposure; In the first case, the determination of the unchanged inhalant in blood is preferred, but in some cases, (trichloroethylene inhalation) metabolites (trichloroethanol and trichloroacetic acid) can be searched in urine; for biological monitoring, the determination of inhalants in blood is preferred too, but the determination of metabolites in urine is commonly done. The technique of choice to perform inhalants analysis is headpace gas chromatography with FID, electron capture detector (ECD) and MSD. FID provides a good linear range detection, while MSD unequivocal identification. ECD is only recommended in trace analysis for halogenated hydrocarbons. Both packed columns such as carbowax and capillary columns such as DB-1 have been used to separate and quantify volatiles. Usually, the headspace gas chromatography method to analyze ethanol in blood can be modified for a preliminary screening before a more specialized and extensive method is performed (Sharp & Dautbegovic, 2001; Sharp, 2001). These procedures have typical LOD´s of 0.01 mg/dl and typical linear ranges to 5 to 10 mg/dl (Broussard, 2003). Analytical conditions of some procedures are given in table 6.

### 3.4 Anesthetics

Within the group of inhalants, fluorinated inhalants stand out; that is because they can be potentially dangerous, as their lethal doses are only two or four time their therapeutic doses. Careful handling of anesthesia during surgical procedures has succeeded in reducing morbidity and mortality due to overdose, and usually the case of death in surgical procedures is some other reason. However, the analysis of these substances in forensic environments is justified on any case of death during an operation, and due to their use as drugs of abuse, the availability of fluorinated anesthetics is limited to hospital personnel or those engaged in its manufacturing and distribution. At clinics, the determination of fluorinated anesthetics is used for monitoring patients under anesthesia and medical personnel, but under these circumstances the concentrations detected are below those that cause acute poisoning (Pihlainen & Ojanperä, 1998).

Currently, the most used fluorinated anesthetics are halothane, enflurane, isoflurane, sevoflurane and desflurane; isoflurane stands out as the most popular at present times.

These substances are metabolized in the liver and to a lesser extent in the kidneys and lungs. Metabolism is higher with halothane (20-46%), followed by enflurane (2.4-8.5%), sevoflurane (2.5-3.3%), isoflurane (0.2%) and desflurane (0.02%). So their determinations are intended to find drugs, not metabolites. The following concentrations in blood have been found in deaths related with these substances (Table 7).

| Anesthetic | Detected concentration (mg/L) |
|---|---|
| Halothane | 45-650 |
| Enflurane | 130-710 |
| Sevoflurane | 26 |
| Isoflurane | 9.9-48 |

Table 7. Blood concentration in deaths related to fluorinated anesthetics (Baselt, 2004)

Gas chromatography is the technique of choice for the analysis of these substances, column selection is handicapped by the possibility of analyzing other gases used in anesthesia with it, in which case columns with solid adsorbents are to be used; otherwise, capillary columns with silicon phases can be used, detectors such as FID performed well to these compounds, but if it is necessary to decrease LOD of the method, some other, more sensitive detectors can be used, such as ECD (Pihlainen & Ojanperä, 1998; Uyanik, 1997), in order to obtain a structural identification, MSD or infrared detector (IRD) has been used, and the most commonly used preparation technique is static headspace injection. (Pihlainen & Ojanperä, 1998), due to the fact that these compounds are easily transferred to headspace gas, according to blood/gas partition constants. The relation gaseous phase/aqueous phase normally used in the vial is of 5-20, and the sample volume is usually 10% of the gaseous phase; internal standard calibration has been used, with various substances and deuterated standards. The sensitivity of the method can also be improved by increasing the extraction temperature, by decreasing the gaseous phase or through salting techniques (table 8). It is important to avoid losses by evaporation and contamination of the samples throughout the whole analytical procedure. In the analyses of these substances, the chromatographic conditions employed are very similar to those used for the determination of alcohol and inhalants; therefore, it is possible to design a method that can simultaneously analyze these substances (Kovatsi, et al., 2011); our laboratory identified the presence of sevoflurane with the method used to determine alcohol, without changing the chromatographic conditions whatsoever.

| Additives[1] | Temperature headspace (°C) | Time headspace (Min) | Injection headspace | Column | Temperature Oven (°C) | Carrier gas (ml/min) | Detection | Figures of Merit[2] | References |
|---|---|---|---|---|---|---|---|---|---|
| Enflurane, (I.S.) | 75 | 30 | automatic | Carbowax 1500 on carbopack C, 2m | 100 | ---------- | FID | --------- | Kulhman, et al. 1993 |
| $CH_2Cl_2$ (I.S.) Tween | 55 | 15 | automatic | DB1 30m X0.53mm I.D. | 60 | Helium, 15 | MSD | --------- | Saito, et al. 1995 |
| Enflurane, (I.S.) | 41 | 120 | automatic | Poraplot Q 27m X0.25mm I.D. | 40-140 | Helium, 1.0 | MSD | RSD[3]: < 8.06%, RSD[4]: < 6.19% | Accorsi, et al. 2003 |
| n-propanol, (I.S.) | 25 | 60 | Manual | Carbowax 1500 on carbopack C, 2m. X 2mm I.D. | 100 | Helium, 20 | FID | CV[3]: 13.7% CV[4]: 3.3% | Burrows, et al. 2004 |
| Aceto-nitrile, (I.S.),NaCl | 60 | 30 | automatic | Supelcowax 10, 30mX 0.25mm I.D. | 42-100 | Helium, 1.2 | FID | LOD: 1.7 mg/dl, RSD[3]:1.3-14.6% | Kovatsi, et al. 2011 |

[1]I.S. Internal standard; [2]RSD Relative standard deviation; [3]Interday; [4]Intraday

Table 8. Headspace gas chromatography procedures for fluorinated inhalation anesthetics determination

### 3.5 Carbon monoxide

Carbon monoxide gas is colorless, odorless, tasteless, and is produced by incomplete combustion of organic matter. Carbon monoxide is involved each year in a significant number of deaths around the world, be they accidental or voluntary; the main sources of exposure to carbon monoxide are the exhausts of internal combustion engines, cigarette smoke, heating systems in poor conditions and fires; there can also be endogenous production related to dihalomethane metabolism and heme catabolism (Kunsman & Levine, 2003). Carbon monoxide exerts its toxic effect because it has approximately 220 times greater affinity to hemoglobin than oxygen, therefore, prevents the transport of oxygen to the tissues, and at the same time changes the allosteric structure of hemoglobin, increasing its affinity with oxygen, this way preventing the exchange of oxygen in the tissues (Walch et al., 2010).

The compound formed by carbon monoxide and the reduced form of hemoglobin, carboxyhemoglobin, is therefore a measure of carbon monoxide poisoning, and its determination is performed in all forensic toxicology laboratories in the world on a routine basis. While carboxyhemoglobin saturation levels greater than 50% are indicative of carbon monoxide poisoning as a cause of death, levels between 10% and 50% are indicative of exposure to this toxic and can cause various symptoms.

Different methods have been developed to determine the percentage of carboxyhemoglobin saturation in blood. These methods are based on colorimetry, infrared spectrophotometry, visible ultraviolet spectrophotometry and gas chromatography (Boumba & Vougiouklakis, 2005). Currently, the most widely used methods are those based on ultraviolet-visible spectrophotometry, utilizing conventional spectrophotometers, usually making two readings at two different wavelengths (Maehly, 1962) or by specialized spectrophotometers: oximeters (Mahoney, et al., 1993); these methods are fast and simple, however, they are unreliable in certain circumstances, such as in putrefied samples, where the putrefaction process produces substances that generate spectral interference or the spontaneous formation of methemoglobin and sulfhemoglobin, this way preventing the determination of carboxyhemoglobin, or in blood samples from fire deaths, where methemoglobin is spontaneously produced (Lewis, et al., 2004; Seto, 1994; Walch, et al., 1984). In this regard, static headspace gas chromatography is not affected by these circumstances, because this technique separates the carbon monoxide from blood, and is thus considered a highly specific and sensitive technique, which makes it the referential technique for determining carbon monoxide (Boumba & Vougiouklakis, 2005); however, static headspace gas chromatography has not been usually used for this type of analyses because it takes longer than established techniques and requires skilled personnel (Mahoney, et al., 1993). Nonetheless, that last disadvantage does not apply to forensic environments, where this technique is widely known.

Carbon monoxide determination by static headspace gas chromatography involves mixing the blood with a substance that lyses erythrocytes and releases the carbon monoxide of blood, either by adding an acid (denatures hemoglobin) or ferrocyanide potassium (oxidizes hemoglobin to methemoglobin); in some cases, a reducing agent can also be added to ensure the methahemoglobin that may be present in the sample is reduced to hemoglobin, which is

important in samples from aviation accidents (Lewis, et al., 2004; Walch, et al., 1984). The gas released into the headspace is analyzed by gas chromatography using different detectors. The detection has been carried out using a FID, after a catalytic reduction of carbon monoxide to methane (Cardeal, et al., 1993; Czogala & Goniewicz, 2005; Walch et al., 2010), a thermal conductive detector (TCD) (Lewis, et al., 2004; Van Dam & Daenens, 1994), by the release of mercury vapor resulting from the combination of carbon monoxide with mercuric oxide (Vreman, et al., 1984) and by MSD (Oritani et al., 2000). The separation is usually done through molecular sieve columns.

In order to determine the percentage of carboxyhemoglobin saturation in blood, it is necessary to quantify the total amount of hemoglobin and a calibration curve must be prepared; to do so, different approximations are used. The most commonly used, parts from a blood sample to which carbon monoxide is passed until the carboxyhemoglobin saturation reaches 100%, the other points of the calibration being obtained by diluting this blood in a blood sample with 0% carboxyhemoglobin, passing oxygen to it (Canfield, et al., 1998). Due to the complexity of the above mentioned procedure, other calibration forms have been implemented, using certified gas standards (Czogala & Goniewicz, 2005) or the stoichiometric liberation of carbon monoxide from the reaction between formic acid and hot sulphuric acid (Cardeal, et al., 1993). Detection limits obtained by this technique are less than 0.1% of carboxyhemoglobin saturation in blood, which is much lower than the normal levels reported (Kunsman & Levine, 2003) and less than the detection limits of the spectrophotometric techniques, reported in 1% (Boumba & Vougiouklakis, 2005). Table 9 describes in detail, the chromatographic conditions for carbon monoxide determination by this technique.

In conclusion, the determination of carbon monoxide by static headspace gas chromatography is a very specific method, as it is not influenced by sample conditions; besides, it has an accuracy and sensitivity which surpasses other, more conventional techniques. However, the fact that it is time-consuming, expensive, requiring of excessive sample handling, and, above anything else, the need to measure total hemoglobin and preparing standards (Canfield, et al., 1998), has prevented this technique from being used on a routine basis in most forensic toxicology laboratories.

### 3.6 Cyanide

Cyanide is a potent, fast-action toxin, which effects by reacting with trivalent iron of cytochrome oxidase, inhibiting the respiratory system, resulting in a quick deterioration of vital functions. Blood concentrations of 2 to 3 ug/ml are already considered not compatible with life, while basal levels of cyanide in non smoker's blood of 16 ng/ml have been detected (Baselt, 2004). Cyanide exposure is relatively common; it is known for being ingested with suicidal intentions or homicidal purposes; but accidental intoxications in fires have also been reported, where the pyrolysis of nitrogen-containing synthetic materials, such as polyurethane and polyacrylonitrile produces hydrogen cyanide, exceeding the mortal concentration in some cases (Seto, et al., 1993; Moriya & Hashimoto, 2001), so even if the most common causes of death in fires are burns, injuries and carbon monoxide intoxication, hydrogen cyanide can also be involved. Concentrations above the normal levels have been detected in smokers, but the toxic effects of these concentrations are yet to be fully clarified; other sources of cyanide exposure are cyanogenic glycosides, present in

| Additives[1] | Temperature[2] headspace (°C) | Time headspace (Min) | Injection headspace | Column | Temperature Oven (°C) | Carrier gas (ml/ min) | Detection | Figures of Merit | Reference |
|---|---|---|---|---|---|---|---|---|---|
| $K_3Fe(CN)_6$, saponin, phosphates | 0 | 30 | automatic | Molecular sieve 5A, 90cmX0.53 cm I.D. | 110 | Air, 50 | Mercury vapor | LOD: 0.005%. $CV^3$:<13%, $CV^4$:<10% | Vreman, et al, 1984 |
| $H_3PO_4$, n-octanol | T.A. | 70 | automatic | Porapak Q, 3 mX0.9 mm I.D. | 80 | Nitrogen, 30 | FID | ---------- | Cardeal, et al, 1993 |
| $H_2SO_4$, saponin | T.A. | 40 | Manual | Molsieve 5A, PLOT, 25mX0.32 mm I.D | 80 | Helium, 3.0 | TCD | LOD: <0.02% $CV^3$:13.7%, $CV^4$:3.3% | Van Dam & Daenens, 1994 |
| $K_3Fe(CN)_6$, t-butanol | | | automatic | DB-624 60mX 0.32mm I.D. | 40-80 | Helium, 28 cm/ s | MSD | ---------- | Oritani, et al, 2000 |
| Dithionite, $H_2SO_4$, saponin | T.A. | 40 | automatic | Molsieve 5A 20mX0.32 mm I.D. | 120 | Helium, 35 psi | TCD | CV:< 10% | Lewis, et al, 2004 |
| $K_3Fe(CN)_6$ | T.A. | 30 | automatic | Molsieve 5A, PLOT 30mX0.53 mm I.D. | 30 | Helium, 3 | FID | ---------- | Czogala & Goniewicz, 2005 |
| $H_2SO_4$, saponin | 50 | 30 | automatic | Molsieve 5A, PLOT 50mX0.53 mm I.D. | 80-300 | Helium, 12 | FID | $CV^3$:<11.0%, $CV^4$:<5.0% | Walch, et al, 2010 |
| $HNO_3$, | 60 | 30 | Automatic | Molsieve 5A, 25mX0.53 mm I.D. | 80 | | FID | ---------- | Felby, 2009 |

[1] I.S. Internal standard; [2]T.A. Ambient temperature; [3]Interday; [4]Intraday

Table 9. Headspace gas chromatography procedures for carbon monoxide determination.

untoasted almonds, bay leaves, apple seeds, etc. Cyanide exposure also results from its use as fumigant, as a metabolite of sodium nitroprusside and in chemical industry, where cyanide has found applications in the metallurgical, oil, photographic and plastic industries (Kunsman & Levine, 2003). Because of this and the presence of basal cyanide levels, it is necessary to count with methods to quantitatively determine cyanide in biological samples.

Traditionally, this determination is carried out by spectrophotometric techniques, preceded by a distillation or microdiffusion pretreatment (Seto, 1994); the technique of gas chromatography with headspace injection provides a faster analysis, susceptible to be automated, with high sensitivity, good recoveries (around 90%) and specific detection The procedure involves the release of cyanide from the biological sample in the form of hydrogen cyanide by the addition of an acid, usually into the previously sealed vial, through the septum; different acids have been used, such as phosphoric acid, sulfuric acid, nitric acid or acetic acid, although the most used is phosphoric acid, as it does not form any blood clots; in the reaction mix within the vial, acetonitrile is usually added when internal standard calibration is used (Calafat & Stanfill, 2002; Moriya & Hashimoto, 2001), and ascorbic or acetic acid, with the purpose of avoiding the production of cyanide in blood by action of its metabolite, thiocyanate, which could result in problems with samples by smokers or with chronic intoxications (Seto, 1996); heating times are usually longer than half an hour and temperature below 63°C are recommended in order to prevent the formation of cyanide from blood. Regarding the detection by gas chromatography, due to the small amounts of cyanide to determine and its relatively high partition coefficient, it is necessary to use more sensitive detectors; pertaining this, two strategies are commonly followed, in the first one, cyanide is detected in the gas chromatograph using a nitrogen-phosphorus detector (NPD) (McAuley & Reiver, 1983; Moriya & Hashimoto, 2001; Seto et al., 1993), or cyanide can be detected as cyanogen chloride after a derivatization with chloramin-T using a ECD (Felby, 2009; Odoul, et al., 1994) but a MSD with an isotope as internal standard has also been used (Dumas, et al., 2005). Finally, it is important to consider the rapid disappearance of cyanide from the blood, which was calculated is up to 30% on a day, in refrigerated blood at 4°C (Calafat & Stanfill, 2002), so it is necessary to perform this analysis as soon as possible in order to obtain meaningful results. Table 10 describes the most important chromatographic parameters.

### 3.7 Ketone bodies

Endogenous volatile metabolites are normal by products of intermediate metabolites. Analysis of these metabolites is important in the diagnosis of certain disease states. An example of headspace gas chromatography analysis applied to these metabolites is the determination of ketone bodies (Seto, 1994). Ketone bodies are present in biochemical altered states known as diabetic ketoacidosis, related to diabetics and alcoholic ketoacidosis, a consequence of chronic abuse of alcohol. Studies (Iten & Meier, 2000; Pounder, et al., 1998; Thomsen, et al., 1995) indicate that alcoholic ketoacidosis could be the cause of death in alcoholics, in cases of sudden and unexpected death, where alcohol determination was negative and that an alcoholic ketoacidosis state can be diagnosed with the determination of ketone bodies in postmortem blood samples. Ketone bodies (acetone, acetoacetate and β-hydroxybutyrate) can be measured separately by gas chromatography headspace. Acetone are determined at a headspace temperature below 60°C to avoid decarboxilation of

| Additives[1] | Temperature[2] headspace (°C) | Time headspace (Min) | Inyección headspace | Column | Temperature Oven (°C) | Carrier gas (ml/ min) | Detection | Figures of Merit[3] | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 1-octanol, acétic acid | T.A | 30 | Automatic | Porapak Q, 180cmX2mm I.D. | 110 | Nitrogen, 20 | NPD | LOD 0.05 mg/ L, CV[5]:3.3% | McAuley & Reive, 1983 |
| $H_3PO_4$ | 50 | 30 | Manual | GS-Q, 30mX0.53 mm I.D. | 90 | Helium, 4.7 | NPD | LOD 0.1 µg/ L RSD[5]:6.1% | Seto, et al., 1993 |
| $H_3PO_4$ | 50 | 30 | Manual | GS-Q, 30mX0.53 mm I.D. | 90 | Helium, 4.7 | FID | LOD 0.02 mg/ L RSD[5]:3.3% | Seto, et al., 1993 |
| $H_3PO_4$ | 55 | 60 | Automatic | CP-Sil 8B 50m X 0.23 mm I.D. | 60 | Helium, 2.0 | ECD | LOQ: 0.1 mg/ L RSD[5]:1-8% | Odoul, et al., 1994 |
| $H_3PO_4$, ascorbic acid I.S. | 70 | 15 | Manual | SupelQ Plot 30mX 0.32 mm I.D. | -30-160 | Helium, 3.0 | NPD | LOD: 2 µg/ L RSD[5]:12% | Ishii, et al., 1998 |
| $H_3PO_4$, I.S. | 55 | 15 | Manual | GS-Q, 30mX0.53 mm I.D. | 100 to 140 | Helium | NPD | ---------- | Moriya & Hashimoto, 2001 |
| $H_3PO_4$, ascorbic acid, I.S. | 60 | 5 | Automatic | HP-PLOT Q, 15 m X 0.32mm I.D. | 30 to 190 | Helium, 3.0 | NPD | LOD 13.8 mg/ L, CV[5]:16% | Calafat & Stanfill, 2002 |
| $H_3PO_4$, ascorbic acid | 50 | 30 | Manual | HP-PLOT Q, 30mX0.53 mm I.D. | 170 | Helium, 5.0 | NPD | LOD: 0.7 µg/ L, CV[5]:3.4-5.3% | Shibata, et al., 2004 |
| $H_3PO_4$, ascorbic acid | 60 | 15 | Manual | GS-GASPRO, 30m X 0.32 mm I.D. | 40-250 | Helium, | MSD | LOD: 0.3 µmol/ L CV[4]:3.9%, CV[5]:4.4% | Dumas, et al. 2005 |
| $HNO_3$, | 60 | 30 | Automatic | CPSIL-19 CB, 50m X 0.32 mm I.D. | 80 | Nitrogen, 20 psi | ECD | LOD: 0.01 mg/ L CV 3.3-7.2% | Felby, 2009 |

[1]I.S. Internal standard; [2]T.A. Ambient temperature; [3]LOQ, limit of quantitation, RSD Relative standard deviation; [4]Interday; [5]Intraday

Table 10. Headspace gas chromatography procedures for cyanide determination.

acetoacetate or in the presence of potassium hydroxide (Felby & Nielsen, 1994; Seto, 1994); for acetoacetate determination, headspace temperature is set at 100°C to promote acetoacetate decarboxylation to acetone and β-hydroxybutyrate levels are determined after oxidative conversion into acetone with potassium dichromate (Seto, 1994) or enzymatic reduction to acetoacetate by β-hydroxybutyrate hydrogenase and then thermal conversion to acetone (Felby & Nielsen, 1994). The most used sample to performed this test is blood but it was found a good correlation between blood:spinal fluid and blood:viteous humor ketone bodies concentration, consequently, spinal fluid and vitreous humor can be used as an alternative specimens in this analysis (Felby, et al., 2007).

### 3.8 Other applications

Finally, there are analyses within forensic science, where static headspace gas chromatography can be used as a part of a more complex analysis; as fire debris analysis (Ren & Bertsch, 1999; Sandercock, 2008), where the static headspace analysis provides complementary information to that provided by dynamic headspace techniques, for the determination of amphetamines and methamphetamines by the addition of potassium carbonate to transform the amine of the stimulant in its unprotonated, volatile form (Seto, 1994), and more recently, the study to substances produced during corpse decomposition (Statheropoulos, et al., 2005; Swann, et al., 2010), where different types of separation techniques are used to characterize the compounds produced in the decomposition and determine how they are produced.

## 4. Conclusions

Static headspace gas chromatography is a mature and reliable technique; it is considered the technique of choice for the analysis of ethanol in biological samples, and is therefore present in the vast majority of forensic laboratories around the world with the qualified personnel to operate it; however, the applicability of this technique is not limited to this test and can be used for the analysis of various substances with minimal modifications, providing proper calibration and proper handling of matrix effects, excellent validation parameters, along with a clean injection. So, with this technique, various substances can be analyzed without the need of additional methods, and that would allow forensic laboratories to expand the number of cases they can take care of, with a minimal investment.

To accomplish that, it is necessary to know the fundamentals of this technique, the different chemical and physical phenomena involved, and the potential occurrences in the analysis of a particular substance, in order to develop a method with the required sensitivity, specificity and reproducibility.

In this respect, specialized methods for the analysis of substances have been developed, such as the determination of ethanol, cyanide or carbon monoxide; but other methods can also be designed, methods capable of analyzing large numbers of volatile substances (Sharp, 2001), or systems designed for specific forensic situations, such as the analysis of toxic gases produced during a fire (Felby, 2009), the determination of ethanol in drivers (Jones & Schubert, 1989), the diagnosis of alcoholic ketoacidosis (Felby & Nielsen, 1994), etc. In some of its applications, static headspace gas chromatography faces competition from SPME (Furton, et al., 2000; Snow, 2000), a more versatile method which has begun to substitute

older methodologies in some applications. However, the broad acceptance of static headspace gas chromatography for ethanol determination has consequently caused for such equipment to be present in most forensic laboratories, which can also be used for analyzing other volatile substances in a reliable way, without investing in other technologies.

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# 11

# A Method Using Gas Chromatography – Mass Spectrometry for the Detection of Mycotoxins from Trichothecene Groups A and B in Grains

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## 1. Introduction

Mycotoxins are toxic secondary metabolites produced by several fungi species growing on many agricultural commodities and processed foods (Bennett & Klich, 2003). The term secondary metabolite designates compounds that are not indispensable for the growth and metabolism of the fungi as such, and in contrast to primary metabolites, such as amino acids, fatty acids, saccharides, nucleic acids and proteins, are not essential for living. These metabolites are generally believed to be produced in response to stress or altered conditions of fungal growth related to a variety of plant and environmental factors. Fungal growth requires a readily available carbohydrate source, adequate moisture, oxygen, and appropriate temperatures (often 15-25°C). Plant or fungal stressors such as drought, high ambient temperatures, insect damage, mechanical harvest damage and reduced plant vigour predispose crop plants to infestation by fungi with subsequent production of secondary metabolites (Osweiler, 1999).

Even if there is no generally accepted theory about the biological benefits of mycotoxin production, mycotoxins have been proposed to provide the following functions to fungal development: (1) ecological role in nature, such as substrate competition with bacteria; (2) regulatory role in metabolism; and (3) regulatory role in, or at least coincident with, differentiation (Miller, 2002).

Since toxinogenic fungi are cosmopolitan, mycotoxins are environmental pollutants present in virtually all parts of the world. More than 300 chemically different mycotoxins formed by more than 350 fungal species and causing various diseases (mycotoxicoses) to living organisms are described (Stein, 1998). Moreover, it can be realistically assumed that further mycotoxins are likely to be discovered. Since only a few mycotoxins have been related to important food and feed borne diseases, the potential impact on human and animal health of many of them remains to be elucidated.

The presence of a toxinogenic fungus in a food or feed commodity gives indication of the potential hazard, but definitive conclusions can be made only by the actual identification of the specific toxin, since the presence of a fungus itself provides no evidence that it is a

producer of the toxin. Moreover, a given toxin may persist in a substrate when the fungus that has produced it is no longer present; or a given fungus may be capable of producing more than one toxin and finally, a given toxin may be produced by different genera of fungi (Fink-Gremmels, 1999).

Mycotoxins exhibit variable resistance to food processing such as cleaning and milling of cereals, bread- or pasta-making, roasting of nuts, production of vegetable oils, alcoholic fermentation, cooking and storage of meat, processing of milk to cheese, butter or yogurt. However, they are in general stable and capable of persisting in the final products (Bennett and Klich, 2003).

The mycotoxins that more frequently pose health risks to human and livestock are aflatoxins, ochratoxin A, trichothecenes, fumonisins, and zearalenone. Focusing on human and animals, biological effects, which may vary greatly among species, comprise hepatotoxicity, nephrotoxicity, neurotoxicity, immunosuppressive activity, teratogenicity, mutagenicity, genotoxicity, carcinogenicity, oestrogenicity, and diabetogenicity (Betina, 1989). Moreover, co-occurrence of different mycotoxins may lead to their interactions. Depending on the concentration and time of exposure to a mycotoxin and on the individual factors such as age, sex, diet or overall condition, clinical signs of mycotoxicoses may vary greatly among species (Grenier & Oswald, 2011).

Trichothecenes constitute a mycotoxin family produced by fungal species from several genera, notably *Fusarium, Stachybotrys, Myrothecium, Trichothecium, Trichoderma, Cylindrocarpon, Verticimonosporium, Acremonium,* and *Phomopsis*. Most of the trichothecenes that have been isolated and characterized chemically are from *Fusarium* species that grow in the field and are distributed worldwide, representing the important pathogens of grains and other food and feed plants. Trichothecenes appear as natural contaminants in cereal grains such as wheat, barley, oat, maize, rice, and derived products, such as bread, malt and beer (Scott, 1989).

There are more than 170 known trichothecenes which can be classified in four categories (A, B, C, and D) according to their chemical structure (Krska et al., 2001). All trichothecene mycotoxins have a basic tetracyclic sesquiterpene structure with a six-membered oxygen containing ring, an epoxide group on the 12,13 position, and an olefinic bond on the 9,10 position (Figure 1).

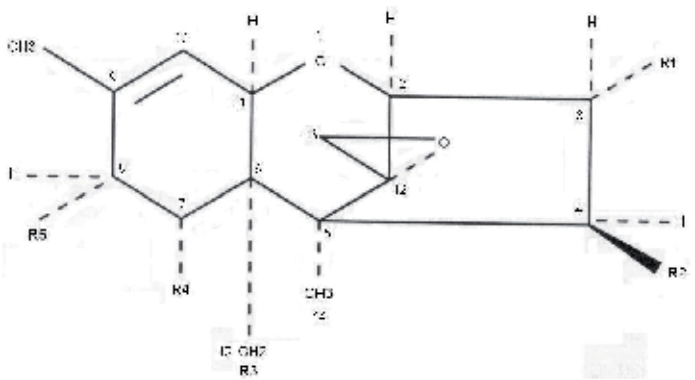


Fig. 1. Chemical structure of trichothecenes.

The trichothecenes may also have side groups as hydroxyl, esterified hydroxyl, keto, or epoxide groups in various combinations. Type-A trichothecenes have a functional group other than a carbonyl group at C-8 and are differentiated by various combinations of hydroxyl or acyloxyl (OAc) groups at C-3, 4, 7, 8, and 15, similarly to the type-B trichothecenes, which have a typical carbonyl bond (keto group) at C-8 as well (Table 1).

| Mycotoxins | Group | | | | |
|---|---|---|---|---|---|
| | R1 | R2 | R3 | R4 | R5 |
| Type-A | | | | | |
| DAS | | OH | OAc | OAc | HH |
| T-2 toxin | OH | OAc | OAc | H | OCOCH2CH(CH3)2 |
| HT-2 toxin | OH | OH | OAc | H | OCOCH2CH(CH3)2 |
| Neosolaniol | OH | OAc | OAc | H | OH |
| Type-B | | | | | |
| DON | OH | H | OH | OH | O= |
| Nivalenol | OH | OH | OH | OH | O= |
| 3-AcDON | OAc | H | OH | OH | O= |
| 15-AcDON | OH | H | OAc | OH | O= |
| Fusarenon X | OH | OAc | OH | OH | O= |

Table 1. The most important trichothecene mycotoxins (DAS: dyacetoxyscirpenol, DON: deoxynivalenol, 3-AcDON: 3-acetyl-DON, 15-AcDON: 15-acetyl- DON).

Group C trichothecenes are characterised by a second epoxide group at C-7, 8 or C-9, 10 (crotocin and baccharin), whereas type-D group includes toxins containing a macrocyclic ring between C-4 and C-15 with two ester linkages (satratoxin G and roridin). While type-A and B trichothecenes are commonly known as contaminants of food and feed, it is assumed that macrocyclic trichothecenes rarely occur in these matrices (Krska et al., 2001). However, these toxins have occasionally been determined in hay or straw. Over the past few years, they have attracted more attention as indoor pollutants (Gottschalk et al., 2009).

Several surveys suggest that the most prevalent trichothecenes are deoxynivalenol (DON), nivalenol, 3-acetyl-DON (3-AcDON) and 15-acetyl-DON (15-AcDON), as type-B trichothecenes, and HT-2 toxin and T-2 toxin, as type-A trichothecenes. They are mainly found on maize, oats, barley, and wheat. The latter, especially durum wheat, which is used nearly exclusively for the production of pasta, is susceptible to *Fusaria* infection and is often highly contaminated with DON. In European agricultural commodities type-A trichothecenes usually occur less frequently and at lower concentrations than DON. The simultaneous occurrence of DON with other *Fusarium* mycotoxins mainly type-B trichothecenes and zearalenone, has been reported for a variety of agricultural commodities (Gareis et al., 1989; Petterson, 1992, as cited in Krska et al., 2001).

*Fusarium graminearum* (teleomorph *Gibberella zeae*) is the main species responsible for the natural contamination by DON and nivalenol; *F. sporotrichioides* is the major producer of T-2 and HT-2, while *F. poe* is a minor T-2 producer but, together with *F. sulphureum, F. roseum, F. equiseti*, and other species, forms diacetoxyscirpenol (DAS) (Krska et al., 2001).

All trichothecenes appear to inhibit peptidyl transferase. Peptidyl transferase is an integral part of the 60S ribosomal subunit and is involved in elongation and termination. All trichothecenes bind to the same ribosomal binding site, but produce different effects of protein synthesis. First, the 12,13 epoxide group is essential for inhibition of protein synthesis, and reduction of the 9,10 double bond reduces the toxic activity, both in vivo and in vitro. Second, substitution at $R_2$ enhances inhibition of peptidyl transferase. Those trichothecenes with substitutions at $R_2$ only are inhibitors of elongation or termination. Third, substitution at $R_1$ and $R_3$ on the same side of the molecule produces initiation-like inhibitors. Substitution at $R_2$, which project away from the other side of the molecule, produces an inhibitor of elongation or termination. The more toxic trichothecenes are initiation-like inhibitors (Feinberg & McLaughlin, 1989).

Several surveys have shown toxicological and immunological effects on farm animals, produced by trichothecenes after ingestion of mould-contaminated cereal grains. The main symptoms observed, are skin and gastrointestinal irritation or necrosis, haematological disorders, diarrhoea, vomiting and feed refusal, decreased body weight gain, damage to the haematopoietic systems in bone marrow, spleen, thymus and lymph nodes and immunological alteration (Ibáñez-Vea et al., 2011).

From the point of view of known incidence, the most important trichothecene is DON. It may also co-occur in grains and feeds with other trichothecenes and zearalenone and other combinations of toxins are possible. The carryover of DON and other trichothecenes into human foods is considerable and a cause of concern for food safety agencies. The carryover of trichothecenes or their metabolites into farm animal products would not appear to be a major problem from experimental studies and no cases of their natural occurrence in meat, milk or eggs have been reported (Scott, 1989).

With regard to human diseases, these compounds have been related to several poison outbreaks such as alimentary toxic aleukia (ATA) and Akakaby-bio or red mould disease (Scott, 1989). During World War II, there was a devastating outbreak of ATA in Russia, and over 100,000 people died from this disease. Clinical signs included severe dermal necrosis, haemorrhaging, leukopenia and bone marrow degeneration. Bread prepared from over wintered grain contaminated by various toxigenic fungi was believed to be the cause of the illness (Trenholm et al., 1989).

Although perhaps outside the scope of this chapter, the controversial topic of the possible role of trichothecenes as biological warfare agents ("yellow rain") should be mentioned as a footnote. T-2, DAS, DON, and nivalenol have been identified by gas chromatography - mass spectrometry (GC-MS) in environmental samples such as leaves, water, and yellow powder from Southeast Asia in 1981 (Scott, 1989).

Trichothecene levels in different matrices vary from μg/kg up to mg/kg depending on a toxin, matrix, climatic condition, as well as other factors. Since toxins can never be completely removed from the food supply, and since they are potential health risks for humans and animals, the European Union has implemented regulations for some of them. The co-occurrence of different trichothecenes (type-A and type-B) in one same foodstuff, could provoke additive or even synergistic effects on human or animal health; however, the knowledge regarding this aspects is still scarce. In order to be able to monitor several toxins,

it is necessary to develop analytical methods for their simultaneous determination that meets the regulatory requirements (Ibáñez-Vea et al., 2011).

According to reports on the incidence of mycotoxins, as mentioned above, one of the most frequently occurring mycotoxin in feed originating from Europe and Asia is DON (Binder et al., 2007). In addition, also the occurrence of T-2 toxin is reported to be significant. However, the indication that the presence of T-2 and HT-2 toxin in products intended for animal feeding could be a reason for concern and the statement that data on the presence of T-2 and HT-2 toxins in products intended for animal feeding are at present very limited are expressed in Commission Recommendation 2006/576/EC (European Commission, 2006b). Thus, the need for the development of a sensitive method and the need for collecting more occurrence data was announced and the guidance values for DON, zearalenone, ochratoxin A, and the sum of fumonisins $B_1$ and $B_2$ for judging the acceptability of compound feed, cereal and cereal products for animal feeding were given (European Commission, 2006b). Reports on analytical methods for the determination of trichothecenes in various matrices using gas chromatography with either electron capture detection (Krska, 1998; Mateo et al., 2001; Radová et al., 1998; Valle-Algarra et al., 2005) or mass spectrometric detection (Ibáñez-Vea et al., 2011; Langseth and Rundberget 1998; Melchert and Pabel, 2004; Schollenberger et al., 1998; Schollenberger et al., 2005; Schothorst et al., 2005; Tanaka et al., 2000), liquid chromatography-mass spectrometry (Berthiller et al., 2005; Binder et al., 2007) and liquid chromatography with fluorescence detection (Dall'Asta et al., 2004; Pascale et al., 2003; Visconti et al., 2005) are available. Because the 12,13 epoxy trichothecenes are a group of closely related compounds, physicochemical methods of analysis are usually intended to determine more than one trichothecene at the same time (Krska et al., 2001).

In the present work, the validation of the procedure for the determination of eight trichothecenes – DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 toxin in animal feed with performance characteristics complying with requirements of Commission Recommendation 2006/576/EC (European Commission, 2006b), Commission Decision 2002/657/EC (European Commission, 2002), and Commission Regulation (EC) No 401/2006 (European Commission, 2006a) is described. However, Decision (European Commission, 2002) and Regulation (European Commission, 2006a) concern the residues in products of animal origin and mycotoxins in foodstuffs, but in view of the absence of suitable regulations, they seem to be a reasonable starting point. The procedure based on analytical methods described elsewhere (Binder et al., 2007; Langseth & Rundberget, 1998; Melchert & Pabel, 2004; Radová et al., 1998; Tanaka et al., 2000; Schothorst et al. 2005) consists of the extraction of trichothecenes with acetonitrile-water mixture, sample clean-up using MycoSep 227 Trich+ columns, derivatisation of trichothecenes with a mixture of bis(trimethylsilyl)acetamide, trimethylchlorosilane, and trimethylsilylimidazole (BSA-TMCS-TMSI) and determination using GC-MS. The problem with unusually high recoveries encountered was obviated with the use of matrix matched calibration suggested elsewhere (Pettersson & Langseth, 2002a, 2002b; Schollenberger et al., 2005). The validation procedure was performed according to Decision 2002/657/EC (European Commission, 2002).

In the years 2007, 2008, and 2009, altogether 175 samples of cereals produced in Slovenia were collected at farms, among them 79 samples of maize, 39 samples of barley, and 34 samples of wheat. In the samples, among analysed mycotoxins, trichothecenes were determined using the analytical procedure mentioned above.

## 2. Experimental

### 2.1 Apparatus

The linear shaker IKA HS 501 digital (IKA Labortechnik, Staufen, Germany) was used for the extraction and the system Syncore Polyvap (Büchi, Flawil, Switzerland) was used for the evaporation under vacuum. Gas chromatograph with mass selective detector 6890/5975B (Agilent Technologies) was combined with the column HP-5MS, 30 m, 0.25 mm I.D., 0.25 μm (Agilent Technologies) and a computer with a program ChemStation (Agilent Technologies) for the system control and data processing. The carrier gas was helium with the column flow rate of 1 mL/min. The injection volume was 1 μL and the splitless injection mode was used. The inlet temperature was 270°C, MSD ion source temperature 170°C, mass filter temperature 150°C and GC-MSD interface temperature 280°C. The column temperature program was: 60°C held for 2 minutes, 25°C/min to 240°C and 5°C/min to 300°C. Electron ionisation (EI) was carried out at 70 eV and spectra were monitored in selected ion monitoring (SIM) mode. Individual trichothecenes were identified by identification points given in Table 2.

| Toxin | Retention Time (min) | Target ion | Qualifier |
|---|---|---|---|
| DON | 12.06 | 422.20 | 512.20 |
| 3-AcDON | 12.90 | 377.10 | 392.10 |
| DAS | 13.00 | 378.10 | 350.20 |
| 15-AcDON | 13.04 | 392.10 | 350.20 |
| Nivalenol | 13.26 | 379.20 | 510.30 |
| Neosolaniol | 14.27 | 350.20 | 436.20 |
| HT-2 | 16.58 | 466.20 | - |
| T-2 | 16.62 | 350.20 | 436.20 |

Table 2. Retention time and ions monitored for trichothecenes.

### 2.2 Reagents

A certified combined crystalline trichothecenes standard of DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 was purchased from R-Biopharm Rhône (Glasgow, Scotland). After reconstitution in acetonitrile, the concentration of each trichothecene in the solution was 100 μg/mL. Working standard solutions with the concentrations of each trichothecene of 0.2 and 2.0 μg/mL were prepared diluting the stock standard solution with acetonitrile. Dichlorodimethylsilane, hexane, acetonitrile and methanol (analytical or chromatography grade purity) were purchased from Merck (Darmstadt, Germany) and MycoSep 227 Trich+ columns from Romer (MO, USA). Phosphate buffer saline (PBS) tablets (Oxoid, Basingstoke, Hampshire, UK) were used for a solution with pH = 7.4 prepared according manufacturer's instructions. Sylon BTZ (BSA:TMCS:TMSI = 3:2:3) was purchased from Supelco (Bellefonte, PA, USA). As the extraction solvent, the mixture of acetonitrile and deionised water (84+16) was used. Prior to use, glass vials for the derivatisation were deactivated with 5% dichlorodimethylsilane solution (25 mL of dichlorodimethylsilane diluted to 500 mL with hexane).

### 2.3 Samples

The samples of raw material were taken directly at the farms. Several incremental samples were taken randomly from the whole lot and combined to the aggregate sample. After homogenisation and grinding of the aggregate sample in the lab, a laboratory sample of 1.5 kg was taken for the examination and stored at 8°C for mycotoxicological analyses.

The validation of the procedure was performed with different types of feed (maize, barley, wheat, oat, soya meal, and compound feeds for pigs, lactating sows, pregnant sows, cattle, laying hens, and chicken reared for fattening) spiked with trichothecenes using the working standard solution with the concentration of 0.2 and 2 μg/mL. The selected volume of the standard solution was added to the weighed portion of a ground feed sample and the spiked sample was kept for half an hour prior to the addition of the extraction solvent.

To follow the contamination of grains with mycotoxins in primary production in Slovenia, altogether 175 samples of cereals produced in Slovenia were collected at farms in the years 2007, 2008, and 2009. Among them, there were 79 samples of maize, 39 samples of barley, and 34 samples of wheat. In the collected samples, mycotoxins were determined. For the determination of trichothecenes, the analytical procedure mentioned above was used. As positive, samples with the concentration of mycotoxins higher than limit of detection (LOD) were designated.

### 2.4 Analytical procedure

The procedure based on analytical methods described elsewhere (Binder et al., 2007; Langseth & Rundberget, 1998; Melchert & Pabel, 2004; Radová et al., 1998; Schothorst et al., 2005; Tanaka et al., 2000) was used. 10.0 g of a ground sample spiked with trichothecenes was extracted with 100 mL of the acetonitrile-water mixture (84+16) by shaking for one hour at the ambient temperature using linear shaker. 10.0 mL of the sample extract was slowly pressed through a MycoSep 227 Trich+ column. 2.0 mL of the purified sample extract was pipetted into a deactivated vial for the derivatisation and the solvent was evaporated at around 60°C under vacuum to dryness. To the dry residue in the vial, 100 μL of Sylon BTZ reagent for derivatisation was added and the vial was capped promptly. It was mixed well and the mixture was let react for 15 minutes at ambient temperature. After 15 minutes, 0.5 mL of hexane and 1 mL of PBS solution were added to the mixture and shaken for 1 minute. The layers were let separate and the upper layer was transferred into GC vial. Trichothecenes were determined by GC-MS at the conditions described above. Matrix matched calibration curves were prepared separately for each matrix type by adding a suitable amount of trichothecenes standard solution into aliquots of the purified matrix extract. Into each of five vials for the derivatisation, 2.0 mL of the purified matrix extract and a suitable amount of a trichothecenes standard solution was pipetted (e.g. 0.01 μg, 0.02 μg, 0.1 μg, 0.2 μg, and 0.3 μg which corresponded to the concentrations of 0.050, 0.100, 0.500, 1.0, and 1.5 mg/kg, respectively) and the solvent was evaporated at around 60°C under vacuum to dryness. The derivatisation and the extraction into hexane were carried out in the same way as with a spiked sample described above.

### 2.5 Validation procedure

For the linearity test as well as determination of LOD and limit of quantification (LOQ), the matrix matched calibration standards corresponding to 0.050 mg/kg, 0.100 mg/kg, 0.500

mg/kg, 1.0 mg/kg, and 1.5 mg/kg were prepared as described above. In the present study, LOD was defined as a concentration giving a signal $b_0 + 3 \times s(b_0)$ and LOQ was defined as a concentration giving a signal $b_0 + 10 \times s(b_0)$, where $b_0$ and $s(b_0)$ were the intercept of the calibration curve and the standard deviation of the intercept, respectively. For the within-laboratory reproducibility test, ten samples of feed were spiked with the trichothecenes standard solution at the concentration level of 0.3 mg/kg, ten samples at the concentration level of 0.5 mg/kg, and ten samples at the concentration level of 1 mg/kg. They were prepared according to the procedure described above. After the extraction, each sample was prepared in duplicates. Along samples, matrix matched calibration standards were prepared as described above. The results of these experiments were used also for testing the repeatability and for the determination of the recovery and measurement uncertainty. The listed parameters for each trichothecene at the concentration of LOQ were tested by measurements performed with six different feed samples spiked at 0.1 mg/kg.

### 2.6 Statistical procedure

To demonstrate the repeatability of the procedure, differences between results of duplicate measurements performed within the within-laboratory reproducibility test were calculated. The within-laboratory reproducibility was expressed with the standard deviation $s_W$, the relative standard deviation $RSD_W$, and the reproducibility limit $R_W$ ( $R_W = 2.8 \times s_W$ ). $RSD_W$ values were compared to the values derived from the Horwitz equation, given as the reference value in Decision 2002/657/EC (European Commission, 2002) and to the performance criteria given in Regulation (EC) No 401/2006 (European Commission, 2006a). Prior to the calculation of the listed parameters, results of experiments were examined by the Cochran test and the Grubbs test to eliminate results with too high difference between duplicate measurements and outliers (Miller & Miller, 2000). The expanded measurement uncertainties (European Co-operation for Accreditation (EA), 2003) at the contamination levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1 mg/kg were calculated from the within-laboratory reproducibility standard deviation ($s_W$) using coverage factor 2 ( $U = \pm 2 \times s_W$ ). Validation parameters, obtained in the validation procedure were compared to parameters given in Recommendation 2006/576/EC (European Commission, 2006b), to parameters given in Decision 2002/657/EC (European Commission, 2002), and to parameters given in Regulation (EC) No 401/2006 (European Commission, 2006a).

## 3. Results and discussion

A procedure for the simultaneous quantification of DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 in feed including different grain samples using the same analysis procedure for all of them along with GC-MS technique has been successfully validated. Most often, samples contained DON; it was present in 121 samples (69.1% of all samples).

Matrix matched calibration curves for DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2 were tested in the range from 0.050 mg/kg to 1.5 mg/kg. The correlation coefficients were higher than 0.99, thus the linearity was considered appropriate. The procedure using pure standards calibration tested previously did not give reasonable results. The problem was unreasonably high recoveries (often higher than 110%, 120% or

130% given in Regulation (European Commission, 2006a). The use of available internal standard(s) seemed to be very expensive and was not expected to solve the problem for all determined trichothecenes satisfactorily, thus the matrix matched calibration was chosen. Concerning compound feedingstuffs, experiments showed that the slope of calibration curves prepared in different types of feedingstuffs were different, but were similar in similar matrices, so the possibility of the calibration using combined matrices (mixture of similar types of feed) was examined. The slope of calibration curves prepared in combined matrices was usually similar to slopes of calibration curves prepared in individual matrices included in the combined matrix, but often outliers (calibration curves with completely different slopes) were detected. Additionally, the concern was raised that in routine analysis it is difficult to judge whether samples are similar or not and whether the combined matrix is justified or not. Therefore, concerning compound feedingstuffs, the decision to prepare the calibration curve on each individual sample was made (standard addition method rather than matrix match calibration). A chromatogram of derivatised barley sample containing trichothecenes at the contamination level of 0.5 mg/kg is shown in Figure 2.

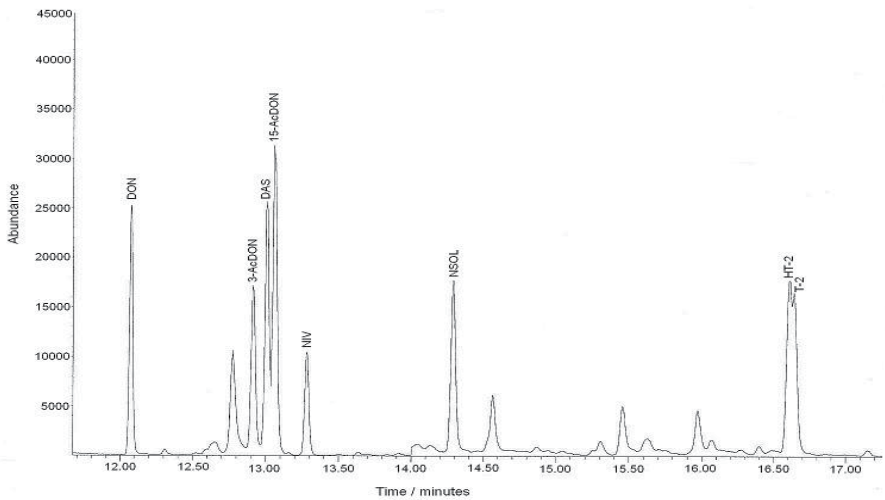


Fig. 2. Total ion chromatogram of barley sample containing trichothecenes at the contamination level of 0.5 mg/kg.

The determined LOD of each trichothecene (DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2, and T-2) in feed was around 0.03 mg/kg. For practical purposes, LOD of 0.05 mg/kg, tested with all examined samples was accepted. LOQ of each trichothecene in feed was 0.1 mg/kg. The ability to determine each of trichothecenes at this concentration level was proven by measurements performed with six different feed samples spiked at 0.1 mg/kg. The obtained parameters are given later on. The determined LOQ is satisfactory in regard to Recommendation 2006/576/EC (European Commission, 2006b) where the lowest guidance value given for DON is 0.9 mg/kg. It means that the determination of such a concentration is possible with the procedure in question.

The repeatability, within-laboratory reproducibility, recovery and measurement uncertainty were tested at the concentration levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1 mg/kg. The levels were chosen according to the lowest guidance value given in Recommendation

2006/576/EC (European Commission, 2006b) for DON and according to the concentrations of DON expected in samples. In Table 3, the average differences between results of duplicate measurements with DON, 3-AcDON, DAS, 15-AcDON, nivalenol, neosolaniol, HT-2 and T-2 are given.

| Analyte | Average differences (mg/kg) | | | |
|---|---|---|---|---|
| | 0.1 mg/kg | 0.3 mg/kg | 0.5 mg/kg | 1.0 mg/kg |
| DON | 0.01 (10%) | 0.04 (13%) | 0.11 (22%) | 0.20 (20%) |
| 3-AcDON | 0.02 (20%) | 0.03 (10%) | 0.10 (20%) | 0.18 (18%) |
| DAS | 0.01 (10%) | 0.02 (7%) | 0.07 (14%) | 0.10 (10%) |
| 15-AcDON | 0.01 (10%) | 0.02 (7%) | 0.09 (18%) | 0.13 (13%) |
| Nivalenol | 0.01 (10%) | 0.03 (10%) | 0.10 (20%) | 0.18 (18%) |
| Neosolaniol | 0.01 (10%) | 0.03 (10%) | 0.06 (12%) | 0.10 (10%) |
| HT-2 | 0.01 (10%) | 0.02 (7%) | 0.08 (16%) | 0.14 (14%) |
| T-2 | 0.01 (10%) | 0.02 (7%) | 0.08 (16%) | 0.12 (12%) |

Table 3. Repeatability of measurements, expressed with the average differences between results of duplicate measurements.

In Tables 4-7, the within-laboratory reproducibility standard deviation ($s_W$), the reproducibility limit ($R_W$), and the relative standard deviation ($RSD_W$), as well as CV derived from Horwitz equation are given for the contamination levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1.0 mg/kg. The results for $s_W$, $R_W$ and $RSD_W$ for each individual trichothecene were calculated from six experiments done in duplicates at the contamination level of 0.1 mg/kg and from ten experiments done in duplicates at the other three contamination levels except those for DON and nivalenol at the concentration levels of 0.3 mg/kg and 1.0 mg/kg which were calculated from nine experiments done in duplicates since one result at each of the two contamination levels was eliminated by the Cochran test. The experimental $RSD_W$ values were compared to the CV values derived from Horwitz equation. Majority of experimental $RSD_W$ values were lower than reference values, only a few exceeded it. However, they were much lower than upper limits for $RSD_R$ given in Regulation (EC) No 401/2006 (European Commission, 2006a) which were 40% for DON and 60% for T-2 and HT-2, thus the determined $RSD_W$ are considered acceptable.

| Analyte | Number of experiments | $s_W$ (mg/kg) | $R_W$ (mg/kg) | $RSD_W$ (%) | Horwitz value (%) |
|---|---|---|---|---|---|
| DON | 6 | 0.01 | 0.04 | 15 | 23 |
| 3-AcDON | 6 | 0.02 | 0.04 | 16 | 23 |
| DAS | 6 | 0.01 | 0.04 | 13 | 23 |
| 15-AcDON | 6 | 0.01 | 0.04 | 14 | 23 |
| Nivalenol | 6 | 0.02 | 0.05 | 21 | 23 |
| Neosolaniol | 6 | 0.02 | 0.05 | 18 | 23 |
| HT-2 | 6 | 0.02 | 0.05 | 21 | 23 |
| T-2 | 6 | 0.02 | 0.05 | 19 | 23 |

Table 4**.** Within-laboratory reproducibility of measurements at the contamination level of 0.1 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation ($RSD_W$).

| Analyte | Number of experiments | $s_W$ (mg/kg) | $R_W$ (mg/kg) | $RSD_W$ (%) | Horwitz value (%) |
|---|---|---|---|---|---|
| DON | 9 | 0.04 | 0.12 | 17 | 19 |
| 3-AcDON | 10 | 0.04 | 0.11 | 14 | 19 |
| DAS | 10 | 0.03 | 0.09 | 12 | 19 |
| 15-AcDON | 10 | 0.03 | 0.09 | 12 | 19 |
| Nivalenol | 9 | 0.04 | 0.12 | 17 | 19 |
| Neosolaniol | 10 | 0.04 | 0.10 | 13 | 19 |
| HT-2 | 10 | 0.04 | 0.10 | 15 | 19 |
| T-2 | 10 | 0.04 | 0.10 | 14 | 19 |

Table 5. Within-laboratory reproducibility of measurements at the contamination level of 0.3 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation ($RSD_W$).

| Analyte | Number of experiments | $s_W$ (mg/kg) | $R_W$ (mg/kg) | $RSD_W$ (%) | Horwitz value (%) |
|---|---|---|---|---|---|
| DON | 10 | 0.09 | 0.24 | 17 | 18 |
| 3-AcDON | 10 | 0.08 | 0.22 | 15 | 18 |
| DAS | 10 | 0.06 | 0.17 | 12 | 18 |
| 15-AcDON | 10 | 0.07 | 0.21 | 15 | 18 |
| Nivalenol | 10 | 0.09 | 0.25 | 19 | 18 |
| Neosolaniol | 10 | 0.07 | 0.19 | 14 | 18 |
| HT-2 | 10 | 0.09 | 0.25 | 18 | 18 |
| T-2 | 10 | 0.07 | 0.20 | 15 | 18 |

Table 6. Within-laboratory reproducibility of measurements at the contamination level of 0.5 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation ($RSD_W$).

| Analyte | Number of experiments | $s_W$ (mg/kg) | $R_W$ (mg/kg) | $RSD_W$ (%) | Horwitz value (%) |
|---|---|---|---|---|---|
| DON | 9 | 0.17 | 0.47 | 19 | 16 |
| 3-AcDON | 10 | 0.17 | 0.49 | 20 | 16 |
| DAS | 10 | 0.11 | 0.32 | 12 | 16 |
| 15-AcDON | 10 | 0.12 | 0.35 | 13 | 16 |
| Nivalenol | 9 | 0.13 | 0.37 | 17 | 16 |
| Neosolaniol | 10 | 0.12 | 0.34 | 12 | 16 |
| HT-2 | 10 | 0.13 | 0.37 | 14 | 16 |
| T-2 | 10 | 0.12 | 0.35 | 13 | 16 |

Table 7. Within-laboratory reproducibility of measurements at the contamination level of 1.0 mg/kg, expressed with the standard deviation ($s_W$), reproducibility limit ($R_W$) and relative standard deviation ($RSD_W$).

The mean recoveries determined at the concentration levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1.0 mg/kg using results obtained in the within-laboratory test are given in Table 8. The number of experiments taken into account is stated above.

The determined recoveries were between 78% and 103% and the mean recovery for individual trichothecenes was between 87% and 97%. This is in accordance to the requirements of Regulation (EC) No 401/2006 (European Commission, 2006b), which demands the recovery of DON from 60 to 110% and from 70 to 120% for different concentration levels and the recovery of T-2 and HT-2 from 60 to 130%.

| Analyte | Mean recovery of the level (%) | | | | Mean recovery (%) |
|---|---|---|---|---|---|
| | 0.1 mg/kg | 0.3 mg/kg | 0.5 mg/kg | 1.0 mg/kg | |
| DON | 99 | 84 | 100 | 88 | 93 |
| 3-AcDON | 94 | 92 | 103 | 88 | 94 |
| DAS | 99 | 93 | 99 | 97 | 97 |
| 15-AcDON | 101 | 91 | 100 | 95 | 97 |
| Nivalenol | 89 | 84 | 96 | 78 | 87 |
| Neosolaniol | 99 | 92 | 96 | 98 | 96 |
| HT-2 | 83 | 83 | 95 | 96 | 89 |
| T-2 | 94 | 86 | 94 | 94 | 92 |

Table 8. Recoveries of trichothecenes.

The measurement uncertainties determined at the contamination levels of 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, and 1.0 mg/kg are given in Table 9.

| Analyte | Measurement uncertainty (mg/kg) | | | |
|---|---|---|---|---|
| | 0.1 mg/kg | 0.3 mg/kg | 0.5 mg/kg | 1.0 mg/kg |
| DON | 0.03 | 0.09 | 0.17 | 0.33 |
| 3-AcDON | 0.03 | 0.08 | 0.16 | 0.35 |
| DAS | 0.03 | 0.07 | 0.12 | 0.23 |
| 15-AcDON | 0.03 | 0.07 | 0.15 | 0.25 |
| Nivalenol | 0.04 | 0.08 | 0.18 | 0.27 |
| Neosolaniol | 0.04 | 0.07 | 0.13 | 0.24 |
| HT-2 | 0.04 | 0.07 | 0.18 | 0.26 |
| T-2 | 0.04 | 0.07 | 0.14 | 0.25 |

Table 9. Measurement uncertainties of results of trichothecenes determination.

From the 175 analysed samples of different cereals, 139 (74.3%) samples contained at least one of the trichothecenes listed above. Most often, samples contained DON; it was present in 121 samples (69.1% of all samples). 15-AcDON and nivalenol were found in 16.0% and 5.1% of investigated samples, respectively. In Table 10, the concentrations of trichothecenes determined in samples of maize, wheat, barley, oats, and triticale are given. Further, 7 samples of fodder pea, one sample of rye and one sample of millet were analysed, but none of them contained any trichothecene. It is obvious, that most often DON was present in wheat, maize, triticale, oats, and barley, which represent the largest part of the meal in the intensive production of poultry and pigs in Slovenia and worldwide (Binder et al., 2007).

| Type | | Maize | Wheat | Barley | Oats | Triticale |
|---|---|---|---|---|---|---|
| DON | 1 | 79/68 | 34/30 | 39/13 | 7/4 | 7/6 |
| | 2 | 0.1-14.4 | 0.1-2.86 | 0.05-0.72 | 0.15-0.52 | 0.13-1.56 |
| 3-AcDON | 1 | 47/2 | 29/0 | 30/0 | 3/0 | 4/0 |
| | 2 | 0.08-0.14 | - | - | - | - |
| DAS | 1 | 47/0 | 29/0 | 30/0 | 3/0 | 4/0 |
| | 2 | - | - | - | - | - |
| 15-AcDON | 1 | 47/28 | 29/0 | 30/0 | 3/0 | 4/0 |
| | 2 | 0.05-0.83 | - | - | - | - |
| Nivalenol | 1 | 47/5 | 29/26 | 30/2 | 3/0 | 4/0 |
| | 2 | 0.05-1.14 | 0.06-0.07 | 0.34-0.51 | - | - |
| Neosolaniol | 1 | 47/1 | 29/0 | 30/0 | 3/0 | 4/0 |
| | 2 | 0.08 | - | - | - | - |
| HT-2 | 1 | 79/3 | 34/0 | 39/1 | 7/0 | 7/0 |
| | 2 | 0.06-2.3 | - | 0.05 | - | - |
| T-2 | 1 | 79/1 | 34/0 | 39/0 | 7/0 | 7/0 |
| | 2 | 0.290 | - | - | - | - |

Table 10. The presence of trichothecenes regarding the cereal type in the period 2007-2009 in Slovenia (1- number of investigated samples/number of positive samples, 2- concentration ranges in mg/kg).

However, besides recommendations on the presence of DON, zearalenone, ochratoxin A, T-2 and HT-2 toxins, and fumonisins in feed (European Commission, 2006b), the legislative limits have been set only for aflatoxin $B_1$ in feed for different species and categories of livestock. For DON the recommended highest concentration per kg cereals and cereal products is 8 mg. Recommended maximum levels for T-2 and HT-2 toxins are not set yet in Europe. T-2 toxin is the most toxic of the naturally occurring trichothecenes ($LD_{50}$ 4.0 mg/kg body weight for rats and swine, oral; 7 mg/kg body weight for mice, oral). Comparison of the $LD_{50}$ of T-2 toxin with those of other toxins, e.g. DON (46 mg/kg body weight for mice, oral), reveals its acute toxicity (Roth et al., 1990, as cited in Krska et al., 2001). But, as it was mentioned, despite the high toxicity of the type-A trichothecenes, only few countries have established legal regulation of recommendations for T-2 toxin (FAO, 1997, as cited in Krska et al., 2001). In our study, T-2 toxin was present in only one sample of maize in the concentration of 0.290 mg/kg.

From the available data (Schuhmacher-Wolz et al., 2010), it can be concluded that raw oats and barley can be highly contaminated with T-2 and HT-2 including high incidence and concentrations. Maize can be contaminated occasionally at moderate concentrations. T-2 and HT-2 contamination of wheat seems to be very infrequent and at low concentrations. Feed products that are of major concern include by-products from oat processing (pellets). Food products generally show low incidence and concentration of T-2 and HT-2, however, oat products may contain some T-2 and HT-2. Field factors that influence T-2 and HT-2 include region-year (climate), variety, sowing date, pre-crop, and organic production. Fungicides seem to have no or only a weak effect. Processing cereals will substantially reduce T-2 and HT-2 contamination in most food products due to redistribution over the various fractions. As a consequence, the levels in the by-products, often used for animal feeding are increased (Schuhmacher-Wolz et al., 2010).

In general, the levels of mycotoxins in our research did not reach the maximum levels set by Slovenian or European legislation. The results are similar to those reported from around the world. DON is the most widely spread mycotoxin, which is confirmed also by the investigation including samples from eleven European countries examined on trichothecenes contents. 57% samples were DON positive and 20% contained T-2 toxin and HT-2 toxin (SCOOP, 2003). Most contaminated were maize samples, with average concentrations of DON 300-3700 µg/kg. Similar results were gained by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2001). Maize is known to be a good substrate for mould infection and production of mycotoxins harmful to both humans and animals (Kumar et al., 2008). Under natural conditions, two mono-acetylated derivatives 3- and 15-AcDON accompany DON, albeit they are produced at lower concentrations (EFSA Journal, 2004). Also in our study, the second most frequent toxin was 15-AcDON. It was found in 59.6% of maize samples in concentration 0.05-0.83 mg/kg. Acetylated toxins are rapidly deacetylated in vivo. DON is therefore discussed together with acetylated forms (Eriksen et al., 2004).

A world-wide survey of DON, nivalenol, and zearalenone, on 500 agricultural samples from 19 countries and districts, reported that approximately 40-50% of the samples were positive for these mycotoxins. Average concentrations of 0.292 mg/kg for DON, 0.267 mg/kg for nivalenol, and 0.045 mg/kg for zearalenone were reported. DON was often found in maize (69%), followed by mixed feed (65%) and wheat (60%) (Tanaka et al., 1988, as cited in Krska et al., 2001).

In Croatia, the neighbouring country of Slovenia, the seven years long investigation of grains (1998-2004) revealed 9.5-66.7% of T-2 toxin positive samples (average 150-410 µg/kg) and 9.1-50% positive for DAS (average 300-130 µg/kg) (Sokolović & Šimpraga, 2006). In 2008 a total of 139 samples of various grains were investigated for the presence and concentration of DON (Jajić et al., 2008). The average incidence rate of DON in maize was 44.7%, in wheat 37.5 %, and in barley 25%. In the positive samples, DON was found in concentration range between 0.04 and 2.46 µg/kg. Recently, Ibáñez-Vea et al. (2011) investigated the type-A and type-B trichothecenes in 44 barley samples. The higher occurrence was found for DON (89% of the samples), although at concentrations below the maximum permitted level. Two or more thichothecenes were present in 41% of the samples.

However, also in our study, quite a number of investigated samples contained more than one trichothecene and other mycotoxins; some of them even up to seven (Jakovac-Strajn et al., 2010). It is stated elsewhere (Tanaka et al., 2000), that subclinical and chronic concentrations as well as synergistic effects of mycotoxins have even higher impact on the health of animals and human than acute concentrations. The response of affected animals to exposure to more than one mycotoxin can be the same as the response predicted from the summation of the response to each mycotoxin individually (additive), less than the predicted response from each toxin individually (antagonistic), or more than the predicted summation of the responses from each individual mycotoxin (synergistic) (Wyatt, 2005). In addition, very little is known about the effects of long-term, low-level exposure, especially with regard to the co-contamination with multiple mycotoxins (Kumar et al., 2008). But, interactions between different mycotoxins are still under investigation (Grenier & Oswald, 2011). Combination of *Fusarium* mycotoxins, in general, result in additive effects, but synergistic and/or potentiating interactions have been observed and are of greater concern

in livestock health and productivity. Synergistic effects between DON and fusaric acid, DON and fumonisin $B_1$, and DAS and aflatoxins (D'Mello et al., 1999, as cited in Kolosova & Stroka, 2011) have been reported. Additive and synergistic effects between known and unidentified mycotoxins may account for enhanced adverse effects observed on feeding mycotoxin-contaminated diet. Furthermore, the toxic effect of any single mycotoxin may be amplified due to synergistic interactions with other substances (Sergent et al., 2008, as cited in Kolosova & Stroka, 2011). The issue of masked mycotoxins should also be taken into consideration. They attracted attention in the eighties already, because in some cases of mycotoxicoses, clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed. The unexpected high toxicity was thought to be attributed to undetected, conjugated forms of mycotoxins that hydrolyse to the precursor toxins in the digestive tract of animals. As reported, combining HPLC with tandem MS results in a powerful tool for characterisation and identification of masked mycotoxins (Berthiller et al., 2005; Binder, 2007).

Except in the case of DON, the concentrations of mycotoxins in our study were mainly not high enough to cause clinically detectable health or production problems. In a range of species, reduced feed intake seems to be one of the most sensitive indicators of dietary exposure to trichothecenes. At higher doses or with repeated exposure, this may be followed by actual feed refusal, emesis in capable species, and possibly the development of perioral, oral, pharyngeal, oesophageal or gastroenteric lesions or combination of these. Perioral and oral lesions have been most thoroughly documented in birds, swine, and horses (Trenholm et al., 1989). Of special interest are trichothecenes immunosuppressive effects and subsequent increased susceptibility to infections. It is clear that the trichothecene mycotoxins suppress the immune network, but the specific function of various cell types affected by trichothecene mycotoxins have yet to be definitively ascertained. As would be expected, newborn animals without fully developed detoxifying mechanisms, and with short-lived energy stores, are even more sensitive to trichothecene toxicoses (Taylor et al., 1989).

In the discussion about results, the problem of representative sampling must be also taken into account. The problem of sampling is less pronounced with *Fusarium* toxins which are regarded as less heterogeneously distributed than the aflatoxins (Larsen et al., as cited in Krska & Molinelli, 2007). Anyway, Hallier et al. (2011) showed recently that within the procedure of DON analysis, the most critical step is grain sampling. Nevertheless, little scientific evidence of mycotoxin distribution and variability have limited the development of alternative sampling plans to be used depending on the mycotoxins and the type and size of commodities, which would reduce the total variability and enable the estimation of uncertainty in the evaluation of mycotoxin concentrations. Nowadays, with relatively few sampling plans available for different commodities, the sampling step contributes to the largest variability in mycotoxin determination (Krska & Molinelli, 2007).

To conclude, DON occurrence is almost exclusively associated with cereals, and the levels of occurrence are in the order of hundreds of µg/kg upwards. DON occurs as a field (pre-harvest) rather than a storage contaminant, and almost always co-occurs with other *Fusarium* toxins. Preventive measures are difficult to implement, and even the effect of fungicide treatment on DON levels is controversial (Edwards et al., 2001). As seasonal variations significantly influence the extent of *Fusarium* infections, levels of DON tend to

vary from year-to-year making it difficult to generalise the typical levels of occurrence (EFSA Journal, 2004).

Prevention of fungal infections during plant growth, harvest, storage and distribution would seem the most rational and efficient way to avoid mycotoxins in agricultural commodities (Huwing et al., 2001; Ramos and Hernandez, 1997, as cited in Kolosova & Stroka, 2011). Recently, European regulation (EC) No 1831/2003 (European Commission, 2003) on additives for use in animal nutrition has been amended. A new functional group defined as "substances for reduction of the contamination of feed by mycotoxins" has been added in the category of technological feed additives (European Commission, 2009). It contains substances most often termed as mycotoxin binders. It should be pointed out that the use of such products does not mean that animal feed exceeding the established maximum limits may be used (Kolosova & Stroka, 2011).

For both, A- and B- trichothecenes there is still a lack of simple and reliable screening methods enabling the rapid detection of these mycotoxins at low cost. Besides the increasing demand for rapid screening methods for both A- and B-trichothecens, the use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) enabling both quantification and identification of several trichothecenes simultaneously can be considered a major future trend in the analysis of these *Fusarium* mycotoxins in cereals (Krska et al., 2001).

## 4. Conclusions

Mycotoxins are secondary metabolites of fungi. It is not possible to predict their presence or to prevent their occurrence during preharvest, storage, and processing operations by current agronomic practices. Therefore, their presence in food and feed represents a constant health risk for animals and humans. To protect the consumer from the harmful effects of these compounds, regulations have been established in many countries, resulting in the development of monitoring methods. The performance characteristics for all grains, obtained from validation in this study, confirmed that GC-MS, a very common technique for the quantification of type-A and B trichothecenes in grains, food, and feedstuff, is well suited for their determination. Among DON, 3-AcDON, 15-AcDON, nivalenol, neosolaniol, T-2, HT-2 and DAS, two mycotoxins DON and 15-AcDON were detected most often. In general, the levels of mycotoxins in the tested samples did not reach the maximum levels set by legislation. Anyway, the long term of chronic exposure to low concentrations of mycotoxins, the co-occurrence of several mycotoxins in the same sample, masked mycotoxins, and the problems connected to sampling must be taken into account when determining permitted levels or carrying out risk assessment.

Experience gained during intercomparison studies clearly shows the need for further improvements in the determination of trichothecenes – more accurate and comparable results are required. Regular maintenance of the GC instrument seems to be of great importance to the achievement of long term reproducibility.

Besides the increasing demand for rapid screening methods for both A- and B-trichothecenes, the use of LC-MS/MS enabling both quantification and identification of several trichothecenes simultaneously can be considered a major future trend in the analysis of these *Fusarium* mycotoxins in cereals.

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# Part 4

## The Other Applications

# 12

# GC-MS Analysis of Volatile Plant Secondary Metabolites

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## 1. Introduction

Knowledge of the identity and relative amounts of the volatile substances released by plants is of great importance to several fields of basic and applied research in biology, chemistry and may other disciplines. Obtaining this knowledge requires overcoming many analytical challenges posed by these complex mixtures, because they normally present large variations in component amounts, chemical structures and functionalities. Gas chromatography (GC) is recognized as the most suitable technique to find out how many components and in what proportion there are in a complex mixture of volatile compounds. When it is coupled to mass spectrometry (GC-MS), additional information arises about each separated compound molecular mass, elemental composition (when high resolution mass spectrometry is used), functional groups, and in certain cases, molecular geometry and spatial isomerism. This chapter covers the most determinant factors which affect biogenic volatile compound (BVOC) GC-MS analysis. Sample preparation, chromatographic separation and mass spectrometry are the main parts explained within the framework of BVOC analysis and practical examples of their application to flower scent and essential oil analysis are presented.

## 2. Isolation techniques

The process employed for plant volatiles isolation strongly determines the final outcome of the analysis. The number of compounds to be found and their relative amounts depend on how the sample was prepared, what extraction technique was employed, whether any clean up step was included, how potential interferences were removed and to what extent the final extract was concentrated. The right selection of extraction technique involves the consideration of aspects such as: (1) the nature of the matrix (origin, aggregation state, homogeneity, stability) and of the analyte (volatility, polarity, reactivity, thermal stability); (2) the purpose of the analysis (qualitative, quantitative); (3) the need to confirm the chemical structure of the analyte (identification using spectroscopic techniques); (4) how urgently the results are needed (for example, to determine the nature of a person´s intoxication); (5) the potential legal implications of the results (forensic, environmental analyses, doping control) and lastly, (6) it is important to know whether the extraction method is well known and is already standardized or it should be established, optimized and validated. GC-MS analysis requires the previous removal of substances which are not

compatible with this technique (ions, gums, polymers and other macromolecules) and an extract of sufficient concentration to have analyte concentrations above the limits of detection/quantitation of the corresponding GC-MS method (Koning et al., 2009). Extraction methods could be classified as: I. Distillative methods, II. Extractive methods and III. Headspace methods. Certain *sui generis* techniques may combine methods from different groups, as in the case of simultaneous distillation-solvent extraction (SDE) (Chaintreau, 2001).

### 2.1 Distillation methods

The isolation of essential oils or volatile fractions for final products, from vegetal material, by means of extractive distillation, may take place under 3 main configurations: (1) Steam distillation (SD); (2) Hydrodistillation (HD), or (3) Water-vapor distillation. Volatile mixtures may be subjected in industrial processes to distillation at reduced pressure in special columns (molecular distillation), in order to isolate or increase the concentration of a particular constituent. However, molecular distillation and related techniques are not appropriate for trace-level components isolation or study. Extracts or volatile fractions isolated by SD, HD or SDE constitute mixtures suitable for direct GC-MS analysis. A drying step (with anhydrous sodium sulfate) may be the only treatment required before their injection into the gas chromatograph. With the aim of increasing extraction efficiency while reducing distillation time, during the last 25 years there has been an increased use of microwave radiation as a heat source (Kingston & Jassie, 1988; Kingston & Haswell, 1997). Microwave-assisted extractions (MAE) employ water or an organic solvent to obtain extracts from solid samples (vegetal material, soil, tissues, etc.) (Rice and Mitra, 2007).

### 2.2 Extraction procedures

The second family of isolation techniques makes use of solubility, adsorption or absorption differences to induce the mass transfer from the vegetal material to a stream or static phase of an extractive agent. Adsorption or absorption-driven extraction processes involve porous polymers (PDMS, Tenax, Porapak, Chromosorb, synthetic resins) or microporous solids (charcoal, silica gel, alumina, molecular sieves). Solubility-driven processes are employed the most. Their main example is liquid extraction, which can be performed in either continuous or batch modes. Careful solvent selection (polarity, boiling point, dielectric constant, H-bond ability, availability, cost, etc.) is the most important determinant of extraction yield and selectivity. Extract cleanup and concentration are frequently required prior to instrumental analysis. Despite the problems associated with liquid extraction (emulsion formation, long extraction times, difficult automation, waste disposal, potential cross-contamination), the number of published research works in which liquid extraction is employed, has continued growing during the last decade (Mustafa & Turner, 2011; Zhang & Li, 2010**)**. Solid-phase extraction (SPE), which appeared around 40 years ago, has been implemented as a competitive alternative to liquid extraction (Fritz, 1999; Thurman & Mills, 1998). The amount of solvent required is reduced substantially and extraction, cleanup and concentration are combined in a single step. Besides, SPE can be automated to increase sample throughput, as exemplified by several commercial devices which include a direct connection with the chromatograph. Despite the high cost of the initial investment, there has been an intense growth in the application of supercritical fluid extraction (SFE) during the

last decade. Extraction selectivity in SFE can be modified by means of operational parameters because analyte solubility depends on supercritical fluid density (pressure, temperature) and the presence of modifier agents (co-solvents) (Westwood, 1993; Wenclawlak, 1992; Huang et al., 2009). When SFE is used with plant material, a cleanup step is required before GC-MS analysis, in order to remove fat, pigments, waxes, or other high-molecular weight compounds. Soxhlet extraction is one of the oldest extractive methods and has been one of the most employed approaches in plant material extraction. However, its low selectivity imposes the need for further extract cleanup steps. A very successful alternative method is accelerated solvent extraction (ASE), in which the use of higher temperature and pressure permit higher selectivity, with important reductions in extraction time and amount of solvent employed. ASE permits the combination of extraction, cleanup and concentration in a single step, with automated operation and the possibility of direct coupling with the GC-MS instrument (Zuloaga et al., 1998; Gan et al., 1999).

### 2.3 Headspace sampling

A true "green revolution" in sample preparation techniques has taken place in the last 2 decades thanks to the introduction of solid-phase microextraction (SPME) (Scheppers, 1999; Wang et al., 2002). In SPME, target analytes are extracted and concentrated on the polymeric coating of a silica fiber under 3 alternative setups: (1) headspace sampling, which consists in the exposure of the SPME fiber to the sample´s vapor phase; (2) direct immersion of the SPME fiber into a fluid sample, and (3) use of a protective membrane around the SPME fiber when it is exposed to the sample. SPME is very compatible with GC-MS and there are commercial devices to perform the SPME sampling process and the direct desorption of the SPME fiber into the injection port of the chromatographic system in a programmable manner. The ionic strength of the sample, the SPME fiber exposure temperature and time, the intensity of sample agitation, and the nature of the SPME fiber coating are variables that the experimenter can modify to change the selectivity of the SPME method and the recovery. Additionally, target analyte derivatization in the sample matrix or on the SPME fiber is a valuable tool to enhance selectivity and sensitivity in an analytical SPME method (Stashenko & Martinez, 2004; Araujo et al., 2008). According to their classical arrangements, the headspace sampling methods are classified into static (S-HS) and dynamic (D-HS) headspace methods. Large capacity sorption techniques, developed in the last 20 years, share features of both setups (Bicchi et al., 2004). The resultant volatile fractions are amenable to direct GC-MS analysis because the vapor phase does not contain macromolecular or ionic interfering species that may be present in the matrix. S-HS is normally employed in cases where the target analyte is present in relatively high amounts and no concentration is required before instrumental analysis. D-HS is preferred in cases where the target analyte is present at trace levels (ppt, ppb) because this sampling method permits enrichment of the collected volatile substances. Basically, the method consists of a purge of the vapor phase of the sample with a stream of an inert carrier gas which removes the VOC´s. They are subsequently removed from this gas by dissolution in a solvent, by cryo-trapping, adsorption or absorption on a highly porous solid material. The final extract is obtained by solvent evaporation (Kuderna-Danish apparatus or removal with a gas flow) in the first case, or thermal desorption, or solvent elution in the other instances. The use of the vapor phase above the sample (headspace) as the interface with the extracting agent has many advantages from the analytical viewpoint, because many possible interferences are

prevented from entering the extract. In natural products studies headspace sampling has permitted in vivo collection of the volatile substances around the whole plant, or directly at its parts (Tholl et al., 2006).

### 2.4 Miscelaneous techniques

New methods for microscale sampling have emerged in recent years after the detection of certain limitations in the application of SPME in different fields (Koning et al., 2009). One of the main improvements sought has been an increase in the concentration capacity of trace-level target analytes. In several cases, a concentration capacity superior to that of SPME is achieved using a larger volume of the sorption phase. In the inside needle capillary adsorption trap (INCAT), a polymeric layer coats the inner wall of a syringe needle or this volume is filled with charcoal (Musshoff et al., 2003 ). A related technique, headspace solid-phase dynamic extraction (HS-SPDE), uses a gas-tight syringe whose inner walls have been coated with a polymer such as polydimethylsiloxane (PDMS), in which the analytes will deposit when the syringe barrel is pushed and pulled repeatedly over the sample (Bagheri et al., 2009). The number of push-pull cycles determines the amount of accumulated analytes in the polymer. Just as in SPME, the analytes are afterwards released by thermal desorption at the injection port of the chromatograph. Stir bar solid-phase extraction (SBSE) and sorption tape extraction (STE) (Bicchi et al., 2005), are examples of sampling techniques in which a much larger volume of sorption phase is used to increase concentration capacity. In SBSE, a magnetic stir bar is coated with at least one type of sorption polymer (PDMS, polyethyleneglycol). Analytes are absorbed or adsorbed in the polymer coating upon stirring the sample solution with this magnetic bar, which is later on transferred to a heating device in order to release the analytes directly into the injection port of the chromatograph. STE is a very simple technique which can be used for in vivo sampling. In STE, a thin PDMS film is placed in close proximity or in contact with a plant part for a fixed period of time. Sampled analytes are then removed by thermal desorption. Liquid-phase microextraction (LPME) employs a hanging solvent drop or the solvent film formed inside a syringe to trap the analytes (Shen & Lee, 2003). Solvent type, syringe size and the number of push-pull cycles are the main operational parameters normally employed to improve the selectivity and recovery of the technique.

## 3. Gas chromatographic methods

Four fundamental blocks comprise a chromatographic system: (1) sample application system (injector); (2) mixture separation system (column); (3) detection system responsive to the analytes eluting from the column (detector), and (4) data system. Analytical quality rests on the combined performance of all basic blocks. For example, the injection system must transfer the sample into the chromatographic column in a quantitative manner, without discrimination due to molecular weight or component volatility and without chemical alteration of any constituent substance. This entrance door into the column must remain clean, contaminant-free, inert and without leaks. Component separation is achieved when the operational parameters (temperature, mobile phase velocity or polarity, pressure) and the column characteristics (length, internal diameter, stationary phase chemical nature and thickness) are such that the distribution constants (between stationary and mobile phases) of all mixture components are different. The detector response (signal) results from the

measurement of a physical property which depends on the amount of analyte molecules emerging from the column (ionic current, thermal conductivity, fluorescence, refractive index, photon emission, etc.). The direct relationship between detector signal intensity and analyte amount constitutes the basis of the chromatographic determination and addresses the question of how many components are there in a mixture and in what proportion they are found (quantitative analysis).

### 3.1 Chromatographic separation

The mixture components are separated in a chromatographic column as a result of their distribution between a mobile and a stationary phase. One of the most relevant performance parameters of a chromatographic column is its resolution, which is its ability to separate compounds with very similar distribution constants. Chromatographic resolution is a function of several operational parameters. Column dimensions (length, internal diameter and stationary phase thickness) constitute an important set of resolution modifiers. The higher the number of mixture components and their structural similarity, the longer the chromatographic column required to achieve their separation. However, for the same purpose, an alternative variation is the reduction of column diameter. Increasing column length notoriously extends the analysis time. While a factor of 2 in column length doubles its resolution, a factor of 0,5 in internal diameter increases resolution 4 times. Micro-bore columns (0,1 and 0,05 mm internal diameter), used in "fast chromatography", permit the separation of complex mixtures in experiments which lasts seconds instead of hours. However, the reduction in column diameter causes a dramatic decrease in sample capacity. Thus, trace-level components could not be determined using fast chromatography. Therefore, the selection of column length and diameter is a compromise between analysis time and the required resolution and sensitivity (Robards et al., 1994; Barceló, 2008). Stationary phase thickness is selected according to sample volatility. Mixtures of highly volatile compounds require columns with higher stationary phase thickness (5 μm), while compounds with high boiling points, such as steroids, are analyzed using columns with thinner coating (0,25 μm or less). Higher stationary phase thickness enhances compound retention, which generates the need for higher column temperature in order to accelerate compound elution. This in turn causes more stationary phase bleeding, which affects the chromatographic profile. Stationary phase polarity is selected according to sample type, its composition and the polarity of the substances to be analyzed. In columns with non-polar stationary phases, e.g. poly(dimethylsiloxanes), compounds elute following their boiling points. In columns with polar stationary phases, e. g. poly(ethyleneglycol), retention times and elution order are determined by intermolecular forces (between analyte and stationary phase polymer) and the dipole moments of the substances. The complete separation of complex mixtures with both polar and non-polar components frequently requires 2 chromatographic columns with orthogonal polarity (polar and non-polar stationary phases). This is the approach regularly employed in essential oil analysis. Figure 1 presents chromatographic profiles of the essential oil isolated from African lemon (*Swinglea glutinosa*) fruit peel, analyzed in 2 columns, with poly(ethyleneglycol) and poly(dimethylsiloxane) stationary phases, and using 2 types of injection conditions. Large retention time differences are apparent between the chromatograms obtained on different stationary phases (Figures 1A, 1B). For example, the retention time of citronellal (peak e), a monoterpenic aldehyde, almost doubles when the stationary phase is changed from non-polar to polar. Since

retention times depend on column dimensions, stationary phase polarity, oven temperature and carrier gas flow, the comparison of retention times obtained with columns of different stationary phase requires the use of retention indices (Zellner et al., 2008). They are calculated from the comparison of the retention times of a homologous *n*-alkanes mixture with those of the sample components, when both are analyzed under the same chromatographic conditions (temperature, gas flow), on columns with different stationary phase (*vide infra*). Retention indices are collected into databases to be used as reference parameters to aid in compound identification.

The amount of sample transferred into de chromatographic column is another factor which affects the analytical results. Solutions with relatively large amounts of components should be admitted into the chromatograph using split injection, in which only a fraction of the injected amount is directed to the column. When the target analytes are present at trace levels, splitless injection is used, in order to transfer the entire injected amount into the column. Figure 1 (C, D) illustrates the large difference in the number of detected components when the same amount of a solution of *Swinglea glutinosa* essential oil is injected in splitless and split (1:30) modes. The splitless mode involves the slow transfer (1 mL/min) of the sample into the chromatographic column, through the hot (~300 °C) injection zone, which may cause thermal degradation of certain components. The trace in Figure 1C shows strong sesquiterpene coelution in the 27-33 min region, owing to structural similarity and possible isomerization at the injection port. Pulsed splitless injection is a modification in which carrier gas pressure is increased just during the injection, in order to substantially reduce the exposure time of heat sensitive substances to the hot areas.

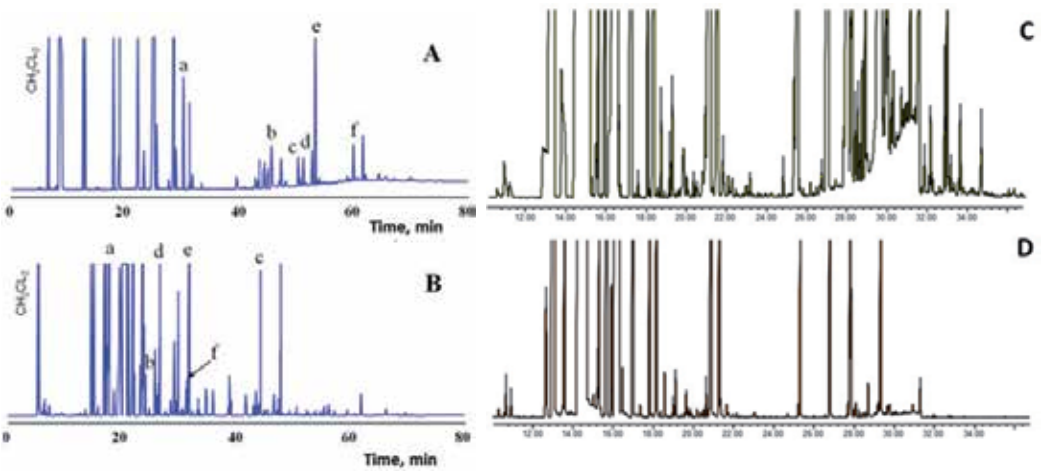


Fig. 1. *Swinglea glutinosa* essential oil chromatograms. A. Acquired on a chromatographic column with polar stationary phase (DBWAX, 60 m x 0,25 mm x 0,25 µm). B. Column with non-polar stationary phase (DB-1, 60 m x 0,25 mm x 0,25 µm). Flame ionization detector, split ratio 1:30, oven temperature program: 40 °C (5 min) @ 3 °C/min to 250 °C (5 min). a – *p*-Cymene; b – Linalool; c – α-Bergamotene; d –*cis*-Allocymene; e – Citronellal; f – Neral. C. Splitless injection. D. Split (1:30) injection. Injection volume - 1 µL (20% in dichloromethane). Mass selective detector (MSD, EI, 70 eV). Acquisition mode: full scan (*m/z* 30 – 400).

On-column injection is another admission mode, which prevents component discrimination due to volatility or molecular weight. The sample is applied directly into the column head. This might be seen as an ideal injection, but with a large number of samples there is the risk of severe column contamination. This is caused by non-volatile sample components which deposit inside the column and eventually clog it. Split/splitless injection ports include a liner just before the column, to act as a filter for these interfering substances. In the case of on-column injection, a pre-column, or retention gap, which is a short capillary with no inner coating, is used for this purpose.

### 3.2 Detection systems

A quantitative and reproducible signal is the main characteristic expected from a detection system. The chromatogram is a plot of the detector signal as a function of time. The chromatographic peak heights or areas (used preferably) are the basis for analyte quantitation, after correcting for the different response that the detector may afford after its interaction with each individual sample component, according to its chemical nature (Novák, 1988). Detectors which generate a signal for each and every eluting substance (except for the carrier gas) are classified as universal. The thermal conductivity detector (TCD) (Kaanta et al., 2010), the infrared detector (IRD) (Wang & Edwards, 2007), the mass selective detector (MSD) operating in full scan mode (Hayward & Wong, 2009) and the flame ionization detector (FID) (de Souza & de Andrade, 2009) belong to this group, although the FID is quasi-universal for not being responsive to permanent gases, water, or compounds without carbon-containing groups. Selective detectors constitute a large group of GC detectors. The nitrogen and phosphorus selective detector (NPD) emits a selective response to all compounds with nitrogen or phosphorus in their structure (Amvrazy & Tsiropoulos, 2009). The flame photometric detector (FPD) releases a signal only in the presence of compounds with sulfur or phosphorus in their structure (Hayward & Thurbide, 2009). The atomic emission detector (AED) expands the domain of chemical elements that can be registered in the eluting substances, to the extent of performing their elemental analysis (Campillo et al., 2010). The electron capture detector (ECD) provides a highly sensitive response to substances with electronegative groups (nitro, halogens, etc.), although it is not very selective (Abhilash et al., 2009). The MSD operated in selective ion monitoring (SIM) mode affords a very selective signal based on the partial ion current produced by a fragment or group of fragments in the mass spectrum of the target analyte. The thermal energy analyzer (TEA) generates a signal for those substances with nitro groups in their structure (Yinon, 1995). This is valuable in the analysis of organic explosive residues or of N-nitrosamines in food. Olfactometric detectors are of great interest in plant volatile analysis. In combination with conventional detectors (TCD, FID, MSD), they permit to go beyond quantification and identification of an aromatic mixture, to perform the organoleptic evaluation of all substances emerging from the chromatographic column (Sasamoto & Ochiai, 2010). The study of semiochemicals, for example, pheromones, involves the use of live detectors, in which the antenna of an insect is part of an electronic circuit which provides a highly specific signal to the pheromones relevant to that insect species (D'Alessandro & Turlings, 2006). Unsaturated and aromatic molecules may be differentiated from other mixture components when the photoionization (PI) detector is used (Lewis et al., 2010). Chemiluminiscense detectors are highly sensitive and selective to nitrogen- or suphur-containing substances (Shearer, 1992). The great limitation of conventional detectors

stems from their incapability to unequivocally identify a problem substance. Absolute or relative retention times and certified standards are employed for the presumptive or tentative substance identification based on GC data from conventional detectors. The confirmatory identification of a substance in a complex mixture necessarily requires a "fingerprint", that is, its spectral signature. The complementary information is provided by the mass spectrum, which presents a unique combination of charged molecular fragments (ions) produced by the fragmentation or dissociation of the target analyte molecules after their ionization. This synergistic match of screening data with confirmatory spectral information is achieved using the combination GC-MSD.

### 3.3 Retention indices

In the middle of the previous century, with the aim of increasing the confidence level of the comparison of chromatographic retention data, Hungarian scientist E. Kovàts introduced a retention index system which is known as Kovàts indices. They are based on the measurement of retention times relative to those of a homologous *n*-paraffin series run under the same experimental conditions as the sample (Kovàts, 1958, 1965). Due to the state of technical development at the time, the chromatographic separations were performed at constant temperature (isothermal regime). The possibility of reliably programming the chromatographic oven temperature was implemented in commercial equipment several years later. Under isothermal conditions, the logarithm of the retention time increases with the number of carbon atoms of the paraffin. E. Kovàts assigned *n*-paraffin retention index values (KI) equal to 100n: 500 corresponds to pentane, 600 to hexane, 700 to heptanes, etc. The isothermal retention indices (KI) are calculated according to equation (1), where n is the number of carbon atoms in the n-paraffin eluting (with retention time $t_{Rn}$) before the compound of interest (with retention time $t_{Rx}$). N is the number of carbon atoms of the n-paraffin eluting (with retention time $t_{RN}$) after the compound of interest.

$$KI = 100n + 100\frac{logt_{Rx} - logt_{Rn}}{logt_{RN} - logt_{Rn}} \quad (1)$$

Retention indices are expected to be independent of most experimental conditions (temperature, carrier gas flow, and most importantly, column dimensions). However, temperature dependence is noticed as well as a clear dependence on stationary phase polarity. Most samples are mixtures of many components with large differences in vapor pressure and molecular weight, which cannot be analyzed in reasonable time with sufficient resolution, using isothermal conditions. The chromatographic analysis of complex mixtures requires programming the oven temperature to mix isothermal periods with periods of heating at a constant rate. This causes *n*-paraffin elution to be linear, not logarithmic. Van den Dool and D. J. Kratz modified equation 1 to calculate linear retention indices (LRI), which are massively employed today according to equation 2 (van den Dool & Kratz, 1963).

$$LRI = 100n + 100\frac{t_{Rx} - t_{Rn}}{t_{RN} - t_{Rn}} \quad (2)$$

Although isothermal (KI) and linear (LRI) retention indices have close values, they are not identical. Therefore, when reporting retention indices it is important to indicate what type of index was calculated. LRI actually depend on the initial column temperature, the heating rate, stationary phase polarity and its state (contamination, activity, and fabricant). In fact,

published retention data have an appreciable dispersion, even for the same stationary phase (Babushok & Zenkevich, 2009). This reduces the reliability of substance identification based solely on retention index matching. Standardization of chromatographic conditions for retention index determination would undoubtedly lead to higher reproducibility and confidence on their use in compound identification. In order to reduce the chance of accidental coincidences, it is highly recommended to determine retention indices on orthogonal (polar and non-polar) stationary phases. This way, the presumptive identification is obtained only when the unknown substance and a standard have very similar retention indices on both types of stationary phase. The confirmatory identification requires the match of a third parameter, their mass spectra.

### 3.4 Multidimensional and comprehensive GCxGC analysis

The frequent coelution of substances with extremely close distribution constants represents the largest challenge in complex mixture separation. Multidimensional chromatography permits the use of a second column to separate the peaks of partially or completely coeluted substances. By means of the "heart cutting" operation, pneumatic commuting valves (nowadays microfluidics devices are available to perform this switching) direct the eluting flow from one to the other column during a fixed time period. Multidimensional chromatography requires at least 2 detectors and may have configurations with 3 columns in the same or separate chromatographic ovens. The idea of using a second column has been extended to transfer not just a portion of the eluting flow, but every single segment during the whole chromatographic run. The resulting technique is known as total or complete chromatography, abbreviated GCxGC. Two columns with orthogonal stationary phases are connected by means of a modulator, which collects the eluting flow from the first column during a short time period and transfers it to the second one, which should be short enough to be transited by the transferred portion before the next one is admitted (Marriot et al., 2001). In contrast with multidimensional chromatography, GCxGC requires only 1 detector. The columns may be in the same or separate ovens. There are different approaches to perform the fractionation of the flow from the first column (1D, 25 – 30 m) into multiple; consecutive slices which are transferred with or without cryoconcentration to the second column (2D, micro bore, 1 m). The modulation time should not be longer than the elution time in the second column. This is the main reason for using a fast chromatography column (short, small diameter) as the second column, in which elution times of a few seconds are regularly obtained. Since this column is connected to the detector, this should have a very high speed of data collection and processing (MSD, FID, μ-ECD). The best choice for fast response is the time of flight mass analyzer (TOF), although it has a relatively high cost (Mondello et al., 2008; Adahchour et al., 2008).

## 4. Gas chromatography coupled to mass spectrometry

A very powerful combination results from the union of the high resolution capacity of gas chromatography with the identification capabilities of mass spectrometry. The resulting data have a tri-dimensional nature, from which retention times, chromatographic areas and mass spectra can be obtained for every single component of a complex mixture. The mass selective detector may operate in 3 acquisition modes (universal, selective, or specific) which facilitate the detailed characterization of complex mixtures such as those normally isolated

from plants. The following sections present several aspects of the GC-MS technique which are important in the analysis of plant volatile compounds.

### 4.1 Ionization techniques

The essence of mass spectrometry stems from the ionization of molecules, accompanied or not by their subsequent dissociation or fragmentation. Molecular ionization may take place in the vapor phase (which requires volatilizable and thermally stable molecules) or in condensed phase (for very polar, or thermo labile, non-volatile or high-molecular weight substances). Molecular ionization requires energy, which may be supplied by accelerated or thermal electrons, by photons (photoionization, crown discharge, laser beam), by accelerated atoms or ions, by a high gradient of an electrostatic field, or by thermal impact. The ionization of vapor-phase molecules may be carried out with various techniques. Electron ionization (EI), chemical ionization (CI), and field ionization (FI) are the most common approaches. EI is the most employed ionization technique for small molecules. Chemical ionization with positive (PICI) or negative (NICI) ions is an analytic complement that is employed when the mass spectra obtained with EI do not contain molecular ions. Electrons are excellent ionization agents for organic molecules. They are easy to generate (just heating a tungsten or rhenium filament) and their energy can be regulated by means of the applied voltage between filament (cathode) and anode. For most organic molecules, the maximum ionization efficiency is obtained with 50 – 60 eV bombarding electrons. Standard mass spectra are obtained with 70 eV electrons because higher repeatability and reproducibility are obtained. Mass spectral libraries are built with spectra obtained by ionization using electrons with 70 eV of energy. This facilitates the comparisons among spectra obtained in different spectrometers and those of different databases. Ionization efficiency depends not only on the energy of the bombarding electrons, but also on the pressure at the ionization chamber or ion source ($10^{-5}$ – $10^{-6}$ Torr), and on their contamination level. Column bleeding, non-volatile interfering substances or their fragments form deposits on the surface of parts of the ionization chamber. This contamination and system leaks (air, water) cause sensitivity decrease as a consequence of the lower ionization efficiency that they ensue.

### 4.2 Mass analyzers

Mass analyzers separate ions according to their *m/z* ratio. Nobel prize winner Wolfgang Paul invented the quadrupole filter (Q) and the quadrupole ion trap (IT), which are the most common analyzers used in GC-MS to determine *m/z* ion ratios (Hübschmann, 2009; Fontana et al., 2010). In recent years the use of the time of flight analyzer has grown considerably (Marsman et al., 2008). Although its cost remains high, there has also been a moderate increase in the applications of Fourier transform ion-cyclotron resonance mass spectrometry (FT-ICR), due to the high resolution it provides. The magnetic sector analyzer was the most employed type during the development of mass spectrometry (McMaster & McMaster, 1999). In the quadrupole mass analyzer the ions formed at the ionization chamber are collimated and accelerated by means of a series of electrodes (with accelerating potentials of 5 – 100 V) which lead them into the space between 4 metallic bars which constitute the quadrupole. Direct and alternating (radiofrequency) voltages are applied to the 4 bars. The resulting electric field determines the complex oscillatory trajectory to be followed by the

incoming moving ions. For a particular combination of DC and alternating RF voltages there will be a particular *m/z* value for which the trajectory will be along the central axis of the quadrupole. All other *m/z* values will have divergent trajectories which will make the ions collide with the quadrupole bars. The systematic variation of the applied voltages permits a continuous scan of an interval of *m/z* values, so that eventually every incoming ion has the appropriate *m/z* value to cross the quadrupole filter and reach the detector (normally, an electromultiplier). Since a scan of the *m/z* interval (50 – 350 *m/z* is typical for VOCs) lasts a fraction of a second, scans are continuously repeated. The value of the detector signal associated with each *m/z* value scanned is stored in the data system after its corresponding electronic amplification. The chromatographic profile in GC-MS is the graphical representation of time variation of the total ion current. The total ion current is a value obtained for every scan after the detector signals for every *m/z* value in the scan are added. A chromatographic peak is thus a set of consecutive scans whose total ion currents increase, reach a high value (modal value), and decrease to a value very close to that of the first scan in the set. For each scan, the graphical representation of detector signal versus *m/z* values constitutes the acquired mass spectrum. GC-MS data systems permit a quick and easy examination of chromatographic profiles and their associated mass spectra. Chromatographic peak purity can be determined by comparing the mass spectra of the scans in a peak. High spectral similarity indicates that the peak effectively corresponds to a single substance, while the detection of different spectral patterns indicates coelution. A metallic ring and 2 end caps with a hyperbolic shape are the basic components of a quadrupole ion trap. Ions are admitted into the trap as a result of an electrostatic pulse. The size of the ion trap (ring radius), the frequency of the voltage applied and the amplitude of the DC voltage modulate the stability of a particular ion in the trap. Collisions with helium (~1 mTorr) present in the ion trap reduce the kinetic energy of the ions and help to focus their trajectories in the ion trap center. Ions of a given *m/z* value form a packet and are ejected as such when the amplitude of the applied radiofrequency is changed. Just as with the Q analyzer, the mass spectrum results from the systematic change of the applied voltage to sequentially eject ion packets from low to high *m/z* values.

### 4.3 Acquisition modes

The MSD may be operated as a universal, a selective, or a specific detector, depending on how the information is acquired. In full scan mode, spectra are obtained for all sample components as they elute from the chromatographic column. These spectra are the basis for substance identification. Scan speed depends strongly on the type of mass analyzer in use (quadrupole, ion trap, time of flight, or magnetic sector) and on the *m/z* range. This mass range is established according to the sample nature, that is, the range of molecular weights of its components. The low mass value for aliphatic compounds, alcohols, and amines, may be set between 30 and 40 Da. Lower values are not recommended because they correspond to background signals. For aromatic structures the low mass may be set at *m/z* 50. The high mass of the full scan should correspond to the molecular weight of the heaviest sample component, plus 40 – 50 units. If a GC-MS analysis of low-molecular weight volatile compounds employs too wide an interval, e.g. *m/z* 30 – 550, the number of spectra gathered per unit time will be small. This affects the quality, reproducibility and reliability of the analytical data. For example, a typical essential oil consists of monoterpenes (Mol. Wt. 136), oxygenated monoterpenes (Mol. Wt. 150, 152, 154), sesquiterpenes (Mol. Wt. 204) and

oxygenated sesquiterpenes (Mol. Wt. 220, 222). For efficient data gathering, the chromatographic run may be split into various intervals corresponding to the retention times of the different substance classes and within these intervals, the mass scan range used could be defined according to the typical molecular weights of the substance class. When instead of a mass interval, a discrete set of *m/z* values is used with the mass analyzer, the MSD acts as a selective or a specific detector. For example, the diagnostic ion in alkylbenzene mass spectra is the tropylium ion, at *m/z* 91, which frequently is also the base peak of the spectrum. In the chromatographic analysis of a complex mixture it is common to find instances of coelution. If only *m/z* 91 ions are allowed to pass the mass analyzer, a simplified chromatographic profile (partial ion current) will be obtained, corresponding to only those substances (alkylbenzenes) whose mass spectrum contains a signal at *m/z* 91. These chromatographic profiles are called mass fragmentograms. In this example, the MSD operated in the selected ion monitoring (SIM) mode was selective to the family of substances whose structures possess the benzyl fragment, which generates the diagnostic *m/z* 91 ion, common to all the spectra in the family. The SIM mode of operation is used not only because of its highly selective character, but also due to the much higher sensitivity achieved by acquiring more data per unit time as a consequence of reading a small number of *m/z* values rather than an interval of several hundred units. The selection of ions to be monitored should attend the following criteria: (1) their signal should be higher than 30%; (2) their mass should be relatively high, since ions with low mass are common to many substances; (3) the selected ion should be structurally representative of the molecule or set of molecules of interest and, (4) the selected ion(s) should not coincide with background signals (*m/z* 17, 18, 28, 32, 40, 43, 44) or with column bleeding fragments (*m/z* 73, 147, 207, 281, 355), septum thermal degradation products (*m/z* 149), or other contaminants. In environmental and forensic analysis it is frequent to monitor 3 ions. One ion is used for quantitative purposes and the other 2 are qualifier ions, distinctive of the substance of interest. An interesting feature that is possible with modern GC-MS instruments is the simultaneous operation in full scan and SIM modes.

### 4.4 Tandem techniques

Just as a chromatographic one-dimensional system (1 column) has resolution and sample capacity that result insufficient in the analysis of complex mixtures, a one-dimensional mass spectrometer with only 1 mass analyzer experiences limitations in resolution, sensitivity, mass range, and principally, the amount of structural information required to elucidate the molecular identity (McLafferty, 1983). Mass spectra with few signals pose a difficult task during substance identification, because valuable structural clues are obtained from the abundance and mass of product ions formed by molecular fragmentation. During the GC-MS analysis of extracts obtained from biological samples, soils, or food, it is frequent to find excessive chemical noise, manifested in the presence of many substances with certain physicochemical properties similar to those of the target analyte. As a consequence, the appearance of additional spectral signals obscures the structural assignment. A successful strategy to reduce the effect of these interferences has been the increase of the method´s specificity. In multidimensional or tandem spectrometry a high level of specificity is obtained by implementing spectral criteria to filter the signals. Just as an extract´s clean up increases its S/N ratio, a series of mass analyzers may include filtering steps to substantially increase an analyte´s spectral S/N ratio. A first analyzer may allow the passage of only the

analyte´s characteristic ions and a second analyzer may perform the actual spectral data collection. An additional analyzer may be included between the previous ones to increase the internal energies of the filtered ions and enhance their fragmentation. This is the classical configuration of a tandem mass spectrometer: MS1, activated collisions chamber, MS2, and a detection system. They are classified into 2 main groups, according to the mass analyzers employed. Linear and quadrupole ion traps, orbitraps and FT-ICR are employed in tandem in time mass spectrometers. Ions produced in the ionization area are confined, isolated, fragmented and subsequently separated according to their *m/z* values, in the same physical space (Yost, 1983). The cascade of dissociation reactions of pre-selected ions, their activation and monitoring, all take place in the same analyzer but happen as a function of time, consecutively, so that it is possible to register daughter ions, granddaughter and subsequent ions, down to $(MS)^n$. Devices of the second type are tandem in space mass spectrometers, in which at least 2 mass analyzers are separated in space. With these spectrometers it is possible not simply the study of product ions, but also of their precursor ions, of the reactions (transitions) between 2 related ions, or monitoring neutral fragment losses (Johnson et al., 1990). The triple quadrupole (QqQ) is a tandem in space spectrometer.

### 4.5 Typical mass spectra fragmentation patterns

The fragmentation pattern (*m/z* and amount, I%, of ions registered in a mass spectrum) reflects the manner in which an ionized molecule dissociates. This is not a random process, but a series of molecular rearrangements and scissions strongly dependent on molecular structure (nature of bonded atoms, bond strength, ring strain, etc.), spatial arrangement, ionization potential and internal energy. Fragmentation patterns are unique to individual chemical structures. Clearly discernible differences exist in the fragmentation patterns of isomers. For example, despite a qualitative similarity, the mass spectra of *cis*-hexatriene, *trans*-hexatriene 1,3-cyclohexadiene and 1,4-cyclohexadiene show differences in the intensity of molecular ion signals, and in the relative intensities of the fragment ions. For any substance, the diverse fragment ions observed in its mass spectrum are formed from molecular ions with different internal energy surpluses. The condition for the appearance of a molecular ion in the mass spectrum is that its internal energy be lower than the appearance energy of its fragment ions. Since the ionization process produces a collection of molecular ions with a distribution of excitation energies, some ions will have sufficient energy to quickly dissociate into daughter ions before reaching the detector and some other ones will remain complete during this same time period, giving rise to a spectral signal at the *m/z* value of the substance´s molecular mass. This signal intensity strongly depends on the ion´s ability to delocalize (stabilize) its positive charge so that no dissociation happens during the path from the ionization chamber to the detector, which lasts ~ $10^{-5}$ s. This fact is noticed when the mass spectra of *n*-decane and naphthalene are compared. Due to the stability of naphtalene´s molecular ion, there are few fragment ion signals in its spectrum and most of the total ion current is represented by the intense molecular ion signal. The decane spectrum shows a weak molecular ion signal and a homologous series of fragment ions at *m/z* 29, 43, 57, 71, 85, typical of aliphatic hydrocarbons. Dissociative ionization processes may be divided into 2 big groups: (1) simple rupture reactions (homolytic, heterolytic) and (2) rearrangement reactions, which may be molecular framework rearrangements, or rearrangements involving hydrogen transposition. The most common simple ruptures are cation formation (for example, in hydrocarbon chains), allyl and benzyl ruptures, the retro Diels-Alder reaction in cyclic monounsaturated systems, and acyl ion formation. As a general

rule, rearrangements require less energy and are more susceptible to steric effects than simple ruptures. A comparison of mass spectra of the same substance, obtained with 70 eV or with 10 – 30 eV electrons reveals that molecular ions and ions resulting from rearrangements and hydrogen transposition dominate the latter spectrum (Ashcroft, 1997). A compact description of the types of ions typically found in electron ionization mass spectra and the structural information that they provide is presented in Table 1.

| Ion type | Information provided | Examples |
|---|---|---|
| Molecular ions | Molecular mass. Elemental composition when high resolution MS is employed. Intense molecular ion signals indicate that the ionic structure can stabilize its positive charge and this normally suggests aromaticity or highly conjugated unsaturations. It is common that mass spectra of branched hydrocarbons, alcohols, and secondary or tertiary amines are devoid of the molecular ion signal. A soft ionization method, such as chemical ionization should be used in these cases. | The molecular ion stability (WM) is calculated as the percent fraction of the total ion current remaining after a substance ionization and fragmentation. WM for hexane, hexene and 1,3-cyclodiene is 2,8, 4,6 and 16,5%, respectively. It is 33,2% for benzene. That is, around one third of the total ion current is represented by benzene´s molecular ion. |
| Fragment ions | They contain the primary information for molecular structure elucidation. These may be cations (even electron number) or radical cations (odd electron number). They may result from simple rupture or rearrangement. Certain common fragment losses are associated with the presence of specific groups in the molecule. | Alcohol molecular ions easily decay with the loss of either an OH group or an $H_2O$ molecule, giving rise to the $(M-17)^+$ and $(M-18)^+$ ions. Fragment ions $C_6H_5^+$ and $C_7H_7^+$ dissociate with the loss of an acetylene molecule ($C_2H_2$) and the generation of signals at *m/z* 51 and 65, respectively. |
| Isotopic ions | The substance´s elemental composition is derived from the relative abundance of isotopic ions. The presence of Cl, Br, S or Si can be easily determined from the isotopic peak pattern. A quality requirement of a mass spectrum is the clear distinction of isotopic ions. | The isotopic distribution pattern permits congener distinction in the analysis of polychlorinated biphenyls (PCB). The successive loss of chlorine atoms and the formation of the corresponding $(M-nCl)^+$ fragments distinguishes individual congeners. |
| Multiply charged ions | They are found in the mass spectra of a limited class of substances, for example, those containing heteroatoms (N, S, O), aromatic or heteroaromatic rings, high unsaturation level, or when several of these elements are combined in the molecule. | Their intensity is relatively low and may have fractional values. The signal at *m/z* 64 in the naphthalene mass spectrum ($M^+$, *m/z* 128) corresponds to a doubly charged molecular ion, $M^{2+}$. In pyrene´s mass spectrum the signal at *m/z* 101 corresponds to its doubly charged molecular ion. |

| Ion type | Information provided | Examples |
|---|---|---|
| Metastable ions | Formed out of the ionization chamber from ions with life time longer than a microsecond, but less than the time required to reach the detector. Their apparent mass m* (a fractional number) relates the masses of parent ($m_1$) and daughter ($m_2$) ions according to $m^* = m_2^2/m_1$. Metastable ions are frequently encountered in mass spectra obtained with magnetic sector analyzers. | A mass spectrum of benzoic acid contains m* signals corresponding to processes of successive loss of radical OH and the CO molecule: $M^+ \rightarrow (M - OH)^+$ and $(M - OH)^+ \rightarrow [(M - OH) - CO]^+$. These signals confirm the genetic link between these ions. The absence of an m* ion corresponding to the direct process $M^+ \rightarrow (M – COOH)^+$ indicates that this process does not take place. |

Table 1. Typical ion types and the structural information they provide.

### 4.6 Compound identification procedure

Two fundamental strategies are employed in GC-MS for compound identification. The first one consist of comparing the unknown substance´s chromatographic (retention indices) and spectroscopic (mass spectra) data against those of certified standard substances. However, in the field of natural products research, many standards are not available. The second strategy combines several approaches: (a) retention indices obtained on stationary phases with orthogonal polarity, together with (b) experimental mass spectra (EI, 70 eV) are compared with (c) published data compiled in databases. The identification may be tentative (preliminary, presumptive) or confirmatory. Confirmation (unequivocal, positive) requires in many occasions the use of a certified standard substance. The coincidence of chromatographic (retention times, retention indices) and spectroscopic (fragmentation pattern) parameters for the problem substance and the standard constitutes a complete or confirmatory identification of a compound in a mixture. When the comparison is not made against a standard´s data but against data published in specialized literature (Adams, 2007) or spectral databases (NIST, WILEY, Adams), the match of parameters is not yet an absolute identification. When a standard is not available, the problem substance must be isolated from the mixture, purified and characterized by means of spectroscopic data (UV, IR, XRD, NMR, MS, or high resolution MS). The data from each spectroscopic method provide pieces of the structural puzzle, which the researcher must solve (McLafferty & Turecek, 1993, Silverstein et al., 2005). Nevertheless, many GC-MS analysis of essential oils and plant extracts reveal that a considerable number of components with potential interest are present in amounts so small that their isolation and purification is a formidable, practically impossible task. In these cases, as well as in those of substances whose mass spectra lack a molecular ion, important advances in characterization can be achieved with the combined use of selective detectors (GC-ECD, GC-NPD, GC-MS in SIM mode). Further advances are possible with tandem mass spectrometers (GC-QqQ) and with high resolution mass spectrometry (GC-Q-TOF).

## 5. Applications

GC-MS Analysis of plant volatiles is the main research tool commonly employed for determining the composition of these complex mixtures. A few examples are presented next, to highlight some of the special features of the mass selective detector in the analysis of flower volatiles and essential oils.

### 5.1 Flower scent analysis

Figure 2 illustrates the experimental setup employed for in vivo and in vitro flower scent sampling and the chromatographic profiles (total ion current) obtained. A total of 40 compounds were identified in *Sansevieria trifasciata* flower scent and their relative amounts changed during the day. There is no single, dominant component of the scent. Aldehydes (hexanal, heptanal, heptenal, nonanal, octenal, nonadienal, decanal), alcohols, ketones (6-methyl-5-hepten-2-one, 3-octen-2-one), acetates (benzyl, hexyl, octyl, 2-ethyl-1-hexyl, decenyl, decyl, dodecenyl, dodecyl and tetradecenyl), methyl and benzyl benzoate, methyl salicilate, eugenol, and *cis,trans*-α-farnesene, were identified as the main scent constituents.

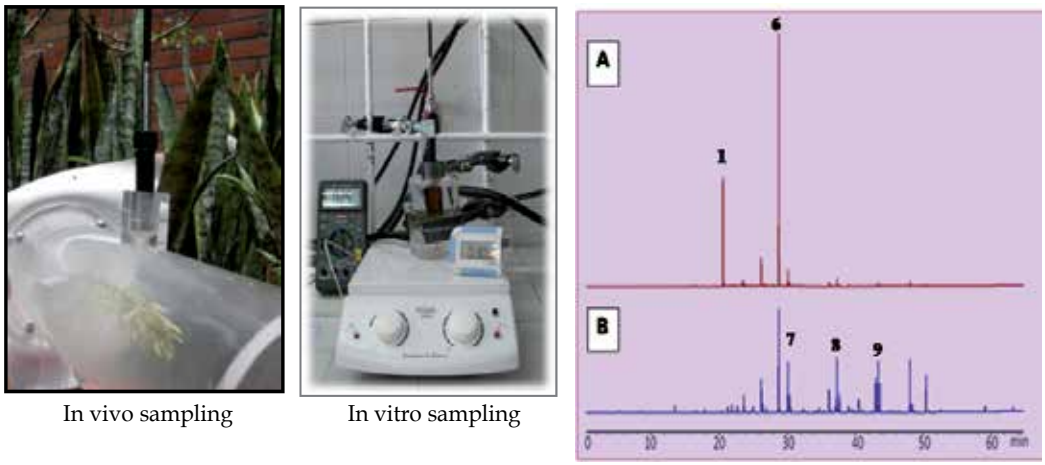


Fig. 2. Setup employed for HS-SPME sampling *Sansevieria trifasciata* flower scent. Typical chromatographic profiles for (A) *In vivo* analysis; (B) *In vitro* analysis. Tentative identification by comparison of mass spectra with databases. Main components: benzaldehyde (1), benzyl acetate (7), *trans*-3-decen-1-yl acetate, (8), and *cis*-7-dodecen-1-yl acetate (9).

Figure 3 contains the chromatographic profiles (GC-ECD) obtained when the carbonyl compounds were derivatized on the SPME fiber with pentafluorophenylhydrazine. The upper trace (3A) reveals the carbonyl compounds present in *Sansevieria trifasciata* flower scent and the lower trace (3B) shows the result obtained with a solution of homologous aliphatic aldehydes using the same on-fiber derivatization procedure. Figure 3C presents the results of applying principal component analysis to the composition data of all samples taken during day and night. The plane formed by the first 2 principal components permits a comparison of the compositions of *Sansevieria trifasciata* flower scent as a function of time. Among the samples collected at different hours, that taken at 9 pm showed the largest difference in number of components and total chromatographic area.

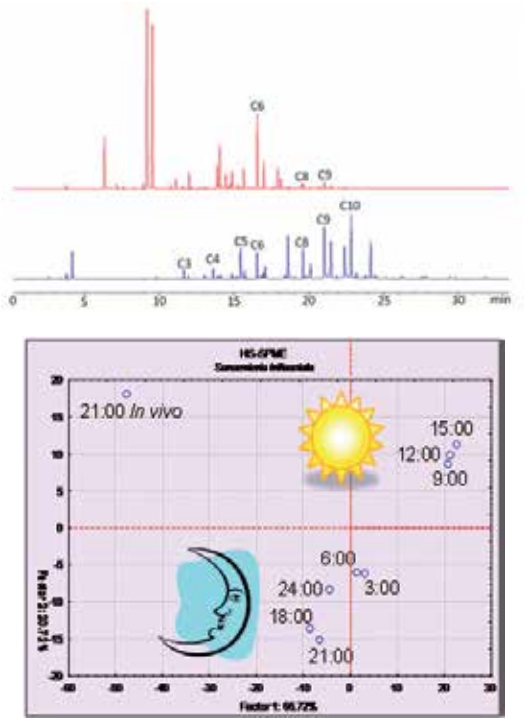


Fig. 3. Typical gas chromatographic profiles obtained after on-fibre derivatization of carbonyl compounds with pentafluorophenylhydrazine (A) from *In vitro* sampling of *Sansivieria trifasciata* flowers and (B) from a standard aqueous solution (1,58 mM of each aliphatic aldehyde, $C_3$-$C_{11}$). (C) Graphical representation of *Sansevieria trifasciata* flower scent composition change during the day. Principal component analysis of the compositional data permitted to discern a coordinate system with 87% of the information.

An important use of GC-MSD-SIM is the selective detection of a substance of interest within a complex mixture. One example is the use of GC-MSD-SIM to determine the presence of a psychotropic alkaloid, scopolamine, in a supercritical fluid (SFE, $CO_2$) extract from *Brugmansia suaveolens* flowers. Figure 4A presents the total ion current (full scan) from the SFE flower extract. Multiple interferences, coelutions, and baseline elevation prevent the extraction of a good quality mass spectrum (Figure 4B) of the candidate component at 33.6 min. Thus, the presence of scopolamine in the extract cannot be confirmed reliably. The search in the NIST spectral database produced a scopolamine mass spectrum (Figure 4C) with characteristic signals at *m/z* 303 (molecular ion, M+), 138 and 94 (base peak). These ions can be used as filters to determine scopolamine in the extract using GC-MS-SIM. Figure 4D presents the corresponding fragmentogram, interference-free, where the alkaloid is detected selectively and with much higher sensitivity than when the full scan GC-MS was used. The experimental spectrum (Figure 4E) contains the selected signals, *m/z* 94, 138 and 303, with the same intensity relations observed in the mass spectrum of the reference substance (Figure 4C). This example illustrates an advantage of the SIM acquisition mode for the selective and sensitive (10 to 100 times higher) detection of a substance of interest in a complex mixture. Scopolamine quantitation and its confirmatory identification would require the use of a certified standard.

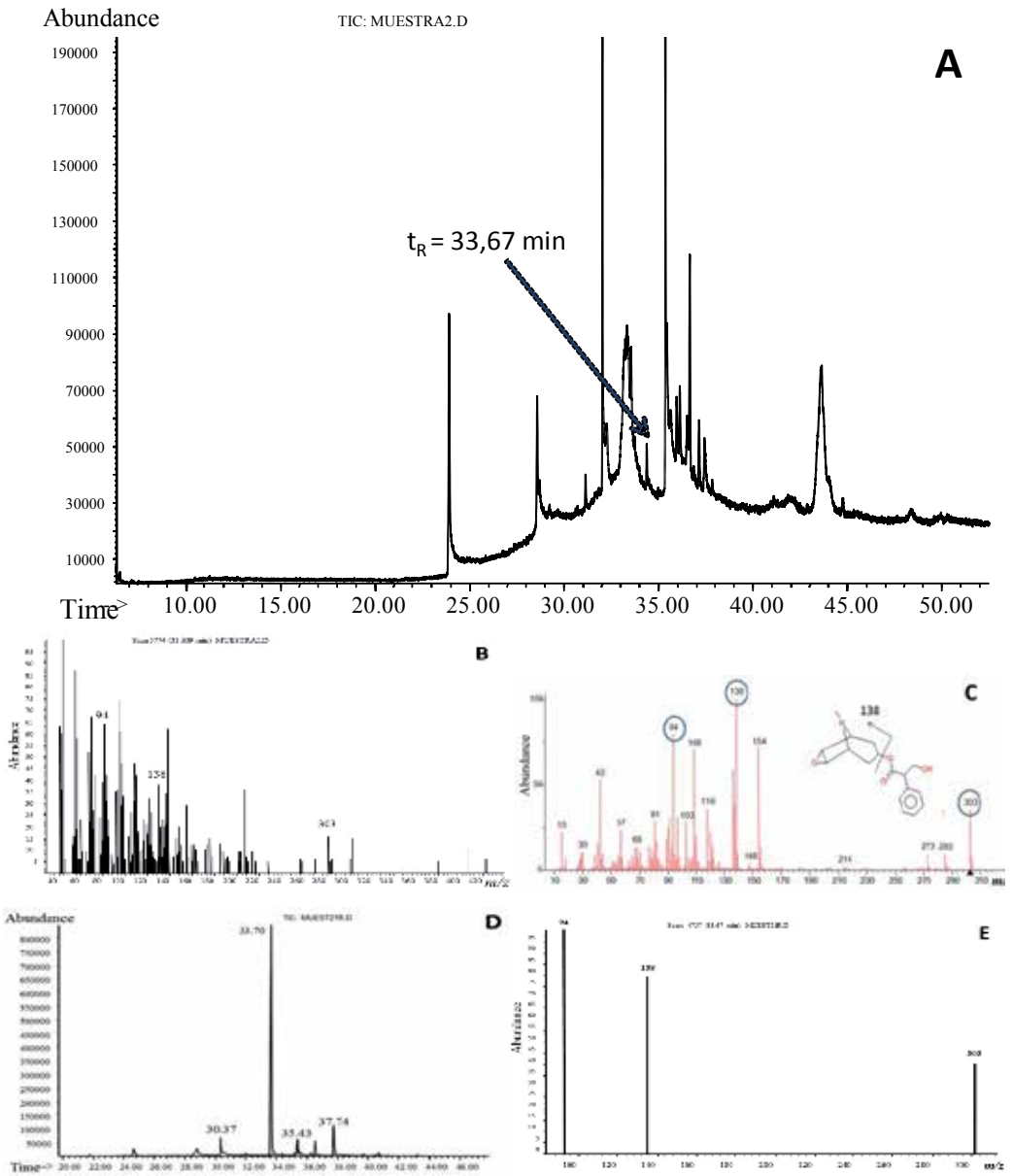


Fig. 4A. Total ion current (chromatogram) of the SFE $CO_2$ extract of *Brugmansia suaveolens* flowers. **4B.** Mass spectrum corresponding to the chromatographic peak with retention time of 33,64 min. Poor quality spectrum with large amount of noise. **4C.** The scopolamine mass spectrum available at the NIST spectral database. **4D.** Mass fragmentogram of the same *Brugmansia suaveolens* flower extract, obtained with GC-MS-SIM using the selected diagnostic ions for scopolamine, i. e. *m/z* 303, 138 and 94. **4E.** The experimental spectrum for the chromatographic peak at 33,64 min. The intensity ratios of these signals are the same as those in the mass spectrum of reference (4C).

### 5.2 Essential oil analysis

*Lippia alba* (Verbenaceae family) is an aromatic shrub that grows in Central and South America. The carvone- and citral-rich chemotypes of this plant are found in Colombia. Plant development, cultivation conditions, plant part, post-harvest treatment, and extraction time are some factors which affect essential oil composition. Freshly collected or dried young and mature leaves and flowers of both *L. alba* chemotypes grown in an experimental garden were subjected to microwave-assisted hydrodistillation for 30, 45, 60, and 90 min. GC-MS (*Agilent Technologies* 6890 Plus/5973/5975, DB-5MS and DB-WAX 60 m x 0,25 mm, D.I columns) analysis of the resultant oils showed large variations in composition. Figure 5 summarizes the composition variations registered as a function of extraction time and plant age. Carvone relative amount varied between 28,9 and 53,9%, while citral relative amount changed from 22,5 to 89,2% in the oils of the 2 chemotypes, respectively. Dried young leaves (3 weeks), afforded the oil with the largest carvone yield after 30 min of hydrodistillation. For the other chemotype, the largest citral yield was obtained from dry young leaves (3 weeks) after 90 minutes of hydrodistillation. These oils are interesting raw materials for carvone and citral isolation, which are extensively used as flavoring agents and as starting materials in fine organic synthesis.

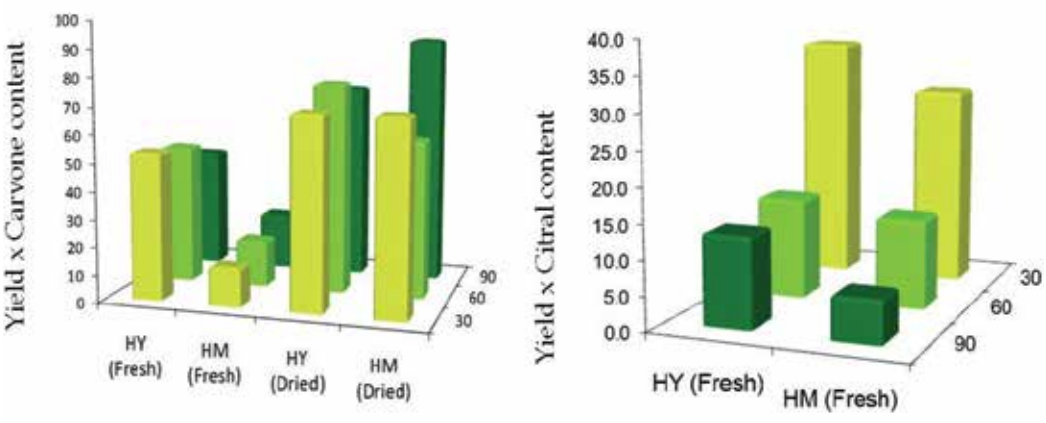


Fig. 5. (A)Variation of the product of carvone content (%) and essential oil extraction yield, with distillation time and vegetal material maturity and moisture, for the carvone-rich *Lippia alba* chemotype. (B)Variation of the product of citral content (%) and essential oil extraction yield, with distillation time and vegetal material state, for the citral-rich *Lippia alba* chemotype.

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13

# The Qualitative and Quantitative Determinations of Volatile Constituents in Some Herbal Medicines by Gas Chromatography

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## 1. Introduction

Many herbal medicines pertain characteristic odour which indicates the volatility of some compositions. Most of the volatiles constituents have low molecular weight, typically, monoterpenes, sesquiterpenes and phenylpropanes and their oxygenated derivatives (Harnone, 1998). Some herbs contain high content of volatile oils from which the oil themseleves are used as drugs, for example eucalyptus oil, eugenol etc., but some are very low. Volatile oils are some times called essential oils due to the believed that it is the essence of the herb. At present, qualitative or identification by gas chromatography (GC) is well established due to the availability of mass spectrometer (MS) detector. When constituents are identified, the interested components can be quantitatively determined by the flame ionization detector (FID).

The purpose of this chapter is to simplify technique of GC for new users in qualitative and quantitative determinations of herbal medicines. The advantage for the method compared to other chromatographic methods is that it can be used to determine complex mixtures with very minute amounts and accurate. This chapter will be emphasized on practical points of view and will be elucidated by examples from literatures.

## 2. Gas chromatographic system

As we already know that GC is used to separate mixtures which vapourise at the operating temperatures. The main parts of gas chromatography are injector, column oven and detector. Running gas chromatography requires accessories one of which is a column which is the crucial part of the separation.

### 2.1 Injector

Injector usually set 50°C above the boiling point of the highest boiling point of the component in the mixture. For separation volatile oils it is usually set around 170-290 °C. Usually sample is injected directly into the injector through the injector rubber septum. Sample will be flushed through the column with a carrier gas. The whole amount of injected sample can be loaded or splitted into 1:10 or 1:100 or 1:500 as desired onto the column which depended on the concentration of the sample.

Helium, hydrogen argon, nitrogen and air can be used as a carrier gas. Hydrogen and nitrogen can be generated from the gas generators which are available in the market. Helium and argon cost more. However, air and hydrogen are usually avoided since they are reactive to some analytes.

### 2.2 Column

Glass capillary column coated with methyl silicone, carbowax, 5%-phenyl-methylsiloxane are usually employed for the separation. Column temperature programming can be preliminary set by using medium ramping time of each step of increments, if the components are eluted unresolved or too far apart, the programme can be optimised. The maximum temperature for each type of column has to be carefully checked.

### 2.3 Detector

There are many types of detector available for a GC system. In this chapter, only MS and FID will be referred.

#### 2.3.1 Mass spectrometer detector

Mass spectrometer is a very useful detector since the mass spectrum can be acquired which is a further step to confirm the compound being analysed. Besides that the intensity of the fragment which is the base peak of the spectrum can be used to increase the sensitivity when the parent peak is very low. The industry standard for sensitivity of GCMS instruments is based on octafluoronaphthalene (OFN); the sensitivity of an instrument is defined as the signal to noise (S/N) obtained from a 1μL injection of a solution containing 1pg/μL OFN (Shimadzu, 2007). However this sensitivity is not always implied to our interested compounds, the chromatographic conditions, the MS conditions employed as well as the fragmentation of the compound itself involves in the sensitivity of the GC-MS.

High purity of helium is usually employed as a carrier gas for a GC-MS. For the mass spectrometer detector, the interface temperature usually set at not lower than the injector temperature, the ion source is also high closed to the interface. Scan mode for positive or negative ions, range of mass required, rate of the scanning and solvent delay can be set as required. The setting methods can be found in the instrument manual. Example of setting these parameters are shown in the analysis of *Mimusop elengi* and *Curcuma aeruginosa.*

The mass spectra of constituents in volatile oils which are monoterpenoids, sesquiterpenoids, and phenylpropanoids have been extensively studied, well documented and collected as a data base. Data base of mass spectra of various volatile components especially which are main constituents in most fragrance, essential oils and many other odorous components are organized systematically as libraries *i.e.* NIST (National Institute of Standard and Technology) or FFNSC (Flavour and Fragrance Natural. and Synthetic Compounds). Thus qualitative analysis of volatile oil in these days is easy to perform. However, similarity of the same group of compounds or variation of the GC system might cause some difficulty in the identification. In ionization a compound in a mass spectrometer of the GC instrument, an electron impact is used. The energy usually employed is 70 eV, which is quite strong thus parent peaks might not be detected. But if they do exist, the molecular weight of many compounds are equal.

Fragmentation of these compounds are also similar. Therefore assignment of the individual component requires additional information, linear retention index or Kovat's index and confirmation with authentic standards. Manual interpretation of the mass spectrum is some time needed, when there is no fitted matching. Isotope abundance is very useful to postulate the identity of molecule or fragment.

### 2.3.2 Flame ionization detector

The flame ionization detector is the most commonly used for GC. It is considered an economical detector. The gas used for the detector is hydrogen and air. Signal out put of the flame detector is very sensitivity, 1pg/s, however the sensitivity also depends upon the sample preparation and the GC instrument. For example for benzene with the headspace GC-FID of one model is 0.02 ppm and 0.06 ppm for detection and quantitation limits, respectively which is more sensitive than the other model of the same manufacturer (Agilent technology, 2007). In quantitative analysis of a known compound by a GC, a MS detector can be used but usually avoided for routine analysis due to the running cost.

## 3. Isolation of volatile oil from its matrix

Isolation of volatile oil by solvent extraction, steam distillation or enfleurage have been employed for a long time. Steam distillation seems to be the method of choice since non-volatile components are excluded. However, decomposition of some components causing the alteration of its original odour is the main drawback. These decomposed components are considered as an artefact. At present, headspace can be used as an *in situ* separation which required an extra part to transfer the sample into the gas chromatographic column. However, steam distillation and solvent extraction are still in use these days, since they are simple and the methods can be done in any laboratory.

### 3.1 Steam distillation

This method, specimens and water are heated in a steam distillation apparatus, once the water is boiled, volatile constituents will be carried along with the water vapour and then condensed in to a trap containing water and an immiscible solvent. Water and the solvent will separate into two phases. The volatile oils is dissolved in the solvent. A standard method described in British Pharmacopoeia (BP) 2010, Essential oil in Herbal Drugs, is very useful in determining the total content of volatile oils in herbs. The volatile distillate thus obtained can be used for determination of the constituents either qualitatively or quantitatively.

### 3.2 Solvent extraction

Diethyl ether, acetone and low molecular weight hydrocarbons, pentane or hexane, are commonly employed. Ether and acetone is more advantage due to its better ability to dissolve more oxygenated compounds. The advantage of these solvents are their high volatility, 34.6°C, 56°C for ether and acetone and 36°C and 54°C for pentane and hexane, respectively. The tendency of these solvents interfering the elution of the peaks of the volatile components is low. Further more if the extract has to be concentrated, these solvents are easily expelled.

### 3.3 Enfluerage

The methods is to allow the volatile substances to be absorbed by fat and then dissolved into alcohol. This method is usually applicable for the old process in fragrance manufacturing.

### 3.4 Headspace

This method, sample containing liquid or solid is sealed in a closed container with an empty space for vapour to be volatilised and collected. The sample is warmed up for a period of time to reach equilibrium in the container. The vapour above the liquid is sampled and injected into the column using pressure as the injection mode. This technique started about 30 years ago. It is already one technique used in the USP 34 to determine the residual solvents in pharmaceutical ingredients as well as in BP 2010. In herbal medicine, this technique becomes very popular due to the ease, convenience, requiring low amount of specimen and less time consuming. The drawback is that an extra cost for the installation of the headspace part. At present rapid headspace solid phase extraction were employed for determination of volatile components in herbs, flowers and wine flavour (Cha, 2009; Won, 2009; Boutou, 2007).

## 4. Qualitative determination

The availability of the GC-MS makes qualitative analysis of volatile oil simple. If the volatile oil of a herb has been investigated and our herb is in same species and we would like to check only the varieties of the herbs that deviated from the previous investigation, the GC-FID can be employed. However, some misinterpretation or discrepancy results might occur. The whole constituents have to be redone and GC-MS is most recommended. In identifying a compound, a mass spectrum itself is very useful, theoretically the parent mass and fragmentation of compound is due to its structure. However, volatile components are similar, for example all monoterpenes consist of 10 carbons. Fragment masses are also similar. Thus additional confirmations is required, such as Linear retention index (LRI) or Krovat's index. Confirmation with authentic compound is also recommended, since it is not only confirm the identity of the compound. It can be used to relate to others constituents in the prior or subsequent eluted components. This is because the sequence in eluting of these compounds on the same type of column are the same. In the qualitative assay, libraries of the volatile components are available and installed in the GC-MS instrument, NIST (National Institute of Standard and Technology) or FFNSC (Flavor and Fragrance Natural. and Synthetic Compounds). At present, even the linear retention time can be confirmed from the library. Identification of the component from the mass spectrum can be done by comparing the obtained mass spectrum with those stored in the library. Similarity index or percentage of fitting indicates the probability of the identification. If the LRI fitting is not available in your instrument, LRI of such compound can be checked with those in literatures, *i.e.* Davies, 1990 and Adams, 2007.

### 4.1 Linear retention index calculation

The volatility of the series of *n*-alkanes (HC) depends on their molecular weight which in turns the chain length. They are nonpolar, their interaction with the GC column are the same, their retentions on the GC solely depend upon the molecular weight. Therefore, it is useful to use as references for gas chromatographic system.

A mixture of *n*-alkanes (HC), $C_{10}$-$C_{25}$ are usually used, 10 µL or 1 mg each of the *n*-alkanes in 100 mL of pentane or hexane or the solvent used for sample. Commercial products of the combination of these *n*-alkanes are available. Temperature programme for the column oven is available in nearly all GC instrument. If the initial temperature of the programme is high, the low molecular weight HCs can be omitted since they will be eluted simultaneously with the solvent front. Most of the linear retention of constituents for volatile oils starts from $C_{10}$. On the contrary, lower molecular weight HC can also be used if constituents pertains low vapour pressure, initial temperature should be set accordingly low. The time of each constituent eluted from the column related the series of HCs are used to calculate the LRI. For example, if the retention time of $C_{14}$ and $C_{15}$ is 11.4 and 12.9 minutes, and the retention time of a component is 11.7, the LRI of the component is 1420, by using the following equation.

LRI $= 100[(t-t_n/t_{n+1}-t_n) +n]$
t = retention time of the constituent
n = carbon number of the preceding *n*-alkane
n+1 = carbon number of the subsequent *n*-alkane
LRI $= 100\ [(11.7-11.4)/(12.9-11.4) + 14]$
LRI $= 1420$

Example 1. Qualitative determination of volatile oil extracted from *Mimusop elengi* flowers:

Dried *M. elengi* flowers are used in many Thai traditional medicine recipe'. The degree of dryness of the flowers was not specified. In establishing the standards for this flower for Thai Herbal Pharmacopoeia, analysis of the left over constituents is one of the aspect to be carried out. Fresh and 4-week air dried flower were GC-MS examined. Fig.1 is the GC-MS

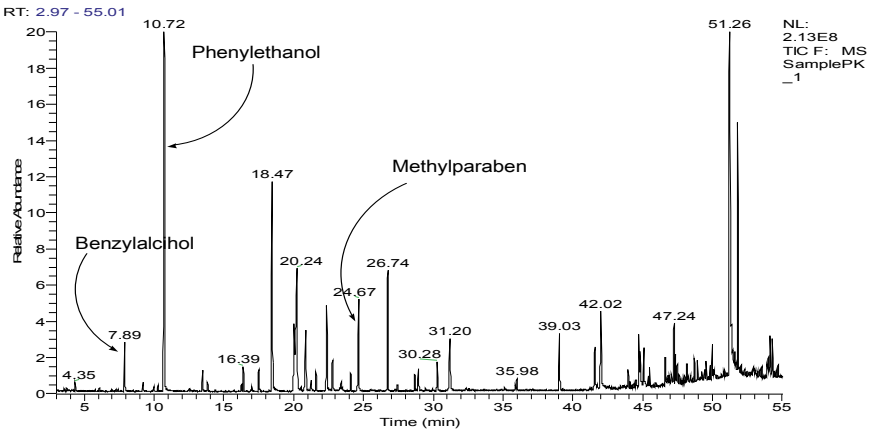



Fig. 1. Chromatogram of fresh *Mimusop elengi* flower. GC system: Column: TR-5 (5%phenyl-95%dimethylpolysiloxane), 30m, 0.25µm, 0.25 mm ID; Oven temperature program: 60 °C to 180 °C at 3 °C.min$^{-1}$, to 280 °C.min$^{-1}$ at 10 °C.min$^{-1}$ (hold 5 min); Run time: 55.00 min; Carrier gas flow rate (Helium): 1 ml/min; Transfer line temperature (Interface temp.): 275 °C; Ion source: 220 °C. Scan mode: full scan 35-650 m/z; Scan rate: 2000amu/s; Solvent delay time: 3min. Instrument: TraceGC ultra, Model K05200B20000070, Italy/MS Model TraceDSQ,USA; Software: Xcalibur 1.4; Library: NIST MS Search

chromatogram of fresh *M. elengi* ethereal extract and Fig. 2 is the dried flowers' ethereal extract. Fig. 3 is the chromatogram of *n*-alkane series, $C_{10}$-$C_{23}$. From Fig. 3 the retention times of $C_{11}$ and $C_{12}$ are 10.08 and 14.00 min, respectively. The LRI of the component at 10.72 min was calculated to be 1116. Its mass spectrum is shown in Fig. 4. From the matching to the NIST-MS library, the compound is 2-phenylethanol, see also Fig. 4. Authentic standard of 2-phenylethanol was injected to confirm the identity (Aromdee, 2009). Langlois (1996) found that the LRI of 2-phenylethanol was 1280. However, the column used was DB1701 which is coated with 14%-cyanopropyl-phenyl–methylpolysiloxane. As we can see that LRI of compounds also depended on the analysis system used, thus mass spectrum and checking with authentic compound is some time necessary. Anyway, there are many components in a volatile oil, checking all components with authentic standard is not possible. However one compound confirmation can be useful in the confiding of the GC system being used.

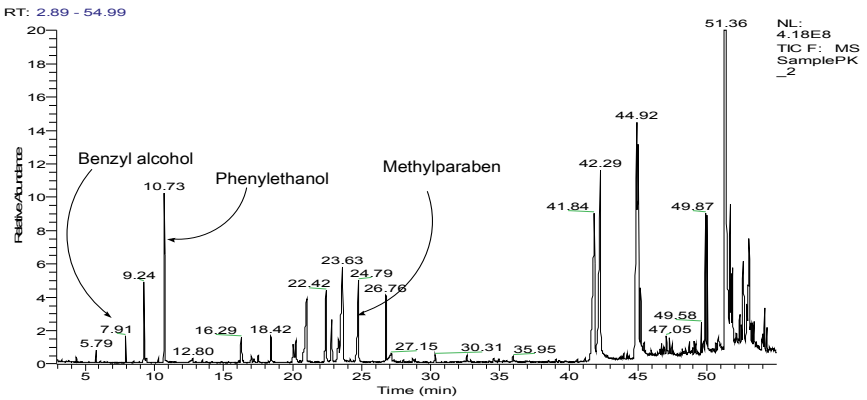



Fig. 2. Chromatogram of dried *Mimusop elengi* flowers. System was the same as described in Fig 1.

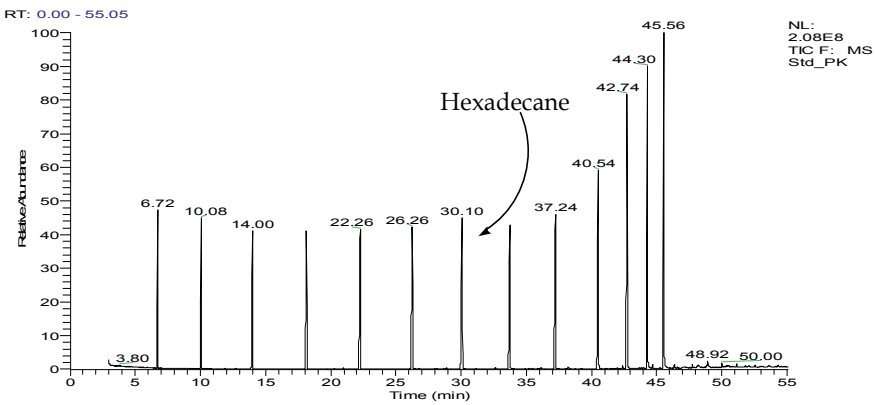



Fig. 3. Series of *n*-alkane $C_{10}$-$C_{23}$. Retention time of $C_{10}$ is 6.72 and $C_{23}$ is 45.56 min. GC system was the same as fresh flowers, Fig. 1.

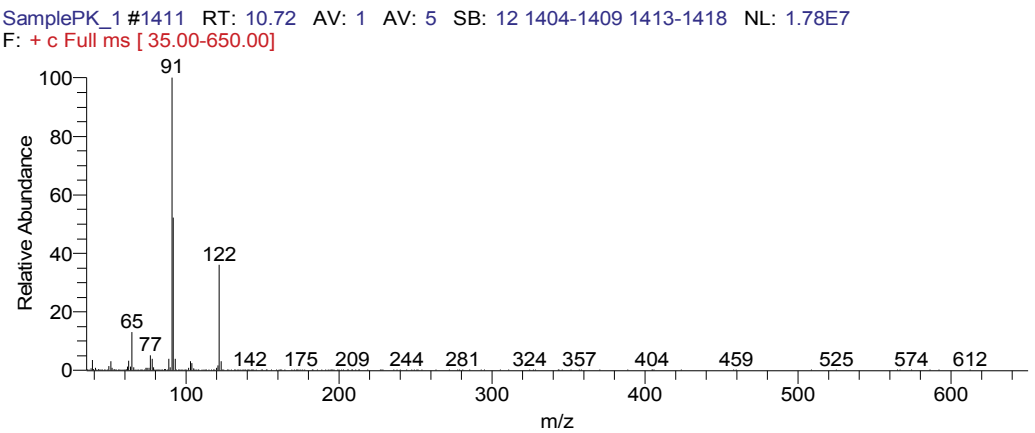



Fig. 4. Mass spectrum of the peak with the retention time of 10.72 min. From the NIST library search, the compound was matched with 2-phenylethanol (phenylethyl alcohol) with 932 similar index (SI).

| Components | Retention time | | LRI | % Relative amount | | Method of identification |
|---|---|---|---|---|---|---|
| | Fresh flower | Dry flower | | Fresh flower | Dry flower | |
| Benzyl alcohol | 7.89 | 7.91 | 1040 | 0.79 | 0.38 | MS, LRI, Std |
| 2-Phenylethanol | 10.72 | 10.73 | 1116 | 10.49 | 3.29 | MS, LRI, Std |
| 3-Phenyl-2-propene-1-ol | 18.46 | 18.43 | 1308 | 6.17 | 0.53 | MS, LRI |
| 4-Hydroxybenzene methanol | 20.24 | 20.25 | 1354 | 8.69 | 0.41 | MS, LRI |
| Methyl 4-hydroxybenzoate | | | | | | |
| 2-Butyl-phenol | 24.67 | 24.78 | 1462 | 2.69 | 1.74 | MS, LRI, std |
| Hexadecanoic acid | 31.20 | - | 1632 | 1.74 | - | MS, LRI |
| Long chain carboxylic acid | 42.02 | 42.02 | 1968 | 2.43 | 0.07 | MS, LRI |
| Unidentified | - | 42.29 | 1980 | - | 5.37 | MS, LRI |
| (Z)-9-Octadecenoic acid | - | 44.92 | 2150 | - | 6.65 | |
| Octadecanoic acid | - | 45.02 | 2158 | - | 4.71 | MS, LRI |
| | - | 45.18 | 2170 | - | 0.66 | MS, LRI |

Table 1. Volatile constituents in fresh and dried *Mimusop elengi*

If we look back at Fig. 3 the elution of *n*-hexadecane was at 30.10 min which does not interfere with any component in dried flowers extraction (Fig. 2), it was later selected as the internal standard for quantitative analysis. The tenderly odour of the flowers still exists in most of the dried flowers purchased from traditional medicine stores in Thailand. Some active constituents of which the pure chemicals are commercial available were determined to represent existing components in the dried flowers, which will be described in section 5 "Quantitive Determination". There are more than 50 components identified in *M. elengi*. Some components in the fresh flowers and 4-week dried flowers were compared as can be seen in Table 1.

Example 2. Qualitative determination of steam distilled volatile oil from *Curcuma aeruginosa* rhizome:

Steam distilled volatile oil of *C. aeruginosa* was similarly determined for the composition. The GC-MS system employed was similar to that of *M. elengi* except the ramping rate and final temperature. The content of volatile oil in *C. aeruginosa* is very rich, the injection was splitted 1:100. The chromatogram is shown in Fig. 5 and the *n*-alkane series is in Fig 6.

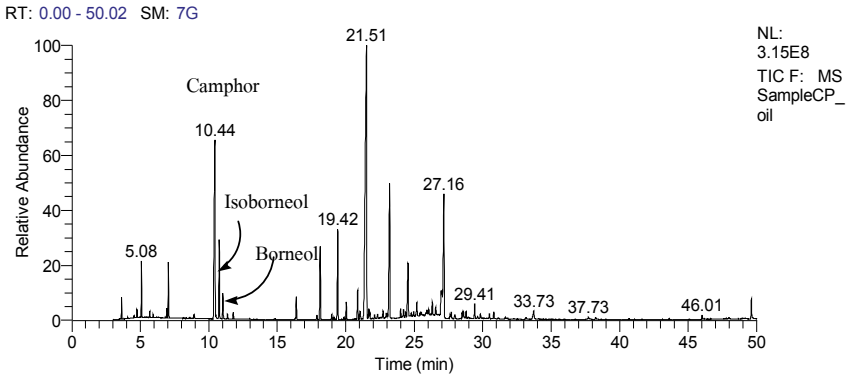



Fig. 5. GC-MS chromatogram of the steam distilled volatile oil of the rhizome of *C. aeruginosa*. Camphor, isoborneol and borneol were eluted at 10.44, 10.65 and 10.99min, their LRI were 1150, 1160, 1177, respectively. GC-MS system: 1 µL (split mode 1:100) at 270 °C onto a TR-5 column using helium as a carrier gas at a flow rate of 1 mL.min$^{-1}$. The oven temperature was programmed for 60 – 240 °C (4 °C.min$^{-1}$) and 240 – 270 ° C (10 °C.min$^{-1}$) then held for 2 min. The detector and interface were maintained at 275 °C and the ion source at 220 °C and the MS scanned in positive ion mode over 35 – 650 m/z. Obtained from the same instrument as Fig. 1.

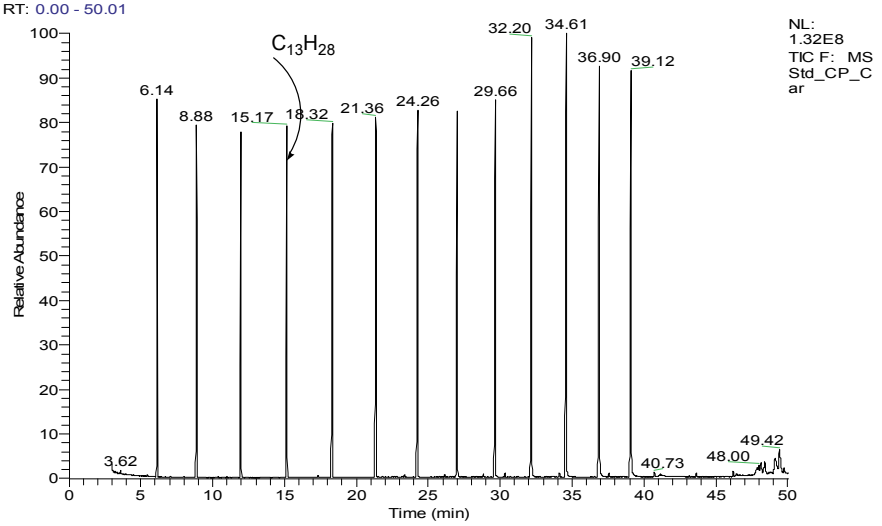



Fig. 6. GC chromatogram of $C_{10}$-$C_{22}$ running in the same conditions as Fig. 5

In matching the mass spectrum obtained from experiment and data base of monoterpenes sesquiterpenes were usually found. It is quite often that mass spectra of components were not matched with any one in the library, thus interpretation from parent peaks and fragmentations is necessary. For example in determination of the volatile oil of *Azadirachta indica* obtained by steam distillation (Aromdee, 2006), terpenoids were indentified as well as some dimers of sulphides were found, dipropyl disulphide, propyl propenyl disulphide, they were elucidated from the LRI and the MS. Chemical dimerisation of sulphide is common. However, some other sulphur containing compounds with no availability of LRI were found. Thus, interpretation from mass spectra, fragmentation and isotopic abundance was carried out. The natural isotopic abundance of sulphur, its atomic mass +1 (A+1) is 0.79% whereas the A+2 is 4.4% (McLafferty, 1993). In the study, the isotopic mass of one of the components with the retention time of 19.52 minutes (m/z 166) was found to contain 3 atoms of sulphur. The peak was assigned as $HSSSC_5H_9$. The mass spectrum details are as follows: m/z (ion mass, relative intensity): 41 ($C_3H_5^+$, 83), 45 ($CH{\equiv}S^+$, 32), 47 ($S^{\bullet+}CH_3$, 11), 59 ($SC_2H_3^+$, 71), 60 ($S^{\bullet+}C_2H_4$, 86), 64 ($2S^{\bullet+}$, 59), 69 ($C_5H_9^+$, 94), 73 ($C_3H_5S^+$, 50), 74 ($C_3H_6S^{\bullet+}$, 40), 92 ($C_2H_4S_2^{\bullet+}$, 48), 101 ($M^+$ - SSH, 53), 102 ($M^+$ - 2S, 69), 106 ($M\text{-}C_2H_4S$, 34), 166 ($M^+$, 100). The isotope peak (M+2) at m/z 168 (13.2% of m/z 166) indicated the existence of 3 sulphur atoms and 5 carbons and the compound/fragment could be an artifact due to the vigorous isolation, steam distilled, and the high temperature GC conditions. Anyway, the definite identities have to be confirmed. The odour of *A. indica* is pungent and strong, thus it is not surprising that *A. indica* contains many sulfur containing compounds.

Some other elements with the high % isotopic mass is also easy to elucidate, for examples, A+2 of chlorine is 32.0 % and of bromine is 97.3%. Unfortunately, not many volatile components in herbs were detected to contain these two elements.

## 5. Quantitative determination

### 5.1 Total content of volatile oil

Herbs which are rich in volatile oil for example anise seed, cinnamon, caraway, curcuma species, *etc*. The total content of volatile oil in the herbs indicate the quality of the herbs. Thus the lower limit of volatile oil content is one of the specification for these herbs. Many official monographs of drug containing essential oil or volatile oil in British Pharmacopoeia and European Pharmacopoeia limit the content of volatile oil as one of the specification of the drug, for example "Ginger BP 2010" contains essential oil not less than 1.5 %v/wt (anhydrous drug). Besides that the chromatographic profile of essential oils were also imposed in these pharmacopoeiae. The limits of relative contents of some characteristic essential components of the oil are specified.

Steam distillation is the standard method used for the determination of volatile oil. In British Pharmacopoeia, the dimension of the essential oil apparatus was specified, since all parts of the apparatus affect the yield of the volatile oil and avoid any controversy in the quality of the herbs. This method is usually employed to determine high volatile oil content herbs. Aromdee *et al* steam distilled various species of curcumas, *Curcuma aromatica*, *C. aeruginosa* by the method described in BP using xylene as an organic entraptor. They found the volatile oils in *C. aromatica* was 0.88-0.96% v/wet wt (Aromdee, 2010), where as *C. aeruginosa* was 0.55-0.42%v/wet wt (Aromdee, 2007). The content of volatile oil in other species of curcuma,

*C. longa*, which is official in Thai Herbal Pharmacopoeia 1 (THP1,1998) is not less than 7%v/w. In establishing the standard limit of content of volatile oil of herbs in national or international pharmacopoeiae, 12-20 authentic samples from various parts of the country or reliable sources have to be collected. Replicate determinations of the volatile oil content in each of these samples have to be carried out according to the pharmacopoeial standard method. Mean and standard error of the results are used to set the limit of content of volatile oil.

By the way, some low content of herb was also studied by steam distillation, *i.e. Azadirachta indica* gave very minute amount of the volatile components (0.001% v/wet wt).

### 5.2 Quantitative determination of constituents in volatile oil

For quantitative determination of some components in volatile oil, internal standard usually required for gas chromatography since the injector is set at high temperature, uniformity of the injection through the septum, time delay in the injection involving in the irreproducibility of the method. At present, although auto-injector improves the reproducibility of the injection, but internal standard is still in used for the accuracy and precision of the analysis. Analytical method developed to determine the constituents in volatile oil has to be validated. International Conference of Harmonisation (ICH), the quality guidelines are widely used for the validation since it is accepted universally. The guidelines are revised periodically, thus updating the guidelines should be caught up occasionally. However, not much alteration in each revision since it have to be relied on basic concepts which are accuracy, precision, limit of quantitation and limit of detection. These parameters are important for the reliability of the method and result.

#### 5.2.1 Finding for an internal standard for quantitative determination of volatile oils

If the LRI of the component to be acquired is known, it is easy the find the internal standard. The *n*-alkane closed to, but does not overlap or interfere the adjacent components or peak of the volatile oil, can be used. For example Polrat used tridecane as internal standard to determine camphor, isoborneol and borneol in some curcuma spp. (Aromdee, 2007). Aromdee (2010) used *n*-hexadecane as an internal standards for determination of benzyl alcohol (1040), 2-phenylethanol (1119) and methylparaben (1525) in *M. elengi*. *n*-Alkanes are universal used for internal standard for gas chromatographic determination of volatile constituents, but not limit to the herbal volatile oil.

To quantify an interested component in the volatile oil, standard solutions of the component have to be prepared in a series in the range that cover the expected concentration of the sample. Once volatile oil is isolated, an exact portion of volatile oil will be taken for the determination. An exact amount of internal standard can be added directly to aliquots of the sample and the standard solutions, then the mixtures are diluted to the appropriate concentration. By the way, internal standard can be added to the diluting solvent and an equal volume of this diluting solvent must be used in the standard series and sample preparations.

Aromdee *et al* quantitatively determined the benzyl alcohol, 2-phenylethanol and methylparaben which are believed to be preservatives for preparations containing dried mimusop's flowers. *M. elengi* flowers was extracted with ether and concentrated under the

nitrogen atmosphere. The residue was reconstituted with ethyl acetate. *n*-Hexadecane was used as an internal standard as it does not interfere with any peak of the extract, see Figs.2 and 3. Validation of the method was carried out for accuracy, precision, limit of quantitation, limit of detection and linearity range as directed in ICH guidelines.

Example 3: Quantitative determination of benzyl alcohol, 2-phenylethanol and methylparaben in dried *M. elengi* flowers:

**Stock standard solutions (SS):** Pipette 10 and 100 μL of benzyl alcohol and 2-phenylethanol respectively into a 10-ml volumetric flask, add 10 mg of methylparaben. Dilute to the volume with ethyl acetate. Standard solutions are prepared as shown in Table 2.

| Solution | Volume used (μL) | | | |
|---|---|---|---|---|
| | Sample | SS | IS | Ethyl Acetate |
| 1 | | 0 | 100 | 200 |
| 2 | | 20 | 100 | 180 |
| 3 | | 100 | 100 | 100 |
| 4 | | 150 | 100 | 50 |
| 5* | Sample residue | 20 | 100 | 180 |
| 6* | Sample residue | 100 | 100 | 100 |
| 7* | Sample residue | 150 | 100 | 50 |

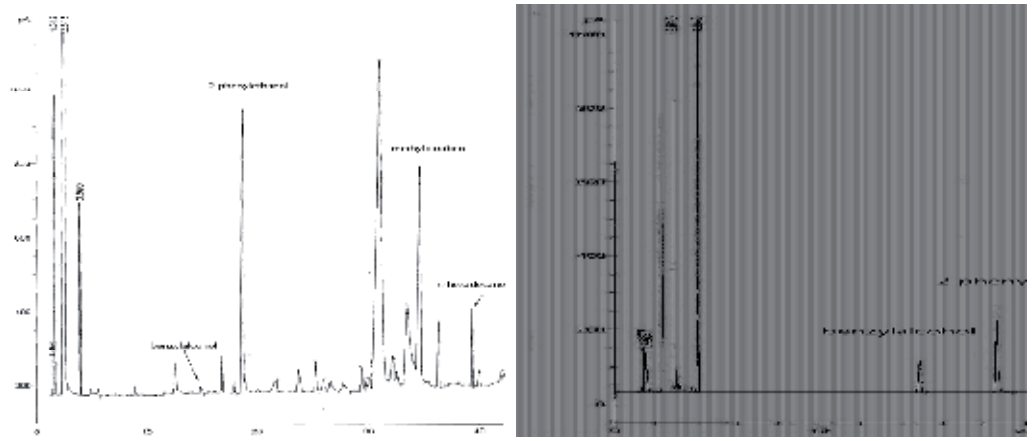
Table 2. Preparation of standard solutions for *M. elengi* (* Used for accuracy validation)

**Internal standard solution (IS):** Pipette 100 μL of *n*-hexadecane and diluted with ethyl acetate to 100 mL.

**Sample preparation:** Extract the finely ground powder of dried *M. elengi*, 3 g, with ether, 12, 5, 5 mL of ether successively. Evaporate the combined extraction solutions under the steam of nitrogen. Add 200 μL of ethyl acetate and 100 μL of internal standard solution to the residue. Vortex for 5 minutes, centrifuge, and inject the supernatant.

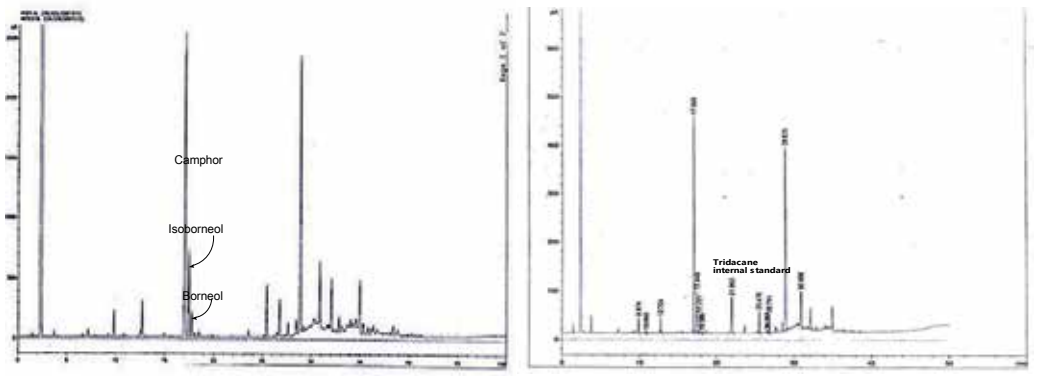
Three sample solutions of the specimen were prepared and each was injected trice as well as the standard solutions and the standard addition samples. The standard curve was constructed for the interpolation of the three components. Reduction of the contents found in the sample solution from the results of standard addition sample, the percent recoveries were obtained which are 91.66, 104.59 and 105.28% benzyl alcohol, 2-phenylethanol and methylparaben, respectively. This dried sample of *M. elengi* was found to contain 13, 196 and 232 ppm of benzyl alcohol, 2-phenylethanol and methylparaben, respectively. Other parameters were also carried out which are the reproducibility, the limits of detection and quantitation. Fig.7 is the chromatogram of dreid *M. elengi* flower extract acquired by the GC-FID.

Example 4: Quantitative determination of camphor, norborneol and borneol in *Curcuma aromatica* and *C. aeruginosa* rhizomes. From Fig. 6 we found that $C_{13}H_{28}$ is suitable to be used as an internal standard, thus it was selected. Fig. 8 showed the chromatograms of the quantitative analysis. Preparation of standard solutions in shown in Table 3. Validation of the method was also carried out according to the ICH guidelines.



a) Chromatogram of *M. elengi* b) Chromatogram of standard solutions

Fig. 7. Gas Chromatograms of a dried *M. elengi* flowers and standard. The specimen was purchased from a Thai traditional medicine store (a). sample extract with *n-hexadecane* as an internal standard; and (b) standard solution containing benzyl alcohol, 2-phenylethanol, methylparaben and the internal standard. Gas chromatographic system: GC (Hewlett Packard HP 6890, USA)/ FID detector; Column: (5%-Phenyl)-methylpolysiloxane, 30m, 0.25µm, 1 mm ID; Oven temperature program: 60 $^{o}$C to 180$^{o}$C at 3 $^{o}$C.min$^{-1}$, to 280 $^{o}$C.min$^{-1}$ at 10 $^{o}$C.min$^{-1}$ (hold 5 min); Run time: 55.00 min; Carrier gas flow rate (Nitrogen): 2 m-l/min.




a) *C. aeruginosa* by the GC-FID system b) *C. aeruginosa* with internal standard $C_{13}H_{28}$

Fig. 8. Gas Chromatogram of volatile oil from rhizomes of *C. aeruginosa,* 1 µL of volatile oil solutions was injected (split mode 1:50) at 270°C onto a HP-5 column using nitrogen as carrier gas at a flow rate of 2 mL.min$^{-1}$. The oven temperature was programmed for 60–240° C (4°C.min$^{-1}$) and 240–270°C (10°C.min$^{-1}$) then held for 2 min. The FID detector was maintained at 275°C.

**Stock Standard Solutions (SS):** Accurately weigh about 0.5g camphore, 0.075 g of isoborneol and borneol into a 5 mL volumetric flask, diluted to volume with ethyl acetate.

**Internal Standard Soltion (IS):** Pipette 100 µL of tridecane and diluted with ethyl acetate to 100 mL.

**Sample preparation:** Dilute 100 μL of volatile oil to 10 mL with ethyl acetate.

| Solution | Volume used (μL) | | | |
|---|---|---|---|---|
| | Sample | SS | IS | Ethyl Acetate |
| 1 | | 0 | 500 | 500 |
| 2 | | 20 | 500 | 480 |
| 3 | | 100 | 500 | 400 |
| 4 | | 150 | 500 | 350 |
| 5* | 30 | 20 | 500 | 450 |
| 6* | 30 | 100 | 500 | 370 |
| 7* | 30 | 150 | 500 | 320 |

Table 3. Preparation of standard solutions for camphore, norborneol and borneol in *Curcuma spp*. (*For accuracy validation)

## 6. Conclusion

Gas chromatography is very useful for the determination of complex mixtures of analytes. The detectors employed for the qualitative analysis is a mass spectrometer. Whereas the quantitative analysis, both detectors are useful. Factors affect the separation are the type of the column, temperature programming of the column oven, meanwhile sensitivity is mostly depended on detector as well as the isolation and injection method of the volatile constituents. In qualitative analysis of volatile oil in herbal drugs, a GC-MS is required and LRI of the constituents have to be determined. For quantitative determination both GC-MS and GC-FID are applicable but FID is more economical. Separation of the volatile oils from herbs, steam distilled is still the method of choice for conventional method. Headspace is now widely available and convenient. In this chapter, examples of both qualitative and quantitative analysis of *Mimusop elengi* flowers and rhizomes of *Curcuma spp.* were illustrated in details.

## 7. Acknowledgement

I am indebt to the School of Chemistry, University of New South Wales, Australia, where I first learnt the gas chromatography, Dr. Joseph Brophy who inspired me about the GC-MS.

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# 14

# Designing Novel Functional Food Using Gas Chromatography

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## 1. Introduction

Fish and fish oils are well known to possess many protective properties against cardiovascular diseases (Kris-Etherton et al., 2003) due to high content in the long-chain ω-3 polyunsuturated fatty acids (PUFA); eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) which are thought to have anti-thrombotic (Din, Newby & Flapan, 2004) and anti-inflammatory properties (Rennie et al., 2003) and also due to micro constituents with anti-thrombotic properties (Nasopoulou et al., 2007; Nomikos et al., 2006; Kristensen et al., 2001; Panayiotou et al., 2000; Mori et al., 1997) making seafood an important component of human's diet.

In addition, the population growth and rising consumer demand result in a continuously increasing demand for fish supply and aquaculture seems to satisfy these requirements at present. Regarding aquacultured fish, one of the basic dietary ingredients of the compounded feeds is fish oil, because of its high digestibility and sufficient content of essential fatty acids, in particular ω-3 PUFA. At the moment, the aquaculture industry uses an estimate of approximately 40% and 60% of the global production of fish meal and fish oil, respectively (Tacon, 2005) while the production of fish meal has remained more or less stable from the late 1980s at about 6 million metric tons/annum (Food and Agriculture Organization (FAO), 2004) suggesting that food grade fisheries providing fish oil and fish meal may reach their limit of sustainability within the next few years (Pike & Barlow, 2003; Shepherd et al., 2005).

Fish oil substitution in compounded fish feeds by plant oils could be a promising solution in order to limit fish oil demand for fish feed formulation as well as to reduce costs since plant oils have steadily increasing production, high availability and better economic value. Thus several studies have been carried out to clarify whether certain plant oils such as soybean, linseed, rapeseed, sunflower, palm oil, olive oil and mixtures of them can be used as partial substitutes for fish oils in compounded fish feeds (Alexis, 1997; Benedito-Palos et al., 2008; Caballero et al., 2002, 2004; El-Kerdawy & Salama, 1997; Figueiredo-Silva et al., 2005; Fountoulaki et al., 2009; Izquierdo et al., 2003, 2005; Kalogeropoulos et al., 1992; Montero et al., 2003, 2005; Mourente et al., 2005**,** 2006; Rosenlund, 2001; Torstensen et al., 2000, 2004; Wassef et al., 2009).

Soybean oil appears to be the preferred plant lipid source regarding fish growth while considerable savings in feed costs could be achieved if used as a partial dietary substitute for fish oil within compound feeds. The same goes to linseed and rapeseed oil but to a lesser extent (Wassef et al., 2009; El-Kerdawy & Salama, 1997). Furthermore, the use of palm oil and olive oil in fish feeds has given growth and feed utilization efficiency comparable to fish fed with equivalent levels of fish oil (Caballero et al., 2002; Mourente et al., 2005; Rosenlund, 2001; Torstensen et al., 2000, 2004).

However, replacement of marine fish oils with alternate oils of plant origin in the farmed fish feeds should occur not only to provide the sufficient quantities of lipids that meet fish essential fatty acid requirements for optimum growth, but also to maintain proper immune function in fish (Montero et al., 2003). Thus, the use of vegetable oils as a sole lipid source is limited.

Many researchers seem to believe that 60% fish oil substitution in compounded fish feeds by plant oils is the preferable percentage in order not to compromise growth performance or feed utilization efficiency of fish (Alexis, 1997; Caballero et al., 2004; Izquierdo et al., 2003, 2005; Montero et al., 2005; Mourente et al., 2005, 2006; Wassef et al., 2009).

Olive pomace and olive pomace oil are natural by-products of olive oil production, which contain micro constituents with antithrombotic properties (Karantonis et al., 2008) and phenolic/polyphenolic molecules with antioxidant and other pleiotropic actions. Recent data from our research team reported for the first time that partial replacement of fish oil in gilthead sea bream grow-out diet by lipids obtained from olive pomace resulted in sufficient fish growth similar to the one of fish fed with 100% fish oil diet combined with improved antithrobotic properties of fish (Nasopoulou et al., 2011).

## 2. Research goals

Our survey over the last years focus both on the *in vitro* and *in vivo* study of the nutritional value - in terms of cardioprotection - of foodstuffs containing micro constituents that block (either inhibit or antagonise) Platelet Activating Factor's activity. Platelet activating factor (PAF) (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine) (Demopoulos et al., 1979) is a potent inflammatory phospholipids mediator that is implicated in the mechanism of atherogenesis (Demopoulos et al., 2003). According to this mechanism, PAF is produced during LDL oxidation (Liapikos et al., 1994) and causes in situ inflammation. It is also known that PAF is a compound of atheromatic plaque and is essential for the activation of leukocytes and their binding in the endothelial cells (Mueller et al., 1995). Furthermore, evidence of the implication of PAF in atherogenesis is provided by studies in animals, which indicates that constituents that blocks PAF's action have protective effect on atherosclerosis (Feliste et al., 1989; Subbanagounder et al., 1999), while PAF-acetylhydrolases (PAF-AHs), the main enzymes responsible for the degradation of PAF, are active in native LDL but converted inactive forms in ox-LDL, leading to higher levels of PAF (Liapikos et al., 1994). Therefore, the presence of PAF-antagonists thus PAF-inhibitors or PAF-agonists (molecules that prevent PAF binding to PAF's receptor causing platelet aggregating far less potent than the one that PAF induces) in various foodstuffs is very important in terms of cardioprotection.

European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) were the core of our survey and considering a) the fact that one of the most common cause of mortality in industrialized countries is heart disease, b) the problem of fish oil dependence

for farmed fish feed formulation and c) of environmental degradation by the olive oil production by-products we tried to design a novel fish by partial replacement of fish oil by olive pomace in the fish diet. By this attempt we aim to reinforce the anti-thrombotic properties of fish – by using olive pomace which contain proven anti-thrombotic properties in the fish diet – to contribute in the resolution of fish oil dependence for farmed fish feed production and to exploit a natural by-product.

### 2.1 *In vitro* studies

Regarding the *in vitro* studies lipids of many traditional foods of the Mediterranean diet, such as fish (Nasopoulou et al., 2007; Nomikos et al., 2006; Panayiotou et al., 2000; Rementzis et al., 1997), olive oil (Koussissis et al., 1993), honey (Koussissis et al., 1994), milk and yogurt (Antonopoulou et al., 1996) and red wine (Fragopoulou et al., 2000) were studied for their inhibitory and /or agonistic properties against PAF.

Total lipids of the aforementioned foodstuffs were extracted according to the Bligh-Dyer method (Bligh & Dyer, 1959). In brief, an appropriate amount of chloroform/methanol/water 1:2:0.8 (v/v/v) solution was added to each sample and mixtures were shaken well and filtered. Phase separation of mixtures in the separatory funnels was achieved by adding chloroform and water to obtain a final chloroform/methanol/water ratio of 1/1/0.9 (v/v/v). Total lipids were obtained by the chloroform phase (lower phase) that was evaporated to dryness under nitrogen's stream and lipids were weighed and redissolved in 1 ml chloroform:methanol 1:1 (v/v). One tenth of the total lipids were stored under nitrogen in sealed vials at -20 $^{o}$C until used - after a short period of time - for biological assay, while the rest of it was further separated into polar lipids and neutral lipids using the counter-current distribution method (Galanos &

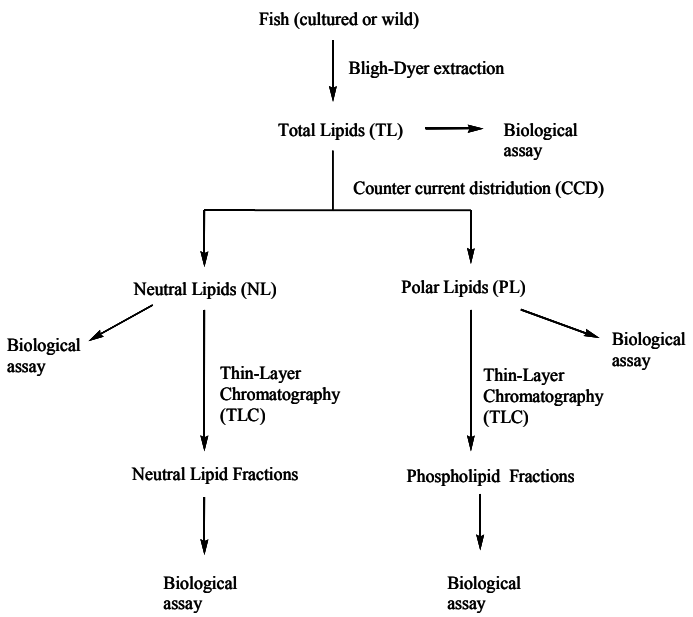



Fig. 1. Schematic diagram of the extraction and purification procedure.

Kapoulas, 1962). In brief, this method is based on polar and neutral lipids different solubility in pre-equilibrated petroleum ether and ethanol (87%). Polar lipids were soluble in ethanol while neutral lipids were soluble in petroleum ether. The obtained lipid fractions were weighed and stored under nitrogen in sealed vials at -20 $^{o}$C until used - after a short period of time - for the biological assay.

Regarding european sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) the polar and neutral lipids of farmed and wild fish specimens were further separated by preparative thin layer chromatography (Nasopoulou et al., 2007). The lipid fractions obtained were stored under nitrogen in sealed vials at -20 $^{o}$C until used - after a short period of time - for the biological assay (Figure 1).

The biological activity of total, polar and neutral lipids, as well as purified fractions of each lipid class after thin layer chromatography separations (Nasopoulou et al., 2007) was studied against washed rabbit platelets according to the method of Demopoulos et al. (1979) as shown in Figure 2.

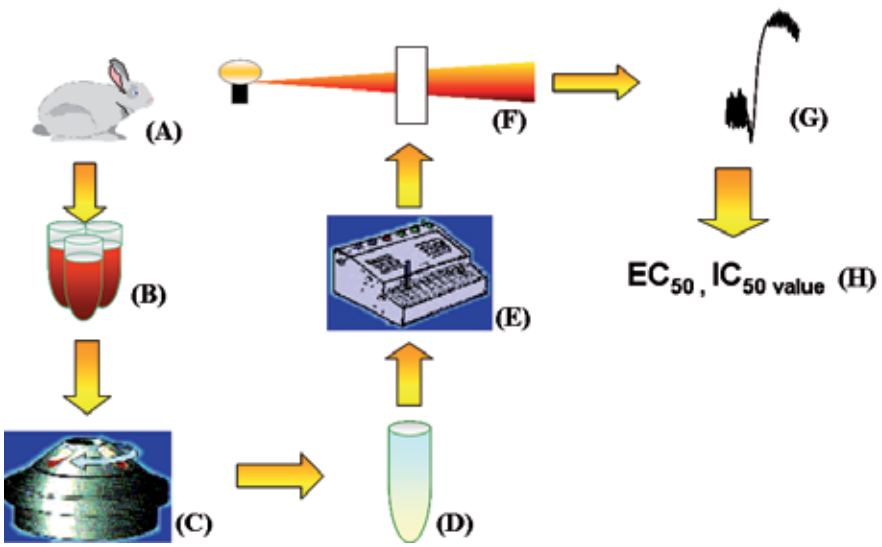



Fig. 2. Biological assay procedure.

Briefly, blood was collected through the main ear artery of the rabbit and was placed in polyethylene tubes containing a ratio of blood/anti-coagulant of 9:1 (v/v) (Figure 2 (A and B) and after adding the appropriate buffer solution and a sequence of centrifugations (Figure 2 (C)) according to the method of Demopoulos et al. (1979) washed rabbit platelets were collected (Figure 2 (D)). Following that the samples being examined and the PAF were dissolved in 2.5 mg of bovine serum albumin (BSA) per ml of saline. Various amounts of the sample being examined were added into the aggregometer cuvette - containing the washed rabbit platelets - in the aggregometer (fig 2 (E)), their absorbance was measured (fig 2 (F)) and thus, the permeability was recorded (fig 2 (G)) and their ability to aggregate washed rabbit platelets or to inhibit PAF-induced aggregation was determined (fig 2 (H)). In order to determine the aggregatory efficiency of either PAF or the samples being examined, the maximum reversible aggregation was evaluated and the 100% aggregation was determined.

The plot of the percentage of the maximum reversible aggregation (ranging from 20% to 80%) versus different concentrations of the aggregatory agent was linear.

From this curve, the concentration of the aggregatory agent, which induces 50% of the maximum reversible aggregation, was calculated. This value is defined as $EC_{50}$, namely equivalent concentration for 50% aggregation.

In order to determine the inhibitory properties of the samples, various amounts of the sample being examined, ranging from 0.0012 to 0.16 mg, were added into the aggregometer cuvette and their ability to inhibit PAF-induced aggregation was determined. The platelet aggregation induced by PAF ($2.5 \times 10^{-11}$M, final concentration in the cuvette) was measured as PAF-induced aggregation, in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various amounts of the sample being examined. Consequently, the plot of % inhibition (ranging from 20% to 80%) versus different concentrations of the sample is linear. From this curve, the concentration of the sample, which inhibited 50% PAF-induced aggregation, was calculated. This value is defined as $IC_{50}$ namely, inhibitory concentration for 50% inhibition.

Furthermore part of our study was to clarify the impact of the seasonal and geographical variation of european sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) on fatty acid content of fish fillets and on the anti-PAF activity of fish fillet lipids.

Briefly farmed sea bass (*Dicentrarchus labrax*) obtained from marine farms situated in Chios Island during winter (January) and summer time (June) and gilthead sea bream (*Sparus aurata*) obtained from marine farm situated in sea region of Nafpaktos during both seasons and from marine farm situated in Chios Island during summer time.

In January the average seawater temperatures of marine farm situated in Chios Island and in sea region of Nafpaktos were 13 and 15°C, respectively, while in June the average seawater temperature of marine farm situated in Chios Island and in sea region of Nafpaktos were 23 and 21°C, respectively. During the experiment, water salinity was 3.5 – 3.8% at both farms. Feeding ratio ranges from 0.8 - 1.1% of body weight per day. Fish of the same species were fed with the same commercial feed, during both winter and summer. Five specimens of each species, each season and each marine farm were analyzed. Their diets were also analysed.

Fatty acid methyl esters of fish and commercial feed total lipids prepared using a solution 0.5N KOH in $CH_3OH$ 90% and extracted with n-hexane.

The fatty acid analysis was carried out using the internal standard method (Nasopoulou et al. 2011). A five point calibration curve was prepared using five solutions of heptadecanoic (17:0) acid methyl ester and heneicosanoic (21:0) acid methyl ester in ratios of 500:1000 (v/v), 500:500 (v/v), 500:200 (v/v), 500:100 (v/v) and 500:50 (v/v), respectively.

Five injects of 1 μL of each solution were analyzed with a Shimadzu CLASS-VP (GC-17A) (Kyoto, Japan) gas chromatograph equipped with a split/splitless injector and flame ionisation detector. The ratio of the mean area of (21:0) to that of the internal standard (17:0) is used as the y-axis variable of the calibration curve, while the concentration (mg $kg^{-1}$) of 21:0 is used as the x-axis variable of the calibration curve. The equation that described the calibration curve was:

$$y = 0.0012x + 0.0210, \quad r = 0.996.$$

The ratio of the area of the analyte peak to that of the internal standard represents the y value at the above equation and subsequently x value represents the analyte concentration of the fatty acid in the unknown mixture.

Separation of fatty acid methyl esters was achieved on an Agilent J&W DB-23 fused silica capillary column (60 mx0.251 mm i.d., 0.25 μm; Agilent, Santa Clara California, USA). The oven temperature program was: initially 120 $^{o}$C for 5 min, raised to 180 $^{o}$C at 10 $^{o}$C min$^{-1}$, then to 220 $^{o}$C at 20 $^{o}$C min$^{-1}$ and finally isothermal at 220 $^{o}$C for 30 min. The injector and detector temperatures were maintained at 220 and 225 $^{o}$C, respectively. The carrier gas was high purity helium with a linear flow rate of 1 ml min$^{-1}$ and split ratio 1:50. Fatty acid methyl esters were identified using fatty acid methyl esters standards (Sigma, St. Louis, Mont, USA) by comparison of the retention times of the relative peaks (Nasopoulou et al., 2011).

### 2.2 *In vivo* studies

As mentioned before evidence of PAF implication in atherogenesis is provided by studies in animals, which indicates that PAF antagonists have protective action against atherosclerosis (Feliste et al., 1989; Subbanagounder et al., 1999), while PAF-acetylhydrolases (PAF-AHs), the main enzymes responsible for the degradation of PAF, are active in native LDL but converted inactive forms in ox-LDL, leading to higher levels of PAF (Liapikos et al., 1994). Recent *in vivo* study in animals is performed by our research team (Nasopoulou et al., 2010). More specific twelve healthy male New Zealand rabbits of specific weight 3129 ± 216 g and age 2.7 ± 0.2 months were purchased from a commercial breeder and were individually housed in atomic stain-less steel cages in constant conditions of temperature (19 + 1 $^{o}$C), relative moisture (55 + 5%), and air conditioning (12 full changes of air per 1 h). The light/darkness ratio was 12 h/12 h. Rabbits were acclimatised for 5 days before the beginning of the study. Living conditions and animal handling were according to the European Regulation 609/86. The local veterinary authorities and animal ethics committee approved the study. Rabbits were randomly divided into two groups of six animals each and were given specific diets for 45 days. Group A was given atherogenic diet 1% cholesterol, while group B was given atherogenic diet enriched with gilthead sea bream polar lipids GSBPL (0.06% w/w) (Figure 3) (Nasopoulou et al., 2010).

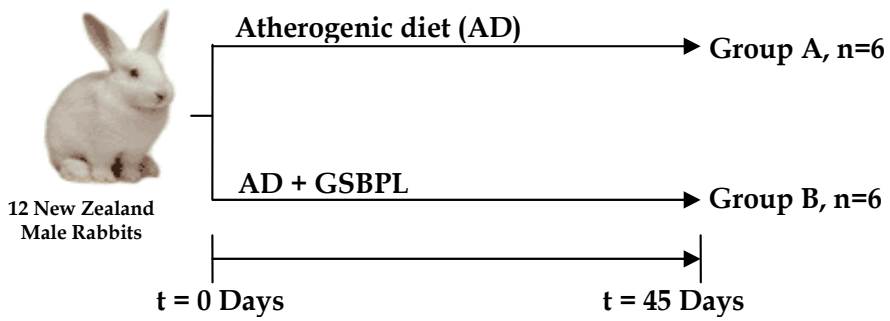



Fig. 3. Experimental design of the *in vivo* trial.

The experimental diet of group A was prepared by dissolving 30 g of cholesterol in 1000 ml of diethyl ether, whereas the experimental diet of group B was prepared by dissolving 30 g of cholesterol and 2 g of GSBPL in 1000 ml of diethyl ether. Each mixture was added to 3 kg of commercial available food for rabbits, placed on large plates, and the solvent was evaporated. This procedure was carried out every three days and food consumption was recorded every three days by measuring the weight of the required food that was added. In this series of experiments, each rabbit consumed 200 g/day, on average, so this resulted to a consumption of 133 mg of GSBPL/rabbit/day. On the 45th day, rabbits were anesthetised by intramuscular injection of 5 mg/kg body weight of xylazine (Rompun, Bayer, Leverkusen, Germany) and 25 mg/kg body weight of cetamine (Fort Dodge Laboratories Inc., Fort Dodge, Iowa, USA). Afterwards euthanasia took place by intravenous injection of 20 mg/kg body weight of pentothal (Hospital Products Division, Abbott Laboratories Abbott Park, IL, USA). The thoracic and peritoneal cavities were opened through a median longitudinal incision and the aorta was dissected from the aortic valve down to the aortic bifurcation (Nasopoulou et al., 2010).

The biochemical measurements that took place were the followings: at the beginning (0 days) and at the end of the experimental time (45 days), blood was collected from all rabbits through the main ear artery and was placed in polyethylene tubes containing a ratio of blood/anti-coagulant of 9:1 (v/v). Plasma cholesterol concentrations – total cholesterol, LDL-cholesterol and HDL-cholesterol – were determined enzymatically by CHOD-PAP, using commercial enzymatic kit (Elitech Diagnostics, Sees, France) and triglycerides concentration was determined enzymatically by GPOPAP, using a commercial enzymatic kit (Roche Diagnostics GmbH, Mannheim, Deutschland). Analyses were performed on a RA-XT autoanalyzer (Technicon Instruments, NY, USA).

In vitro $CuSO_4$-induced plasma oxidation (Schnitzer et al., 1998) was assessed by a Helios b (Spectronic Unicam, Cambridge, UK) spectrophotometer equipped with a 7 position automated sample changer. In quartz cuvettes, 20 μl of plasma were added to 880 μl of phosphate buffer solution, pH 7.4, with 146 mM NaCl at 37 $^oC$ for 5 min. The oxidation reaction was started by the addition of 100 μl $CuSO_4$ 1mM, and the absorbance of resulting conjugated dienes, at 245 nm, was continuously recorded for 3 h at 37 $^oC$.

Platelet-rich plasma (PRP) was obtained by centrifugation of blood samples at 562g for 13 min, while platelet–poor plasma (PPP) was obtained by further centrifuging the specimens at 1750g for 20 min. The centrifugation was performed on a Heraeus Labofuge 400R (Hanau, Germany) at 24 $^oC$. PRP concentration was adjusted to 300,000 platelets/ml using the respective PPP. PRP was used to test the aggregation induced by various concentrations of PAF in 2.5 mg of BSA/ml saline. The maximum reversible (or the minimum irreversible) PAF-induced platelet aggregation was determined as the 100% aggregation, and then various PAF concentrations were added, so as to achieve aggregations between 20% and 80%. These PAF-induced aggregations were of linear response to the respective PAF concentration; therefore, the $EC_{50}$ value was calculated. $EC_{50}$ accounts for the PAF concentration inducing 50% of the maximum aggregation. These studies were performed using a Chronolog aggregometer (model 400) coupled to a Chronolog recorder at 37 $^oC$ with constant stirring at 1200 rpm.

Plasma PAF-AH activity was determined by the trichloroacetic acid (TCA) precipitation procedure (Antonopoulou et al., 1994). Plasma (diluted 1:30 v/v in Tris buffer, pH 7.4) was

examined in a final volume of 200 μl. The mixture was incubated at 37 ºC for 2 min and the reaction was initiated by adding 5 μl of 800 μM [3H]-acetyl PAF/PAF solution in BSA (10 μg/μl in Tris–HCl buffer pH 7.4). The PAF-AH assay was performed at 37 ºC for 15 min. Unreacted [3H]-acetyl PAF was bound to an excess of BSA (final concentration, 0.75 mg/ml) for 0.5 min and precipitated by addition of trichloroacetic acid (final concentration, 9.6% v/v). The samples were then placed in an ice bath for 15 min and subsequently centrifuged at 16,000g for 5 min, at 4 ºC. The [3H]-acetate released into the aqueous phase was measured on a liquid scintillation counter (1209 Rackbeta, Pharmacia, Wallac, Finland). Blank assay was performed with no added plasma. The enzyme activity was expressed as pmol of PAF degraded per minute per μl of plasma.

Histopathological examination of the atherosclerotic lesions performed after comparable areas of the thoracic aortas were sectioned into 1 cm segments, fixed in 10% buffered formaldehyde, embedded in paraffin blocks, and stored at room temperature. For the histopathological examination, 5 μm thickness tissue slide sections were then cut, transferred to slides and stained with haematoxylin and eosin. The thickness of atherosclerotic lesions was measured blindly in five sections from each artery. Conventional measurements of early atherosclerosis lesions in the histopathological tissue sections of resected aortas were performed, using an automated image analysis system. The apparatus comprised a Sony-Exwave HAD Color Video Camera (Sony Corporation, Japan), fitted to a Zeiss Axiostar light microscope (Zeiss, Germany), a host computer (Pentium 90 MHz, 32 MB RAM) and Sigma Scan version 2.0 image analysis software (Jandel Scientific, Erkrath, Germany). Foam cell formation is characteristic of the early atherosclerosis lesions. In the present study, early atherosclerosis lesions were observed as foam cell layers developed inside the blood vessels (Nasopoulou et al., 2010).

### 2.3 Design of novel functional fish

Two plant oil sources, namely olive pomace and olive pomace oil were selected by our research team because of their content in athero-protective substances (such as PAF inhibitors) and phenolic/polyphenolic molecules (Karantonis et al., 2008), null cost since they are natural wastes of olive oil production, and fatty acid profile, which makes them likely candidates to partially substitute fish oil in compounded fish feeds. The reference diet (fish oil diet) contained 100% fish oil (cod liver oil) and its chemical composition is shown in Table 1, while the two experimental diets: olive pomace and olive pomace oil diet were compounded by substituting 8% of fish oil of reference diet, respectively (Nasopoulou et al., 2011).

All the diets were formulated at the facilities of the marine farm where the experiment took place using a twin-screw extruder creating pellets, followed by the addition of oil mixtures. The pellets were dried, sealed and kept in air-tight bags until use (Nasopoulou et al., 2011). Olive pomace and olive pomace oil originated from a local oil industry and obtained after the olive oil production procedure.

Each of the three tanks of each fish species contained fish fed with each dietary treatment (fish oil, olive pomace and olive pomace oil diet).

Two different fish species, namely gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) of initial mean body weight 340 g obtained from a commercial marine

| Fillet composition (% wet weight) | Fish oil diet |
|---|---|
| Crude protein | 46±4.3 |
| Fat | 21±3.2 |
| Moisture | 9.1±1.3 |
| Dietary fiber | 1.8±0.3 |
| Ash | 8.3±0.9 |
| Energy (MJ/Kg) | 23±2.6 |
| Digestibility (%) | 90±6.2 |
| Vitamin A (IU/Kg) | 20 000±410 |
| Vitamin D(IU/Kg) | 3 000±120 |
| Vitamin E(mg/Kg) | 258±19 |
| Vitamin K3(mg/Kg) | 33±7.3 |
| Vitamin C(mg/Kg) | 168±14 |
| Cu(mg/Kg) | 7±1 |

Table 1. Chemical composition of fish oil diet (FO diet) (% wet weight)

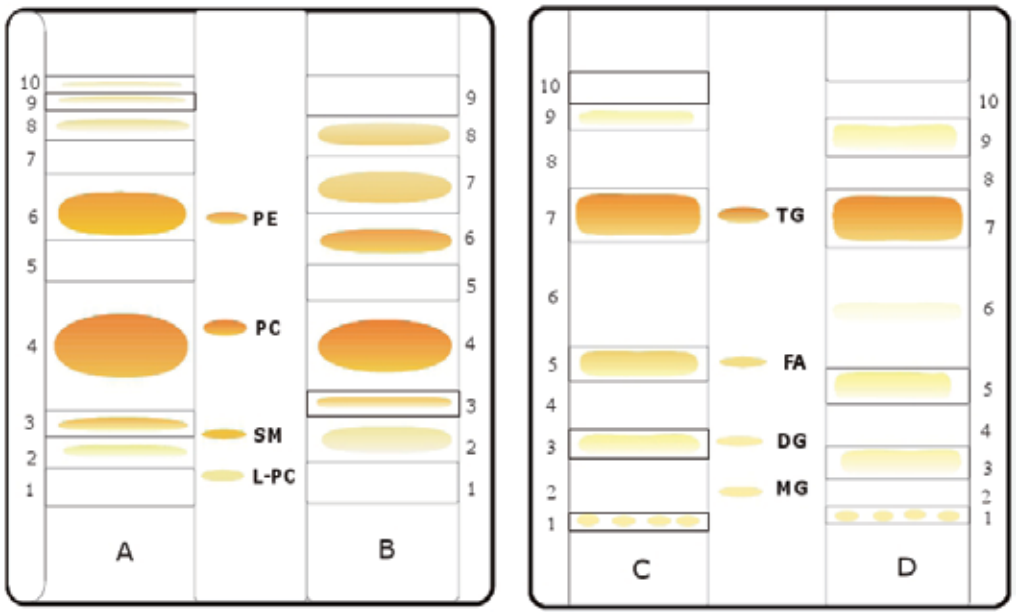
farm. The fish of each species were randomly distributed into three tanks (15 $m^3$ each) in groups of 600 fish per tank at the facilities of the farm. All the tanks were supplied with flow-through natural sea water system and were provided with continuous aeration and filters. The water temperature was 19–21 $^oC$ and oxygen content was kept close to saturation. The fish were forced to fast for three days before being transported to the tanks and then were acclimatised to the new environment for three days. The fish were fed with 1% diet of body weight per day. The fish were acclimatised for 7 days to the experimental diets, prior to feeding trial initiation. The fish were weighed under moderate anaesthesia twice throughout the experimental period (at the begging and at the end) which lasted for 90 days (May–July). The feed conversion ratio, specific growth rate, fatty acid profile of fish muscle and biological activity of fish lipids were calculated. Feed intake and morality were recorded daily. At the beginning of the on-growing period, the fish were fed only with fish oil diet (reference diet) and fifty fish samples were collected and weighed. After 60 days, 200 fish samples were collected from each fish species and dietary treatment and 10 of them provided the muscle samples to estimate the biological activity of fish lipids. At the end of the on-growing period (90 days), 200 fish samples were collected from each fish species and dietary treatment, 50 of them weighed and 10 of them provided the muscle samples to estimate the biological activity of fish lipids and the fatty acid analysis. Fish lipids obtained according to methods described above and the biological assay and fatty acid analysis performed as mentioned before (Nasopoulou et al., 2011).

### 2.4 Results

Regarding the *in vitro* studies lipids obtained from many traditional foods of the Mediterranean diet, such as fish (Nasopoulou et al., 2007; Nomikos et al., 2006; Panayiotou et al., 2000; Rementzis et al., 1997), olive oil (Koussissis et al., 1993), honey (Koussissis et al., 1994), milk and yogurt (Antonopoulou et al., 1996) and red wine (Fragopoulou et al., 2000) demonstrated the existence of compounds with PAF-inhibitory and/or PAF-agonistic activities in washed rabbit platelets.

More specific the content of total lipids in farmed gilthead sea bream and sea bass is increased compared to that of the respective wild species. This increase is attributed to both neutral and polar lipids' elevated levels, while the contribution of polar lipids is much higher than that of neutral lipids (Nasopoulou et al., 2007). The high amounts of total lipids in farmed fish may be attributed to the diet of the cultured fish and the confined swimming area compared to the wild fish.

The typical profile of polar and neutral lipids separation of the fish species on preparative thin layer chromatography is shown in Figure 4.




(**a**) (**b**)

Fig. 4. (a) Typical profile of the polar lipids separation of the fish species on preparative thin layer chromatography. A: wild, cultured sea bass, B: wild, cultured gilthead sea bream, L-PC: lyso phosphatidylcholine, SM: sphingomyelin, PC: phosphatidylcholine, PE: phosphatidyl ethanolamine. (b) Typical profile of the neutral lipids separation of the fish species on preparative thin layer chromatography. C: cultured gilthead sea bream, D: wild gilthead sea bream, E: wild, cultured sea bass, MG: monoglycerides, DG: diglycerides, FA: fatty acids, TG: triglycerides. The elution system used for the separation of total neutral lipids was petroleum ether:diethyl ether:acetic acid 75:30:1 (v/v/v), while the elution system used for the separation of total polar lipids was chloroform:methanol:water 65:35:6 (v/v/v).

Polar lipid fractions of farmed and wild fish of both species exhibited more potent antithrombotic activity, compared to the one of neutral lipid fractions (Nasopoulou et al., 2007), underling the more profound and beneficial effect of fish polar lipids. In addition polar lipids of both farmed and wild fish of both species demonstrated strong antithrombotic properties especially lipid fractions 4 and 6. Lipid fraction 4 corresponded to phosphatidylcholine which does not possess aggregating properties such the aforementioned lipid fraction had therefore this biological activity could be due to oxidized-

PC, which has a similar $R_f$ value to that of phosphatidylcholine, while lipid fraction 6 exhibited potent inhibitory action against PAF activity (Nasopoulou et al., 2007). This study highlights for the first time that fish polar lipids have more potent antithrombotic effect compared to neutral lipids and that both wild and farmed fish contain strong antithrombotic properties, pointing out that both farmed and wild fish are of high nutritional value in terms of cardioprotection (Nasopoulou et al., 2007).

Regarding the impact of seasonal and geographical variation on fat, fatty acid content and the antithrombotic properties of fish lipids the study showed that total lipids obtained from gilthead sea bream during summer and winter time exhibited no statistical differences, probably due to the fact that the increased feeding intensity observed at the beginning of the summer is not sufficient enough to induce statistical changes in fat deposition in comparison with the feeding intensity at the end of the summer (Table 2), which according to the literature, induce statistical higher fat deposition (Grigorakis et al., 2002). On the other hand, total lipids obtained from sea bass during summer time were significantly reduced in comparison to total lipids obtained from sea bass during winter time (Table 2), which is in agreement with the literature (Grigorakis et al., 2004). Regarding geographical impact in gilthead sea bream fillet fat depots there was no significant difference between gilthead sea bream of Nafpaktos and Chios region (Table 2), since water temperature in both marine farms are similar during the same season.

| Fish species or fish feed | Season/ Temperature °C | Fish origin | No. of individuals analyzed | TL (% ) | NL (%) | PL (%) |
|---|---|---|---|---|---|---|
| Gilthead sea bream | December/15 | Nafpaktos | 5 | 2.91±0.11 | 1.39±0.05$^{a,\dagger}$ | 1.52± 0.07†† |
| Gilthead sea bream | June/21 | Nafpaktos | 5 | 2.72±0.08 | 1.19±0.02$^{b,\dagger}$ | 1.53± 0.07$^{b,\dagger\dagger}$ |
| Gilthead sea bream | June/23 | Chios | 5 | 2.60±0.06 | 1.26±0.04$^{a}$ | 1.34± 0.04$^{a}$ |
| Sea bass | June/23 | Chios | 5 | 2.67±0.07$^{a}$ | 1.24±0.03$^{a,\dagger}$ | 1.53± 0.15 †† |
| Sea bass | December/13 | Chios | 5 | 3.68±0.30$^{b}$ | 1.88±0.10$^{b}$ | 1.80± 0.22 |
| Gilthead sea bream feed | | | | 18.20±0.40 | 9.52±0.17 | 9.66± 0.23 |
| Sea bass feed | | | | 18.14±0.42 | 9.18±0.04† | 9.96± 0.33†† |

a, b in each column: indicates significantly different values within the same fish species and the same lipid fraction seasonally and geographically, according to the Wilcoxon test ($p < 0.05$).
†,†† in each row: indicates significantly different values within the same fish species, the same season and marine farm and different lipid fraction (NL vs PL), according to the Wilcoxon test ($p < 0.05$).

Table 2. Total lipid (TL), polar lipid (PL), neutral lipid (NL) content in farmed sea bass (*Dicentrarchus labrax)* and gilthead sea bream (*Sparus aurata*) fillets and in their commercial feed (% lipid fraction), expressed as mean±SD, (n=3)

The dominant fatty acids in the commercial feeds of sea bass and gilthead sea bream were 16:0, 18:1 ω-9, 18:2 ω-6, 20:5 ω-3 and 22:6 ω-3 (Table 3) which is in accordance with the literature (Grigorakis et al., 2002). The content of 20:4 ω-6 PUFA in the commercial feed of both farmed fish was minimal (Table 3), result which is in agreement with previous findings (Sargent et al., 1999), while 18:2 ω-6 PUFA detected in high levels in both commercial feeds (Table 3) due to the fact that this fatty acid is a constituent of plant oils used for farmed fish feed manufacture (Owen et al., 1975; Yamata et al., 1980).

| Fatty acids | Gilthead sea bream feed (mg $kg^{-1}$) | Sea bass feed (mg $kg^{-1}$) |
|---|---|---|
| 14:0 | 36.7 ± 7.33 | 25.8 ± 5.17 |
| 16:0 | 293 ± 58.5 | 445 ± 89.0 |
| 16:1 (ω-7) | 15.8 ± 3.17 | 18.3 ± 3.67 |
| 18:0 | 20.8 ± 4.17 | 48.3 ± 9.67 |
| 18:1 cis (ω-9) | 119 ± 23.8 | 145 ± 29.0 |
| 18:1 trans (ω-9) | 6.67 ± 1.33 | 20.8 ± 4.17 |
| 18:2 (ω-6) | 284 ± 56.8 | 335 ± 67.0 |
| 18:3 (ω-3) | 48.3 ± 9.67 | 12.5 ± 2.50 |
| 20:4(ω-6) | - | - |
| 20:5 (ω-3) | 60.8 ± 12.2 | 80.8 ± 16.17 |
| 22:6 (ω-3) | 117 ± 23.3 | 207 ± 41.3 |
| Total SFA | 350 ± 70.0 | 519 ± 104 |
| Total MUFA | 141 ± 28.2 | 184 ± 36.8 |
| Total ω-3 PUFA | 226 ± 45.2 | 300 ± 60.0 |
| Total ω-6 PUFA | 284 ± 56.8 | 335 ± 67.0 |
| 22:6 (ω-3)/20:5 (ω-3) | 1.9 ± 0.3 | 2.6 ± 0.7 |

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 3. Fatty acid profiles, as determined by GC, of sea bass and gilthead sea bream commercial feeds (mg $kg^{-1}$ of TL), expressed as mean ± SD, (n=3)

Fatty acid profiles in the fillets of the farmed gilthead sea bream and sea bass reflected fatty acid profiles of commercial feeds thus the main fatty acids in the fillets of the farmed gilthead sea bream in summer and winter time were 16:0, 18:1 (ω-9), 18:2 (ω-6), 20:5 (ω-3) and 22:6 (ω-3) (Table 4) and for sea bass were 16:0, 18:1 (ω-9), 20:5 (ω-3) and 22:6 (ω-3) (Table 5). Moreover there were no significant differences among the fatty acid profiles in seasonally obtained samples of farmed gilthead sea bream. Regarding sea bass there were a few significant differences among the fatty acid profiles in seasonally obtained samples of farmed sea bass ($P > 0.05$), such as 14:0, 16:1 and 18:1 trans fatty acids which levels where significant elevated in winter compared to summer (Table 4). The lack of significant differences in the fatty acid profile of farmed sea bass and gilthead sea bream fillets seasonally was probably because of the fact that fish were fed the same diet throughout the experimental period of our study. Same observations made other researchers (Yildiz et al., 2008), indicating that different seasons did not cause differences in the fatty acid profile of farmed fish fillets. Literature referring to seasonal impact on fatty acid content in sea bass and gilthead sea bream fillets is limited, thus it is difficult to conclude to a clear result (Grigorakis, 2007), however it can be said that fish of both species were good source of 20:5 (ω-3) and 22:6 (ω-3) (Tables 4 and 5).

| Fatty acid | Summer (mg kg$^{-1}$) | Winter (mg kg$^{-1}$) |
|---|---|---|
| 14:0 | 10.0 ± 2.00 | 15.0 ± 3.00 |
| 16:0 | 416 ± 83.2 | 396 ± 79.3 |
| 16:1 (ω-7) | 17.3 ± 3.47 | 25.7 ± 5.13 |
| 18:0 | 89.5 ± 17.9 | 97.3 ± 19.5 |
| 18:1 cis (ω-9) | 138 ± 27.7 | 193 ± 38.6 |
| 18:1 trans (ω-9) | 21.3 ± 4.25 | 28.6 ± 5.72 |
| 18:2 (ω-6) | 95.7 ± 21.8 | 150 ± 33.7 |
| 18:3 (ω-3) | - | - |
| 20:4 (ω-6) | 9.67 ± 1.93 | 11.3 ± 2.25 |
| 20:5 (ω-3) | 98.7 ± 19.7 | 126 ± 25.1 |
| 22:5 (ω-3) | 16.5 ± 3.30 | 27.3 ± 5.45 |
| 22:6 (ω-3) | 393 ± 78.6 | 380 ± 75.9 |
| Total SFA | 515 ± 103 | 509 ± 102 |
| Total MUFA | 177 ± 35.4 | 247 ± 49.4 |
| Total ω-3 PUFA | 508 ± 102 | 532 ± 106 |
| Total ω-6 PUFA | 105 ± 21.1 | 161 ± 32.2 |
| 22:6 (ω-3)/20:5 (ω-3) | 4.0 ± 1.2 | 3.0 ± 0.8 |

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

Table 4. Fatty acid profiles (mg kg$^{-1}$ of TL), as determined by GC, of farmed gilthead sea bream (*Sparus aurata*) fillets during summer and winter, expressed as mean ± SD, (n=3).

| Fatty acids | Summer (mg kg$^{-1}$) | Winter (mg kg$^{-1}$) |
|---|---|---|
| 14:0 | 0.25 ± 0.05$^{a}$ | 22.1 ± 4.42$^{b}$ |
| 16:0 | 282 ± 56.5 | 229 ± 45.8 |
| 16:1 (ω-7) | 3.83 ± 0.77$^{a}$ | 28.8 ± 5.77$^{b}$ |
| 18:0 | 68.2 ± 13.6 | 48.8 ± 9.75 |
| 18:1 cis (ω-9) | 74.3 ± 14.9 | 121 ± 24.2 |
| 18:1 trans (ω-9) | 5.08 ± 1.02$^{a}$ | 9.67 ± 1.93$^{b}$ |
| 18:2 (ω-6) | 12.9 ± 4.98 | 25.3 ± 7.85 |
| 20:4 (ω-6) | 2.75 ± 0.55 | - |
| 20:5 (ω-3) | 92.9 ± 18.6 | 103 ± 20.6 |
| 22:6 (ω-3) | 272 ± 54.4 | 222 ± 44.5 |
| Total SFA | 351 ± 70.1 | 300 ± 60.0 |
| Total MUFA | 83.2 ± 16.7$^{a}$ | 160 ± 31.9$^{b}$ |
| Total ω-3 PUFA | 365 ± 73.0 | 326 ± 65.1 |
| Total ω-6 PUFA | 15.7 ± 3.13 | 25.3 ± 5.05 |
| 22:6 (ω-3)/20:5 (ω-3) | 2.9 ± 0.8 | 2.2 ± 0.4 |

a, b in each row: indicates significantly different values within the same fish species according to the Wilcoxon test ($p < 0.05$).
Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 5. Fatty acid profiles (mg kg$^{-1}$ of TL), as determined by GC, of farmed sea bass (*Dicentrarchus labrax*) fillets during summer and winter, expressed as mean ± SD, (n=3).

The dominant fatty acids in the fillets of the farmed gilthead sea bream in both marine farms were 16:0, 18:0, 18:1 (ω-9), 20:5 (ω-3) and 22:6 (ω-3) and there were no significant differences among the fatty acid profiles in geographically obtained samples of farmed gilthead sea bream (Table 6).

Similarities in the fatty acid fillet content of gilthead sea bream of the same age, fed with the same diet and originated from two different marine farms could probably attributed to the fact that the two marine farms were of similar water temperature and salinity. However gilthead sea bream from both marine farms are good sources of 20:5 (ω-3) and 22:6 (ω-3) (Table 6).

| Fatty acids | Gilthead sea bream Chios (mg $kg^{-1}$) | Gilthaed sea bream Nafpaktos (mg $kg^{-1}$) |
|---|---|---|
| 14:0 | 17.0 ± 3.40 | 10.0 ± 2.00 |
| 16:0 | 419 ± 83.9 | 416 ± 83.2 |
| 16:1 (ω-7) | 30.6 ± 10.5 | 17.3 ± 7.4 |
| 18:0 | 99.8± 20.0 | 89.5 ± 17.9 |
| 18:1 cis (ω-9) | 155 ± 77.7 | 138 ± 27.7 |
| 18:1 trans (ω-9) | 29.4 ± 5.88 | 21.3 ± 4.25 |
| 18:2 (ω-6) | 62.1 ± 12.4 | 95.7 ± 19.1 |
| 20:4 (ω-6) | 18.8 ± 5.77 | 9.67 ± 4.93 |
| 20:5 (ω-3) | 153 ± 30.6 | 98.7 ± 19.7 |
| 22:5 (ω-3) | 26.7 ± 5.33 | 16.5 ± 3.30 |
| 22:6 (ω-3) | 405 ± 80.9 | 393 ± 78.6 |
| Total SFA | 536 ± 107 | 515 ± 103 |
| Total MUFA | 215 ± 43.1 | 177 ± 35.4 |
| Total ω-3 PUFA | 584 ± 117 | 508 ± 102 |
| Total ω-6 PUFA | 80.9 ± 16.2 | 105 ± 21.1 |
| 22:6 (ω-3)/20:5 (ω-3) | 2.6 ± 1.2 | 4.0 ± 1.6 |

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

Table 6. Fatty acid profiles (mg $kg^{-1}$ of TL), as determined by GC, of farmed gilthead sea bream (*Sparus aurata*) fillets obtained from Nafpaktos and Chios marine farms, expressed as mean ± SD, (n=3).

The experimental data showed that total lipids of gilthead sea bream and sea bass in summer contained more potent PAF-agonists – constituents with aggregatory activity less powerful than the one of PAF – and PAF-inhibitors, respectively than the total lipids of the aforementioned fish in winter. Such elevated biological activity of fish during summer time could be due to the increased water temperature. These data show that both fish species posses more potent antithrombotic properties during summer time.

Total lipids of gilthead sea bream from Nafpaktos marine farm exhibited more intense biological activity than total lipids of gilthead sea bream from Chios marine farm possibly due to the elevated polar lipids content of gilthead sea bream from Nafpaktos marine farm (Table 2), which are responsible for the biosynthesis of PAF antagonist.

Regarding the *in vivo* studies gilthead sea bream polar lipids (GSBPL) exhibits an anti-atherogenic effect by increasing HDL-cholesterol levels, despite the fact that total cholesterol, LDL-cholesterol and triglycerides were equally increased in both groups (group A: atherogenic diet and group B: atherogenic diet supplemented with GSBPL) (Nasopoulou et al., 2010).

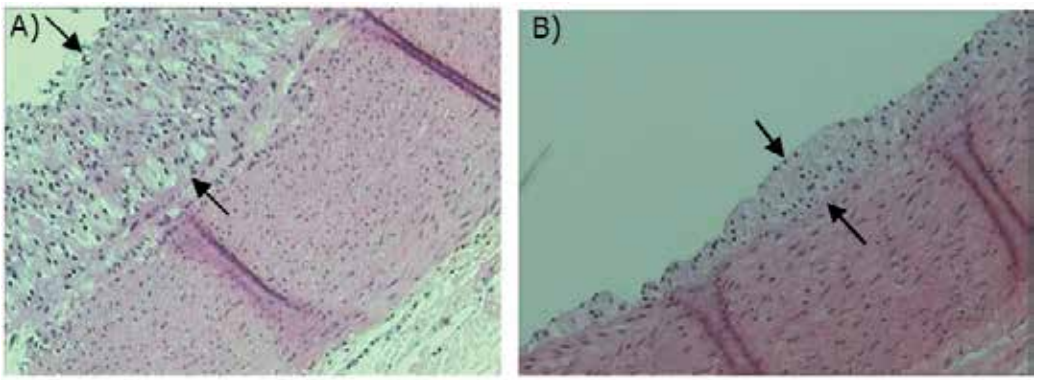
At the end of our experiment (45 days), plasma PAF-acetylhydrolase activity was significantly elevated in group B (atherogenic diet supplemented with GSBPL) compared to group A (atherogenic diet), which may be attributed to the increased HDL-cholesterol levels, since this enzyme is partially located on the HDL particles.

Rabbits of group B (atherogenic diet supplemented with GSBPL) developed early atherosclerotic lesions, of significantly lower degree compared to group A (atherogenic diet) (Figure 5) (Table 7).

| | Early atherosclerosis lesions evaluation<br>Thickness (mm) Surface area ($mm^2$/mm)x1000 | |
|---|---|---|
| Group A | 0.44±0.15[a] | 0.45±0.16[a] |
| Group B | 0.11±0.08[a] | 0.12±0.09[a] |

Results were expressed as mean ± SD.
A: atherogenic diet; B: atherogenic diet enriched with GSBPL.
[a]Donates statistical significance between groups A and B ($p < 0.05$), according to Mann-Whitney *U*-test.

Table 7. Assessment of early atherosclerosis lesions observed in rabbit aortas.




A: Group A (atherogenic diet);
B: Group B (atherogenic diet enriched with GSBPL)

Fig. 5. Representative optic micrographs x 100 of aortic wall sections stained with hematoxylin and eosin from the two experimental groups, where atherosclerotic lesions appear as foam cells (↑).

This result could be explained on the basis of the elevated PAF-acetylhydrolase activity in the plasma of rabbits of group B. This study shows that polar lipids of cultured gilthead sea bream (*Sparus aurata*) contain bioactive micro-constituents, PAF inhibitors, that inhibit PAF activity both in vitro and in vivo, consequently inhibiting early atherosclerosis development. The above data reinforce the beneficial effect of cultured gilthead sea bream polar lipids against atherosclerosis development (Nasopoulou et al., 2010).

Finally, regarding the results of the novel fish designing the growth performance factors of gilthead sea bream fed with fish oil diet in comparison with gilthead sea bream fed with olive pomace and olive pomace oil diet exhibited no statistical differences, indicating similar feed conversion ratio and specific growth rate (Nasopoulou et al., 2011). However, sea bass fed with olive pomace and olive pomace oil diet showed statistical decreased specific growth rate and statistical increased mortality in comparison with sea bass fed with fish oil diet (Nasopoulou et al., 2011), suggesting that gilthead sea bream fed with the experimental diets exhibited satisfactory growth performance – similar to gilthead sea bream fed with fish diet – and better than the one of sea bass.

Comparing gilthead sea bream fed with olive pomace to fish fed with olive pomace oil, fish fed with olive pomace diet showed significant lower ($p < 0.05$) mortality in comparison to olive pomace oil diet, while the feed intake, feed conversion ratio and specific growth rate did not exhibit significant differences (Nasopoulou et al., 2011). Therefore, olive pomace could be used as 8% dietary fish oil substitute, in gilthead sea bream without compromising the growth performance and due to significant decreased mortality it is preferable than olive pomace oil.

All classes of fatty acids, such as saturated (especially 16:0 and 18:0), monoenes (especially 18:1 cis), x-3 (20:5 and 22:6) and ω-6 (18:2) of the fish fed with olive pomace diet were found to statistically decrease compared to the fish fed with fish oil diet (Nasopoulou et al., 2011). Such alterations of the fatty acid content in the fish fillets, especially the reduction of ω-3 HUFA levels, particularly 20:5 ω-3, are in accordance with the literature (Izquierdo et al., 2003, 2005; Montero et al., 2005; Mourente et al., 2005).

The main constraint for the use of plant oils in fish feeds is the lack of ω-3 HUFA, particularly 20:5 ω-3 and 22:6 ω-3. According to the literature, the biological demand of gilthead sea bream and sea bass is at least 0.9% and 0.7% of the diet, respectively (Ibeas et al., 1994; Kalogeropoulos et al., 1992, Skalli & Robin, 2004). In the present study, the ω-3 HUFA content of experimental diets meet the theoretical values of the examined species requirements in these fatty acids (Nasopoulou et al., 2011).

The biological activity of gilthead sea bream total lipids fed with the olive pomace diet was significantly increased in comparison with the gilthead sea bream fed with the fish oil diet at the end of the experimental period (90 days) (Nasopoulou et al., 2011). The experimental data indicate that olive pomace reinforces the anti-PAF biological activity of gilthead sea bream, probably due to the fact that this olive oil production industry by-product contains anti-PAF lipid components (Tsantila et al., 2007). In addition, these anti-PAF lipid components possess antibacterial properties (Nasopoulou et al., 2008), protecting the fish from bacteria and prolonging shelf-life.

Gilthead sea bream is the fish species that absorbed and metabolized better the experimental diets, in particular the olive pomace diet, exhibiting improved biological activity in comparison with sea bass fed with olive pomace diet and with gilthead sea bream fed with fish oil diet, indicating that olive pomace reinforced the anti-PAF biological activity of gilthead sea bream. Moreover gilthead sea bream fed with olive pomace diet had a statistically decreased content ($p < 0.05$) of 20:5 ω-3 and 22:6 ω-3 in comparison with the ones of fish fed with the fish oil. Therefore, the more potent anti-PAF properties and subsequent cardio protective ability of the gilthead sea bream fed with the olive pomace diet

cannot be attributed to the fatty acid content since the amounts of these fatty acids are decreased.

The current work focused for the first time on improving the nutritional value of fish in terms of cardio protection by partially substituting fish oil in fish feed with olive oil industry by-products rich in phenolic compounds and PAF inhibitors, making the management of these by-products cost-effective and producing a new ''variety'' of gilthead sea bream with properties against cardiovascular diseases under the guidelines of E.C.1924/2006.

In order to confirm the cardioprotective and beneficial effects of this novel fish for humans, future research is required by using *in vivo* the biologically active compounds of the novel fish with experimental animals and to examine which compounds inhibit the formation of atheromatic plaque in blood arteries.

## 3. Conclusion

To summarize the scope of our work that has been carried by our group in the past 10 years, it should be highlighted that we work towards the improvement of fish feeds with the ultimate goal to produce aquacultured fish with higher nutritional value. To achieve this goal, the use of Gas Chromatography has been an extremely versatile tool: both the enrichment of fish feed with beneficial fatty acids from olive pomace and olive pomace oil and the enrichment of fish flesh with beneficial fatty acids from fish feed were screened and quantified by Gas Chromatography. In conclusion, Gas Chromatography has been a powerful analytical tool in our attempts to improve the quality of fish feeds and ultimately fish. Gas Chromatography offers the capacity to fine tune our enrichment experiments before carrying out expensive and time-consuming biological experiments. Under this scope, in our work towards novel food, that are described in this chapter, the application of Gas Chromatography has allowed us to perform successful "feed enrichment" experiments and also collect valuable analytical data on the nutritional value of the novel (enriched) fish feeds and fish.

## 4. Acknowledgment

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# 15

# Study of the Chemical Composition of Essential Oils by Gas Chromatography

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## 1. Introduction

Essential oils are complex mixtures, constituted by terpenoid hydrocarbons, oxygenated terpenes and sesquiterpenes. They originate from the plant secondary metabolism and are responsible for their characteristic aroma.

The various applications of essential oils account for the great interest in their study. Such applications may be found in the cosmetic industry, as ingredients of fragrances, decorative cosmetic, fine fragrances and flavouring, in the food industry, as aromas and flavours, in the pharmaceutical industry, as active components of medicines and as antibacterials/antimicrobials, and in aromatherapy. At present, there are many studies in which they are used as intermediaries in fine chemistry reactions, among other applications.

The most common methods used for the industrial extraction of these oils are steam-distillation, extraction with solvents and expression. Their selection will depend on the characteristics of the material from which the oil will be extracted, since they can be present in different parts of the plant, like the roots, the stem, the leaves, the fruits and/or the seeds.

Once the oils are obtained, the fundamental contribution of the organic chemistry to the industry resides in their characterisation, as their chemical composition may vary even whithin one botanical species. These variations might be due to the presence of different chemotypes, according to the plant adaptation to the surrounding environment, as well as its state of development. We have to take into account the fact that it is the composition of the essential oils what provides their intrinsic properties and economic value.

The development of chromatographic techniques has allowed us to make considerable progress in the study of the chemical composition of essential oil. Gas Chromatography (GC) is, by all means, the best method, due to its simplicity, rapidity and efficiency, for both the identification and quantification of essential oil components and composition variations.

The aim of this chapter is to describe the different applications of GC, starting from the quality control up to the identification and quantification of the chemical components of essential oils from different aromatic species that grow in the northeast of Argentina, which have emerged as a result of years of experience in this topic.

In regards to the applications of GC, different studies will be presented, among which we will discuss the optimization of the operational conditions used to separate the different components, the analysis of the variation in the composition of regional essential oils, the measurement, using internal standards, of oils as well as oil modification by fractional vacuum distillation, and the study of semi-synthesis reactions to obtain high added-value compounds, starting from oils or their components.

## 2. Essential oils

Essential oils are natural products that plants produce for their own needs other than nutrition (i.e. protection or attraction). In general, they are complex mixtures of organic compounds that give characteristic odour and flavour to the plants. They are mainly made up by monoterpenes and sesquiterpenes whose main metabolic pathway is through mevalonate leading to sesquiterpenes and from methyl-erythritol leading to monoterpenes. They are located in different parts of the plant. They can be found in the root such as that of the vetiver grass (*Vetiveria zizanioides*), in stems like that of peteribi wood (*Cordia trichotoma*) and incense, in leaves like in eucalyptus trees (*Eucalyptus citriodora*), citronella (*Cymbopogon nardus*), chinchilla (*Tagetes minuta*) and lemon grass (*Cymbopogon citratus*), in flowers like lavenders (*Lavandula officinalis*), in fruit like lemon, orange (*Citrus spp*.) and even in seeds as in the case of anise (*Pimpinella Anisum*), coriander (*Coriandrum sativum*) and pepper (*Piper nigrum*), among others (Baser, 2010). They can work as internal messengers, like defense substances or plant volatiles aimed at natural enemies but also to attract pollinating insects to their host (Harrewijn et al., 2001).

Essentials oils are accumulated in cells, secretory cavities or glandular hairs of plants. They are globules with impermeable cells (stomata) whose interior have essentials oils. In the case of citrus, stomata can be observed at first sight because they are macroscopic. Apart from superior plants, some land and sea animals, insects, mushrooms and microorganisms are also known for the biosynthesis of similar volatile compounds (Berger, 2007).

In general, essential oils have a nice smell, that is why they are used in different industries, especially in perfumes (fragancias and lotions), in foodstuff (like flavoring and preservatives) and in pharmaceutical products (therapeutic action) (Zygadlo & Juliani, 2000).

There are different methods for essential oil extraction. One of the most common is steam-distillation since it allows for the separation of slightly volatile, water-inmiscible substances by means of low temperature distillation, being of particular use when the components boil at high temperature (higher than 100ºC) and are susceptible to decomposition below this temperature. Although this methodology presents several advantages, it is necessary to bear in mind that it is not just a simple steam dragging business. The release of the components present in the stomas is caused by cell-wall rupture as a result of the higher pressure and the oil content expansion of the cell generated by heat. The steam flow gets in through the stomas, breaks them and eventually drags the essential oil (Baser, 2010).

In a nutshell, steam-distillation consists of steaming as a result of a straight current of steam water, which heats the mix as well as it decreases the boiling temperature because of the higher steam tension inherent in water to those of volatile components in essential oils. The steam coming from the distillator gets cold in a condenser and, finally, the inmiscible mix gets separated in a clarifier or Florentine flask. This methodology is more convenient than

organic solvent extraction or straight distillation as water steam has a lower cost compared to organic solvents. Also, it avoids oil heating or the use of sophisticated equipment. Nonetheless, the extraction method depends, among other factors, on the kind of material to be processed and the location of the components within the vegetable structure according to the species and botanical family (Bandoni, 2000).

The extraction technique with organic solvents is based on the distribution balance or selective dissolution of the oil within two inmiscible phases. The starting point is the fresh or dessicated vegetable material. However, the dessicated material, even when the extraction is done at room temperature and in darkness, incurs in a partial loss of the product, because of the steaming of most of the volatile components of the oil. The extraction can be carried out with volatile solvents, for instance petroleum ether, n-hexane, among others. In simple extractions, solid solvents and high quality fat can be used for the extraction of essential oils of flower leaves like violets, jasmine or roses (enfleurage).

In order to get oil from citrus fruits, such as oranges, lemons or tangerines, cold expression is preferred, due to the thermal instability of the main constituents of the essential oil. The oil cells are located below the epicarp surface. The fruit must be washed and sliced into two halves, the pulp withdrawn and then the peel must be softly pressed to break the oil glands, which can be removed with water.

Once the oil is obtained, it must be dried with anhidrous $Na_2 SO_4$, then filtered and stored for its ulterior study or separation of the components (inert atmosphere and protected by light). The oil analysis entails the determination of its physical properties (refractive index, density, and optical rotation) and chemical properties (acid index, esters, carbonyl compounds, fenols, primary and tertiary alcohols), as well as the separation and identification of its major components.

Although the essential oils have a great number of components, the ones of commercial interest are generally those composed of one or two major components, which provides them with accurate features. Nonetheless, in some cases, the minor components are also important because they might provide the oils with a of exquisite perfume, that is why this kind of material must be handled with care. The extraction, preservation and conditioning of such a material are very important in order not to alter its composition. Otherwise the market price would drop sharply. For the same reason, it is vital to study the composition of these oils and, in some cases, even those components present at milligram or nanogram amounts. The organic chemistry, mainly through GC and mass spectroscopy, has a leading role in this area.

From the chemical point of view, the essential oil composition frequently changes in different parts of the plant. Quite often, between the different organs of the plant, phytochemical polymorphism can be produced. As an example, in *Origanum vulgare* ssp. hirtum, polymorphism could be detected, even within one individual plant, between different oil glands of a single leaf (Johnson et al., 2004). However, this kind of polymorphism is not very usual, being the difference in the oil composition between glands usually related to gland age (Grassi et al., 2004; Johnson et al., 2004; Novak et al., 2006; Schmiderer et al., 2008). In general, the different growth stages of the plant create variations in the oil composition within the same organ of the plant (Chamorro et al., 2008).

Polymorphism is also often found when the essential oil composition of individual plants of one species is compared (intraspecific variation, "chemotype") and is based on the genetic background of the species. Sometimes the difference in the complex composition of two essentials oils of one kind are difficult to assign to specific chemotypes or to differences that arise as a result of the plant response to environmental conditions, for instance, different growth locations.

Generally speaking, genetic differences are much higher than those caused by varying environmental conditions. However, many intraspecific polymorphisms are unlikely to have been detected yet or have been recently described, even for essential oils widely used, such as those present in sage (Novak et al., 2006).

Due to its soil and climate characteristics, Argentina is a prime area for growing aromatic plants, both native and foreign. In the northeastern region comprising the provinces of Chaco, Formosa, Corrientes and Misiones, species that grow well contain large percentages (60-90%) of oxygenated monoterpenes such as citronellal, dehydrotagetone tagetone, α-pinene and β-pinene, among others. An important source of those compounds are species like *Eucalyptus citriodora*, *Cymbopogon spp. Tagetes spp.* and *Schinus spp*., which have been studied in various aspects by GC and mass spectroscopy.

As a starting point for the study of essential oils, it is necessary to define the best working conditions in order to get the right separation, identification and quantification of its components. To exemplify the methodology used in regards to this matter, we will describe work done on *Cymbopogon winterianum*.

Within the several uses of this methodology, we can mention the study of the variation of the oil composition in terms of the different stages of the plant, as well as the different origins. In this regard, the methodology used in the study of two effective biocides will be described, the essential oil of *Tagetes minuta* L (Zygadlo et al., 1993), which presents variations in its composition in the different parts of the plant in different stages of its vital cycle; as well as the results obtained in the study of essential oils of *Schinus molle* in the City of Resistencia, Chaco, presenting diverse variations in connection with plants of other origins (Maffei & Chialva, 1990; Menendez et al., 1996).

Also be quantified by gas chromatography, reaction products of semi-synthesis of terpenes from low economic value, such as obtaining isopulegol from the cyclization of citronellal, a major component of essential oils of *Eucalyptus citriodora* and *Cymbopogon winterianus.*

## 3. Determination of chromatographic conditions

The aim of the selection and definition of chromatographic conditions is to achieve a proper separation of the components of the oil, both for the qualitative analysis, as also for the proper quantification. To do so, well resolved peaks and not distorted ones, good relation signal-noise and horizontal base line with absence of drift, must be obtained for each one of the components.

To accomplish this objective, a correct selection of the column is key: the ones which are the most used for essential oils are the polar, and in particular, have chiral stationary phases those essential oils which have components of interest that present optical isomerism.

In general, for the development and selection of stationary phases, it must be considered, among other things, the thermal and chemical stability of the column, the selectivity in the separation of the components, the lining or coating surface, the diameter of the column, as well as the incorporation of more specific components to the stationary phase, or the use of different technologies to optimize the phase available to the specific regions of analyses that require better resolution (Marriott et al., 2001). From all the factors that should be considered in the selection of stationary phases, the thermal stability of the column is of less importance, since the essential oils are eluted prior to the rank of highest temperature defined by the high-temperature GC. However, a greater column stability also implies that the column will be stable over a longer period, and this will translate into a better reproducibility in long-term analysis, which leads to a more precise analytical characterization.

The variable that the analyst most frequently handles at the time of separation of the components of essential oils is perhaps the working temperature.

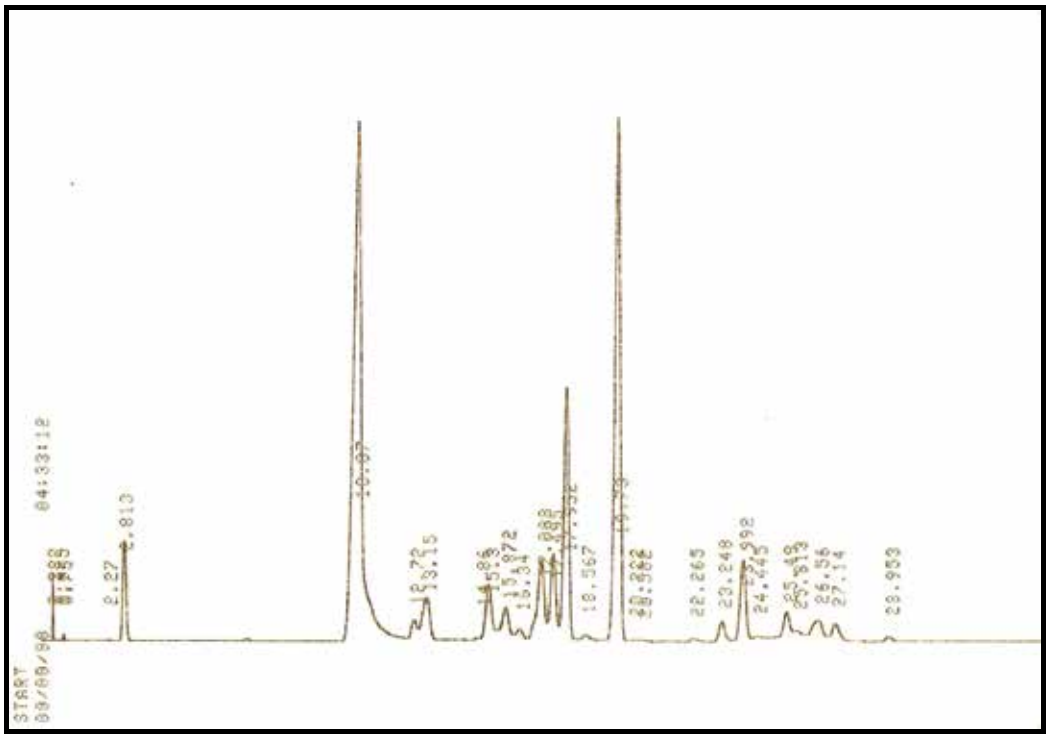


Fig. 1. Chromatogram of citronella essential oil with column temperature of 60 °C for 5 minutes, then a ramp with an increase of 5 °C/minute up to 200 °C, finally leaving this temperature constant for 10 minutes.

Given that essential oils are mixtures of compounds of different molecular weights, from the most volatile hydrocarbons of ten carbon atoms, called monoterpenes, to oxygenated compounds of 15 atoms of carbon, or sesquiterpenes, it is necessary to start with low temperatures that allow the separation of the most volatile ones, then raise it 5 °C or 10 °C per minute to reach the temperature of 200 °C to achieve the elution of the heaviest terpenoids (Francisco et al., 2008).

If the chromatography was performed isothermally at low temperature (60 °C), the components were not separated from each other, migrating as a single, broad peak and employing long elution times. If, on the other hand, the isothermal run was done at high temperatures (200 °C), the peaks were slender and the elution time shorter but proper peak migration and separation was not achieved.

Figure 1 shows a chromatogram of the essential oils of citronella (*Cymbopogon winterianus*) in which the column temperature was kept at 60 °C for 5 minutes, then ramped at 5 °C/minute up to 200 °C and finally kept for 10 minutes at that same temperature. The run was done using a SHIMADZU GC 14B chromatograph, equiped with a Mega Bore DB-WAX P/N 125-7032 column (30 m in length x 0.53 mm i.d. x 1 µm), a Flame Injector Detector (FID) with an operating temperature of 220 °C and an injector with a temperature of 180 °C, manual injection and nitrogen as gas carrier.

## 4. Identification of essential oils components

*Tagetes minuta* L. is an native aromatic plant from South America, which is well known in the Province of Chaco (Argentine), and it is vulgarly called "chinchilla" (Parodi, 1959). It grows naturally from spring until it practically disappears with the beginning of the winter, developing its complete life cycle within this period of time. This oil has wide applications as flavoring and perfume (Vasudevan et al., 1997). In addition, it is well known for its biocide properties (Zygadlo, 1994).

With that purpose, vegetable material, from different locations of the Province of Chaco, was collected during the fall. It is during this season that the plant is likely to be used for essential oil extraction. The oil obtained by steam-distillation, independently from non-bloomed plant leaves, from bloomed plant leaves and from flowers of *T. minuta*.

The identification of the essential oil components were carried out by gas chromatography-mass spectrometry (GC-MS) using a gas chromatograph Agilent 6890 with selective mass detector Agilent 5973, a capillary column of HP-5MS (30 m x 0.25 mm i.d. x 0.25 µm film thickness) and a split/splitless injector, with an automatic injection system ALS Agilent 7683, and the Library NIST Mass Spectral Search Program, version 1.6d. Working conditions were: split injection; ratio 70:1, injecting 0.2 µl. The test was carried out at 250ºC, the oven at initial 50ºC for 2 minutes increasing 10 ºC per minute till 200ºC was reached. The flow was of 0.7 µl/min at a constant speed of 30 cm/s with a 250ºC interface.

Figure 2 shows a chromatogram of the essential oil of *Tagetes minuta*, in which efficient separation of different components, including geometric isomers such as cis and trans-tagetenone (peaks 6 and 7) was obtained. Figure 3 and 4 show the mass spectrum of both these isomers. As it can be seen, both have the same formula and molecular weight (150) and yet show very different mass spectrum. In the same manner, we could identify all the other components of the oil.

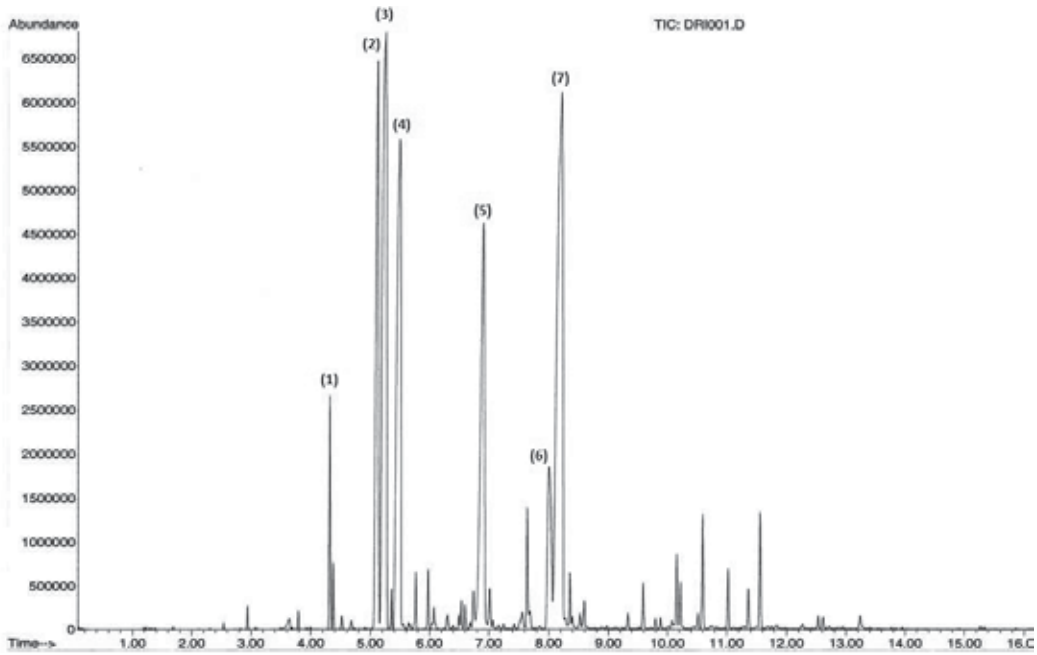


Fig. 2. *Tagetes minuta* essential oil chromatogram carried out using a gas chromatograph-mass spectrometry Agilent 6890 with a capillary column of HP-5MS. Components: β-phelandrene (1), limonene (2), β-ocimene (3), dihydrotagetone (4), tagetone (5), cis-tagetenone (6) and trans-tagetenone (7).

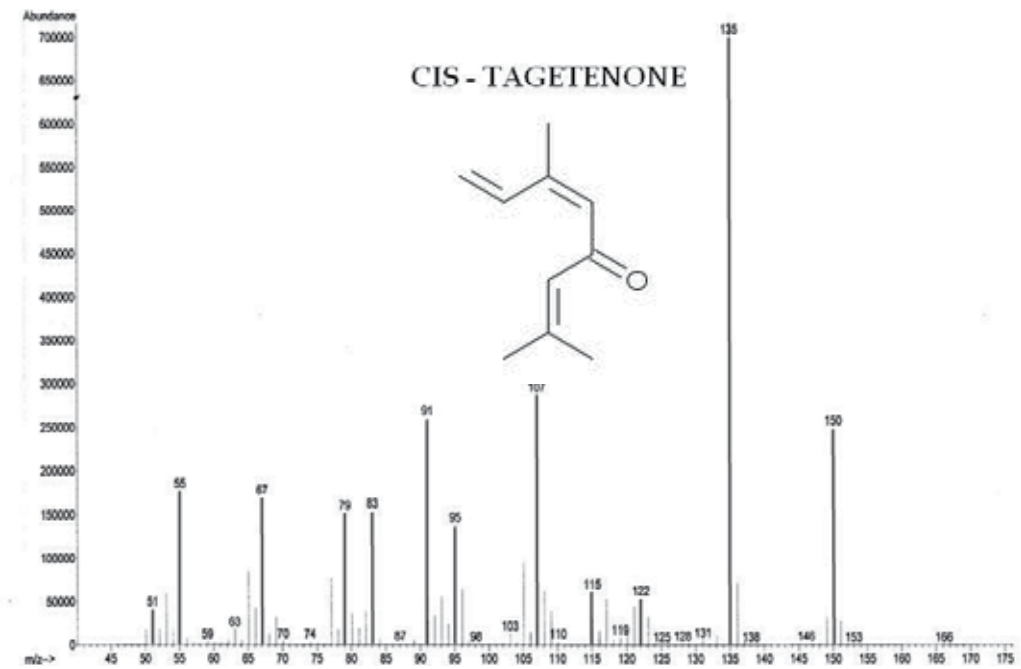


Fig. 3. Mass spectrum of cis-tagetenone.

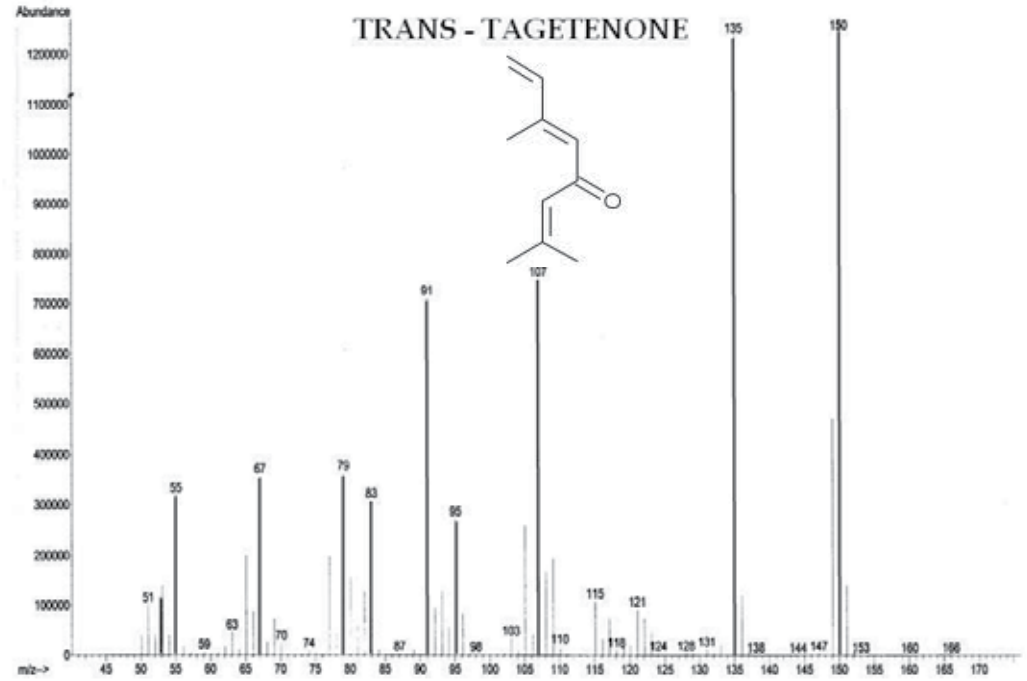


Fig. 4. Mass spectrum of trans-tagetenone.

Various studies on *T. minuta* reported that there are variations in the essential oil composition according to the harvesting location (Zygadlo et al., 1993), the growth stage (Moghaddam et al., 2007) the different parts of the plant (Weaver et al., 1994) and the different chemotypes (Gil et al., 2000). All these facts suggest that a deep study of the native *T. minuta* essential oils is necessary at the regional level.

Given that the applications of essential oils, such as the biocide action, are based on their composition, these variations may explain presence or absence of a certain effect, depending on the material used. With this purpose, we studied the composition of the essential oil of *T. minuta* at different stages of the plant life cycle.

The oil were obtained by steam-distillation, independently, from non-bloomed plant leaves, from bloomed plant leaves and from flowers of *T. minuta*. The essential oil obtained was analyzed qualitatively and quantitatively by means of GC. The quantitative data were determinated from the peak percentage areas. A Shimadzu GC 14B gas chromatograph equiped with a Mega Bore DB-WAX P/N 125-7032 (30 m x 0.53 mm i.d. x 1 µm film thickness) column and FID detector. The column was programmed as follows: 60ºC during 5 minutes, increased to 200°C at 5°C/min. The injector temperature was 180°C and detector temperature was 220°C. Peak area percentages were calculated with a Shimadzu C-R6A Chromatopac Integrator without including response factors or internal standards.

Figure 5 shows a comparison of the chromatograms obtained for the essential oil of *T. minuta* obtained from a) leaves from non-bloomed plants, b) leaves from bloomed plants and c) flowers, whose components had been previously identified by GC-MS. The variations in the relative proportions of the six components identified are clearly shown.

An analysis of the relative composition of flowers oil, shows that the major components are β-ocimene and tagetenone, at the expense of a considerable decrease of dihydrotagetone (major component of leaves from non-bloomed plant). This might explain the increased biocidal activity of flower oil, since its effectiveness is usually associated with the presence of tagetenona in it.

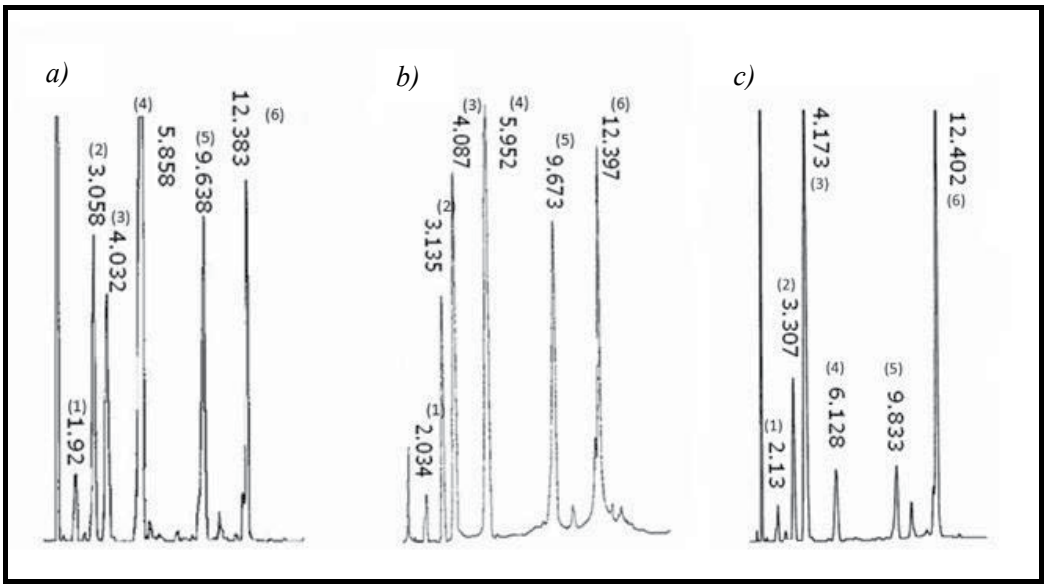


Fig. 5. Cromatograms of *Tagetes minuta* essential oil from *a)* leaves non-bloomed plant *b)* leaves bloomed plant and *c*) flowers. Components: β-phelandrene (1), limonene (2), β-ocimene (3), dihydrotagetone (4), tagetone (5) and tagetenone (6).

| **Peak N°** | **Component** | **leaves non-bloomed plant oil %** | **leaves bloomed plant oil %** | **leaves bloomed / fructified plant oil %** | **flower oil %** | **flower oil %** | **flower and seed oil %** |
|---|---|---|---|---|---|---|---|
| | | **March** | **April-May** | **June** | **April** | **May** | **June** |
| **1** | β-phelandrene | 2.6 | 2.1 | 2.2 | 0.8 | 1.2 | 1.4 |
| **2** | limonene | 12.9 | 13.1 | 11.8 | 4.4 | 6.4 | 8.6 |
| **3** | β-ocimene | 11.1 | 17.6 | 15.4 | **47.4** | **38.2** | **31.2** |
| **4** | dihydrotagetone | **47.0** | **28.1** | **38.3** | 1.5 | 6.6 | 11.7 |
| **5** | tagetone | 15.9 | 18.2 | 12.3 | 3.7 | 8.9 | 11.8 |
| **6** | tagetenone | 8.8 | 18.1 | 11.5 | **36.0** | **34.0** | **28.7** |

Table 1. Variation of the chemical composition of the essential oil from *T. minuta* L. leaves and flowers depending on the growth stage.

*Schinus molle* L. (Common name: bolivian Pepper, molle, aguaribay, huaribay, false pepper, peruvian mastic, anacahuita), plant species of the family Anacardiaceae, native to the Peruvian Andes (Huerta et al., 2010), is widely cultivated in tropical and subtropical countries (Wimalaratne, et al., 1996). The *Schinus molle* L. is widely spread outside their original geographical ranges, grows in North and Central America, Africa, Middle East and is cultivated around the Mediterranean in southern Europe. In Argentina, it is widely used as an urban tree because of its resistance to pollution, easy and economical spread and little need for irrigation.

The leaves of this tree are an important raw material for the extraction of essential oil used in folk medicine as they have antimicrobial, antispasmodic, antipyretic, antifungal and cicatrizing properties (Marongiu et al., 2004, Ferrero et al. 2006; Hayouni et al. 2008; Maffei & Chialvo, 1990). Likewise, it is noteworthy as a repellent and its utlization as a bioinsecticide (Ferrero et al., 2007). All these properties are associated with the presence of certain components in the essential oil, whose composition can change according to several factors: growth stage, geographical factors (location), ecological (habitat), genetic variability (chemotype ), the extraction process, etc. (Bandoni, 2000).

GC coupled with mass spectrometry proved to be a useful application to identify variations in the composition of essential oil and make comparative analysis, in view of their possible use originated on the properties described before. Essential oils obtained by steam-distillation from *Schinus molle* leaves and young tree branches from the city of Resistencia, Province of Chaco were compared with oils from trees of different backgrounds (Maffei & Chialvo, 1990, Menendez et al., 1996, Barroso et al., 2011; Guala et al., 2009).

With this purpose, these oils were analyzed using Shimadzu GC 14B gas chromatograph equipped with a Mega Bore DB-WAX P / N 125-7032 column (30 mx 0.53 mm id x 1 micron film thickness) and a FID detector.

Identification of the components was performed with an Agilent 6890 gaseous chromatograph with Agilent 5973 mass detector, a HP-5MS capillary column (30 mx 0.25 mm id x 0.25 um film thickness) a split / splitless injector, the ALS Agilent 7683 automatic injector, and Library NIST Mass Spectral program.

The major components identified in the essential oil of *S. molle* of Resistencia city were α-pinene (11.5%), β-pinene (14.71%), limonene (9.17%), α-ocimene (3.1%), germacrene D (3.6%), γ-cadinene (6.9%), δ-cadinene (4.9%) and epi-bicyclosesquiphelandrene (18.6%), as shown in the Figure 6 and Table 2. However, the composition of these oils differ in their main components compared to data reported from other sources, such as Liguria (Italy), whose main components are α-phellandrene (30%) and elemol (13.25%) (Maffei & Chialvo, 1990), Uruguay with 30% of Biciclogermacreno (Menendez et al., 1996), state of Rio Grande do Sul in southern Brazil with 40% of limonene (Barroso et al., 2011) and Santa Fe (Argentina) whose major component is limonene (40%) (Guala et al., 2009).

The differences in the oil composition of *S. molle* from distinct sources, probably attributed to different geography and plant chemotypes, strongly support for the need for a thorough evaluation of the biocidal properties of these oils obtained from different locations.

| compounds | Chaco (%) Arg. | Santa Fe (%) Arg. | Brazil (%) | Uruguay (%) | Italy (%) |
|---|---|---|---|---|---|
| α-pinene | **11.51** | **11.7** | 2.70 | - | 1.46 |
| canfene | 0.45 | 0.3 | | - | tr |
| β-pinene | **14.71** | **12.7** | | **13.95** | 0.10 |
| sabinene | | 45.0 | 5.85 | **12.92** | 0.67 |
| β-myrcene | 0.49 | 2.4 | 0.83 | **5.46** | tr |
| α-felandrene | - | | | - | **30.24** |
| limonene | **9.18** | 3.8 | **41.87** | 0.88 | 9.27 |
| β-felandrene | - | | | 0.30 | **9.63** |
| γ-terpinene | | 2.9 | | 1.13 | - |
| β-ocimene | 0.16 | | | - | - |
| α-ocimene | 3.17 | | | - | - |
| linalool | | | | 0.71 | - |
| caryophillene | 1.05 | 1.2 | **15.60** | **7.68** | 0.08 |
| terpinen-4-ol | | 4.4 | | **10.57** | 0.03 |
| azulene Derivative | 3.71 | | | - | - |
| α-humelene | | | | 0.57 | 0.39 |
| α-terpineol | | | | 1.25 | - |
| germacrene D | **3.57** | | **8.86** | **12.08** | **5.21** |
| bicyclogermacrene | | | **11.59** | **29.20** | - |
| isoledene | 3.93 | | | - | - |
| γ-cadinene | **6.87** | | | - | - |
| δ-cadinene | **4.90** | | | 1.26 | 1.71 |
| copaene | 0.26 | | | - | 0.02 |
| elemene | | | | | 0.03 |
| caryophilene oxide | | | | 0.53 | |
| spathulenol | 0.99 | | | - | |
| eudol | 1.11 | | | - | |
| germacrone | | | | 0.75 | |
| unidentified sesquiterpene alcohol | 3.40 | | | - | |
| epi-bicyclosesquiphelandrene | **18.60** | | | | |
| A-cadinol | 1.62 | | | | tr |
| elemol | | | | | **13.25** |
| neril hexanoate | | | | | 2.94 |
| γ-eudesmol | | | | | 3.24 |
| γ-cadinol | | | | | 4.70 |
| unidentified sesquiterpenes | 6.50 | | | | 1.96 |

Table 2. Chemical composition of essential oils of *Schinus molle* Argentina, Uruguay, Brazil and Italy (tr: traces).

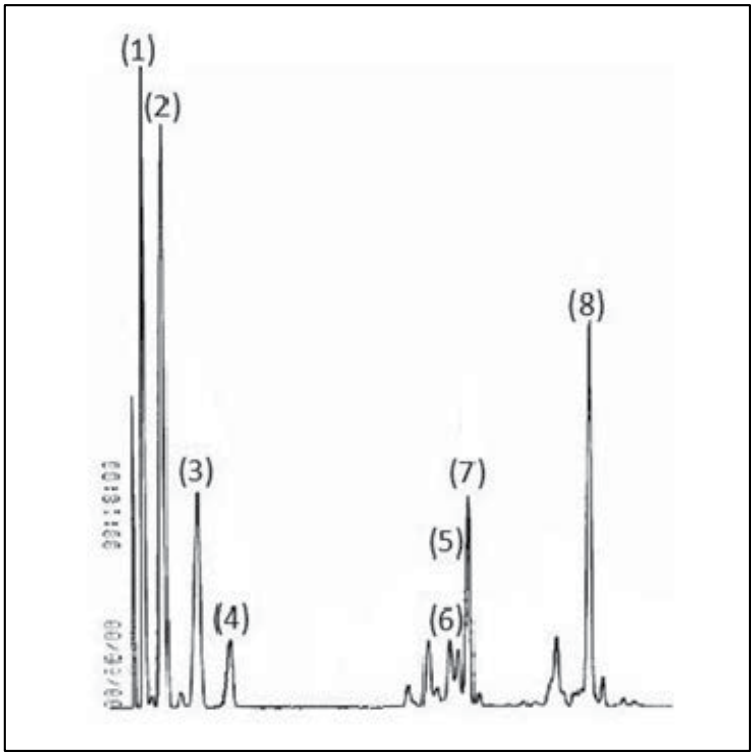


Fig. 6. Chromatogram of essential oil from *Schinus molle*. Components: α-pinene (1), β-pinene (2), limonene (3), α-ocimene (4), germacrene D (5), γ-cadinene (6), δ-cadinene (7) and epi-byciclosesquiphelandrene (18.6%).

## 5. Quantification of essential oil components

In order to quantify a component by GC, it is possible to use the methods of either the internal or external standard. For the particular case of essential oils, one can frequently use the method of relative concentrations related to the total area of the peaks, due to the similarity of reaction factors that the main components of the terpene family have. What follows is a brief description of each one of them, its advantages and limitations.

The relative area method results in a percentage relation of the area corresponding to each component with regards to the total area of the chromatogram, understanding this as the addition of the individual areas of each one of them, as it is shown in the following equation (1) (Orio et al., 1986)

$$\%\text{relative of the component} = \frac{\text{component area}}{\text{Total area}} \text{x } 100 \quad (1)$$

The external standard method is easy to apply, and is the habitual basis for numerous analytical determinations. It allows us to calculate the concentration or percentage of mass of one or many constituents that appear separated in the chromatogram, even in the presence of unsolved peaks.

| Number of peak | Time (min) | Area | Relative concentrations (%) |
|---|---|---|---|
| 1 | 0.382 | 6679 | 0.10 |
| 2 | 2.730 | 176430 | 2.74 |
| 3 | 9.065 | 2343024 | 36.45 |
| 4 | 10.070 | 53522 | 0.83 |
| 5 | 10.385 | 177509 | 2.76 |
| 6 | 11.472 | 184517 | 2.87 |
| 7 | 11.888 | 119067 | 1.85 |
| 8 | 12.618 | 541246 | 8.42 |
| 9 | 12.837 | 689214 | 10.72 |
| 10 | 13.760 | 1464544 | 22.79 |
| 11 | 15.648 | 70466 | 1.10 |
| 12 | 15.990 | 282347 | 4.39 |
| 13 | 16.763 | 197766 | 3.08 |
| 14 | 17.333 | 121177 | 1.89 |
| Total | | 6.427.508 | 100 |

Table 3. Individual areas, their addition and the relative percentage of each component, corresponding to the chromatogram in Figure 3.

Such process is based on the comparison of two chromatograms, one of which corresponds to the established standard and the other one to the component being studied. In such comparisons, we have to dilute the standard (purity ≥ 95.0 % from Aldrich) of the component to be determined. For example, (±)- Citronella GC (Zambón et al, 2011). A known volume of what has been diluted is injected and the reference area (Aref) and the corresponding peak in the chromatogram is measured. Then, without changing any of the analysis conditions, an identical volume of the sample is injected and the area corresponding to the sample (Asample) is measured. Since the volume in both cases is equal, there is proportionality between the areas, which depends on the injected masses and the concentrations. Thus, we can determine the sample concentration with the equation (2) (Rouessac et al., 2003)

$$\text{Sample concentration} = \frac{\text{Asample}}{\text{Aref}} \text{x reference concentration} \quad (2)$$

To exemplify this methodology we show the results of the quantitative study of citronella (the major component of the essential oil of *Cymbopogon winterianus*), during the reaction of the cyclization to isopulegol with acid heterogeneous catalysis. The samples corresponding to tests 1 and 2 show the remnants of citronella after the cyclization of this component to isopulegol, while samples 3 and 4 show initial citronella.

It is important to take into account that, if only one point corresponding to the standard is used, the calibration curve is considered to go through the origin, so the precision will be acceptable if the concentrations of the standard and the samples are similar. This can be considerably improved by using many identical injections for both, the component and the standard. However, instead of taking a great number of measurements, it is preferable to work with calibration with various points, called multilevel calibration.

| Sample | Area | Concentration (%P/P) |
|---|---|---|
| standard of (±)-Citronellal | 42624105 | 95.0 |
| Sample 1 | 43072780 | 96.0 |
| Sample 2 | 41726755 | 93.0 |
| Sample 3 | 13035302 | 29.05 |
| Sample 4 | 12373866 | 27.58 |

Table 4. Areas and citronellal concentrations (initial and remnant) of the reaction of cyclization of isopulegol, by means to the external standard method.

| Sample | Mass isopulegol (g) | Mass solvent (g) | Mass of internal standard (g) | Area of internal standard | Area isopule-gol | Relation A isopulegol/ A internal standard | Concentra-tion of isopulegol (Mol) |
|---|---|---|---|---|---|---|---|
| M0 | 0.0189 | 0.1139 | 0.0361 | 809262 | 442226 | 0.55 | 0.56 |
| M1 | 0.0439 | 0.1115 | 0.0353 | 902541 | 1729214 | 1.92 | 1.14 |
| M2 | 0.0720 | 0.1158 | 0.0365 | 667942 | 2032979 | 3.04 | 1.67 |
| M3 | 0.0893 | 0.1060 | 0.0366 | 610626 | 2220885 | 3.64 | 1.93 |
| M4 | 0.1141 | 0.1038 | 0.0344 | 633292 | 3020415 | 4.77 | 2.24 |

Table 5. Calibration curve of isopulegol by means of the internal standard method.

The internal standard method is based on the use of the relative response factor of each component to be measured with respect to a marker introduced as reference. This avoids the imprecision related to the injected volumes, which is a disadvantage of the previous method. However, it requires the addition of a component to a sample dilution. In general, a calibration curve is built by applying different solutions of increased concentrations of the standard analyte with a constant quantity of internal standard. When injecting such samples, we obtain the relation between the areas of the analyte and the internal standard; then, it is marked in a graph according to the concentration of analyte in each solution. By means of interpolation in the graphic, we get the relation of the areas of an unknown sample, which has to contain the same quantity of internal standard.

The selection of an appropriate internal standard is very important. It has to be pure and must not be present in the sample at the beginning; its elution peak must be well solved in relation to those that conform the chromatogram of the sample; the retention times must be next to those of the solute to be measured; its concentration has to be near or superior to the rest of the solutes, so that it allows for a linear answer of the detector, and must be inert in relation to the constituents of the sample.

Table 5 shows an example of a calibration curve for (-) – isopulegol (purity > 99% GC, addition of enantiomers) where tert-butanol was used as solvent, and the internal standard was methyl ethyl ketone. Figure 7 shows the obtained calibration curve.

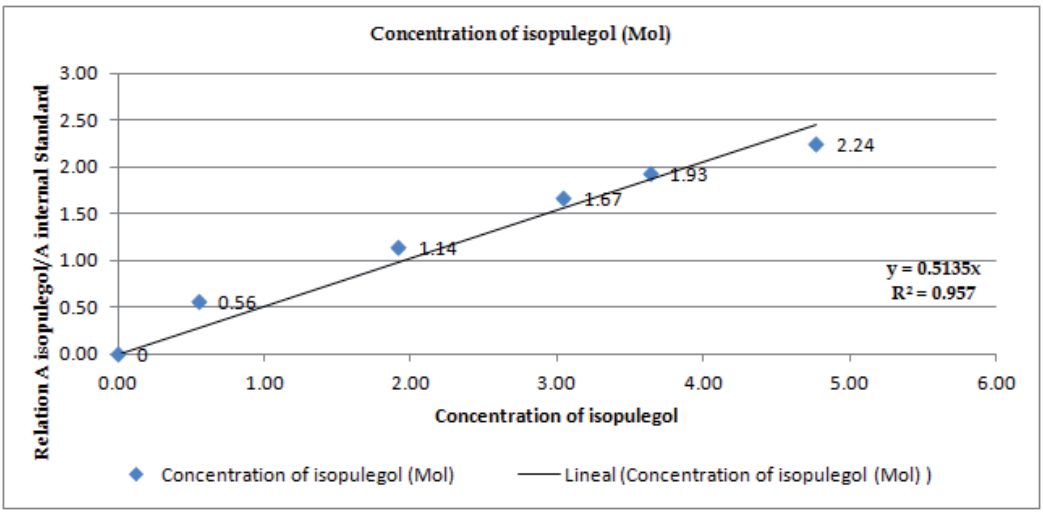


Fig. 7. Calibration curve of isopulegol by means of the external standard method.

By using the obtained calibration curve, it is posible to calculate the isopulegol moles in unknown samples.

| Samples | Area internal standard | Area of isopulegol | Relation A isopulegol/A internal standard | Concentrations of isopulegol of unkown samples |
|---|---|---|---|---|
| Sample 1 | 733318 | 140553 | 0.19 | 0.44 |
| Sample 2 | 789175 | 363884 | 0.46 | 0.55 |

Table 6. Concentration of isopulegol (expressed in moles) in unknown simples.

It is important to remark that the use of automatic injectors increases the reproducibility in the injection, which has allowed for the spread in the use of external standards because of its simplicity. However, in the case of manual injection, the internal standard method turns out to be simple and adaptable to everyone.

## 6. Conclusion

In this chapter, we described the importance of the technique of gas chromatography in the study and analysis of essential oils. It is important to remark that they have extremely varied industrial applications, such as food, pharmaceutical, aromas, flavors, organic synthesis, among other.

This technique has become an indispensable tool for the control of essential oils, as prices of these vary depending on their composition and distribution of components, and the oils do so according to their origin, growth stages, chemotypes, among others.

Currently, there are new stationary phases which significantly improve the separation of the components, especially the most important are chirals phases, which allow for the separation of compounds with optical isomerism, which creates an especially important step for the chemical and pharmaceutical industries.

When searching for the correct separation of the peaks, it is important to consider the selection of detectors. Notably, the most commonly used for identification of the components is the mass spectrometer (MS), while for the quantification of its simplicity and specificity toward organic compounds is the flame ionization detector (FID).

It is important to note that the components of essential oils are what provide them with their intrinsic properties and for which they are used productively.

As important as identifying the components, quantifying them, such as the variation in the distribution of mixture components, modifies the particular properties of essential oils.

As developed in this chapter, there are several measurement techniques, such as the relative percentages, internal or external standards. Each has advantages and disadvantages and are applicable to quantifying components that have previously been identified.

Through the study of the essential oils by GC-MS, we have been able to appreciate the value of this technique in the identification and quantification of their components. With this technique, it was possible to show that the composition of the essential oils of *Tagetes minuta* varies in different parts of plants, as well as in different stages of growth. It was also found that *Schinus molle* essential oil, an important biocide, shows large differences in composition, depending on the origin of plants, been this data a key for assessing their biocidal properties. In a similar way, this technique becomes important in the quantification of oil components and even more when added value products are developed from them, such as the cyclization of citronellal to isopulegol.

Argentina is an important exporter of essential oils because it has favorable agro-climatic characteristics for the development of aromatic plants, both native and foreign. The main destination of these products are European countries that demand high levels of quality and is this sense GC is the technique that ensures compliance with the specifications agreed between the parties.

Then, in a highly demanding market such as essential oils is gas chromatography the technique that provides the industry and science with simplicity, speed and efficiency for the characterization and quantification of components.

## 7. Acknowledgment

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# 16

# Chiral and Nonchiral GC×GC/TOFMS Analysis of Natural Compounds: The Case of Possible Aggregation Pheromones of Chinese Bark Beetles *Ips shangrila* and *Ips nitidus*

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## 1. Introduction

Semiochemicals (compounds used for chemical communication within and between species) are usually produced in tiny amounts that vary from picograms to nanograms. As a consequence, pheromones are rarely isolated in quantities sufficient to carry out the full range of spectroscopic tests ($^{1}$H and $^{13}$C NMR, infrared, and mass spectrometry) routinely used in structural organic analysis. Pheromone identifications predominantly rely on gas chromatography coupled with mass spectrometry (GC-MS) and electroantennographic detection (GC-EAD) (Struble & Arn, 1984). The GC-EAD analysis, where insect antenna is used as specific biological detector, helps to focus the identification effort only on a particular part of the chromatogram. Quite often, however, the GC-EAD indicates an activity in chromatogram areas where FID or MS detects no compound (Svatoš et al., 1999, 2006; Kalinová et al., 2006) or the active compound is overlapped by higher amount of non-active one(s).

Enantiomeric composition of a pheromone is instrumental with respect to the behaviour mediating capacity of the signal. This especially stands for bark beetles (Coleoptera: Curculionidae, Scolytinae) where even different populations of the same species employ pheromone of different enantiomeric composition (Seybold, 1993; Miller et al., 1996). Enantioselective production of, and response to pheromones has been demonstrated in many species of Scolytinae subfamily (Birch, 1984; Borden, 1985; Byers, 1989). Electrophysiological studies have revealed that species such as *Ips pini* Say, *I. typographus* (L.), *I. paraconfusus* Lanier, *Scolytus multistriatus* (Marsham), *S. scolytus* (F.) and *Trypodendron lineatum* (Olivier) and many others have olfactory receptor cells specific to optical isomers of aggregation pheromones (Mustaparta et al., 1980, 1984; Wadhams et al., 1982; Tømmeras et al., 1984).

Conventional chiral analysis provides data of a high complexity with high extent of peak overlap even for moderately complex mixtures. This complexity makes the chiral analysis of

low quantities of insect pheromones quite difficult. The comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC-TOFMS) is the most advanced analytical technique of high sensitivity and selectivity. It is also a technique which provides a substantial enhancement of peak capacity and signal intensity over conventional GC analysis. Signal capacity and intensity enhancements are achieved by using a modulator which is capable to trap and concentrate effluent portions from the first column and deliver these "samples" into the second column (Lee et al., 2001). Several books and reviews focusing on the two dimensional gas chromatography with FID or MS detection have been published to familiarize the reader with the method's fundamentals (Adahchour et al., 2008; Dallüge et al., 2003; Mondello et al., 2008; Panić & Górecki, 2006; Ramos, 2009). The most advanced of these methods, comprehensive GC×GC-TOFMS, was successfully used for separation of many groups of natural compounds or xenobiotics (Dallüge et al., 2002; Gogus et al., 2006, Kalinová et al., 2006, 2009; Matamoros et al., 2010; Rocha et al., 2007 and many others). The using of chiral column in one of two dimensions of the comprehensive two-dimensional gas chromatography thus adds a new "dimension" into the separation process and offers another, more intimate view into 2D/3D-visualizations of chromatograms (Shellie et al., 2001).

China has large mountain regions of conifer habitat with unexpectedly rich and still largely unknown Scolytid fauna (Critchfield & Little, 1966; Cognato & Sperling, 2000; Cognato & Vogler, 2001; Cognato & Sun, 2007). In north-eastern margin of the Tibetan Plateau (NW China), where remnants of the former mountain forest occur, bark beetles cause severe damages. The main target of bark beetle infestation is the thick leaf spruce, *Picea crassifolia* Komarov (Pinaceae). The major *Ips* species in these areas are *Ips nitidus* and newly discovered *Ips shangrila* (Cognato & Sun, 2007), monophyletic sister species of *Ips typographus* (L.) and *Ips amitinus* (Eichhoff), respectively (Cognato & Sun, 2007). Both bark beetle species are serious pests of *Picea crassifolia* in NW China mountain areas.

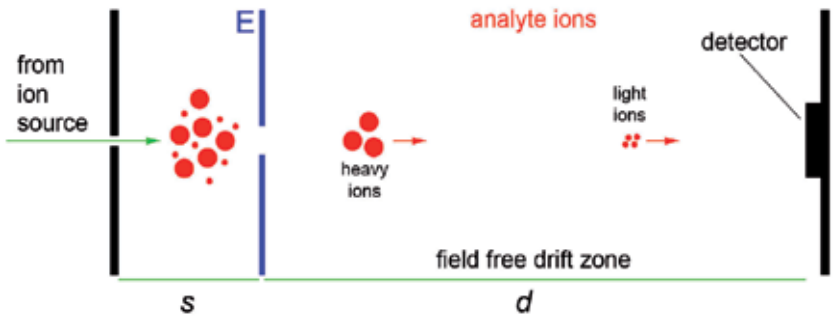
In the present chapter we want to demonstrate in the form of chemo-ecological/analytical study the applicability of the GC×GC-TOFMS for achiral and chiral insect semiochemicals analysis together with another key instrumental technique in this research - the gas chromatography with electroantennographic detection (GC-EAD). The insects we described in the study were the two above mentioned Chinese bark beetle species - *Ips nitidus* and *Ips shangrila*.

## 2. Brief introduction into used instrumental techniques

### 2.1 TOFMS

Time-of-flight mass spectrometry (TOFMS) is probably the simplest method of mass spectrometric measurement by the physical principle. The key features of TOFMS are extreme sensitivity (all ions are detected), practically unlimited mass range and as well as high-speed analysis (recent TOFMS instruments are able to measure hundreds full spectra per second). This all makes TOFMS one of the most desirable methods of mass analysis (Schlag, 1994; Guilhaus, 1995). The general scheme of TOFMS is shown in Scheme 1.

The ions are introduced either directly from the ion source of the instrument as a very short pulse. This results in all the ions receiving the same initial kinetic energy. As they then pass




Scheme 1. TOFMS general scheme

along the field free drift zone, they are separated by their masses, lighter ions travel faster, reaching the detector earlier. This enables the instrument to record all ions as they arrive at the detector and so accounts for the techniques high sensitivity. The equation describing the time-of-flight separation is:

$$\frac{m}{z} = 2eEs\left(\frac{t}{d}\right)^2$$

Where $m/z$ is mass-to-charge ratio of the separated ion, E is the extraction pulse potential, $s$ is the length of the flight tube over which E is applied, $d$ is the length of the field free drift zone and finally, $t$ is the recorded time-of-flight of the ion.

### 2.2 GC×GC-TOFMS

The used comprehensive GC×GC-TOFMS is a recently developed analytical technique which offers a solution to the co-elution problem and provides high sensitivity and selectivity (Dallüge et al., 2003; Dimandja, 2003; Mondello et al., 2008). In principle, the instrumental system consists of two GC ovens equipped with columns of different manner of separation (by a boiling point, polarity, chirality…) connected by an interface equipped with a non-moving quad-jet cryomodulator. The modulator's cryogenic trap (using two cold jets) repeatedly condenses compounds eluting from the primary column and releases them (using two hot jets) periodically as short pulses to the secondary column. Parameters like duration and frequency of both condensation and injection pulses are variable and allow precise tuning of the instrument according to the requirements of the analysis. Since the GC×GC system produces very narrow peaks (mostly narrower than 50 ms, depending on the frequency of modulation) a TOFMS detector with a high acquisition rate (up to 500 full spectra per second) is required. The pulsed nature of the TOFMS source of ionisation further enhances the system accuracy by avoiding spectral skewing common in a continuous ionisation mode. GC×GC with TOFMS detection thus operates with a high precision independent of concentration range.

Another important advantage of the comprehensive GC×GC, especially significant while analyzing the complex mixtures of natural origin with trace concentrations of target analytes, is the second dimension separation of the background from the compounds of our interest. The background components (solvent traces, cyclosiloxanes of septum and column bleed, etc.)

have significantly different retention and elute from the 2nd dimension column much earlier. The high "wall" of column bleed is usually perfectly separated in the second dimension allowing MS analysis without any interference (Kalinová et al., 2006; Rocha et al., 2007).

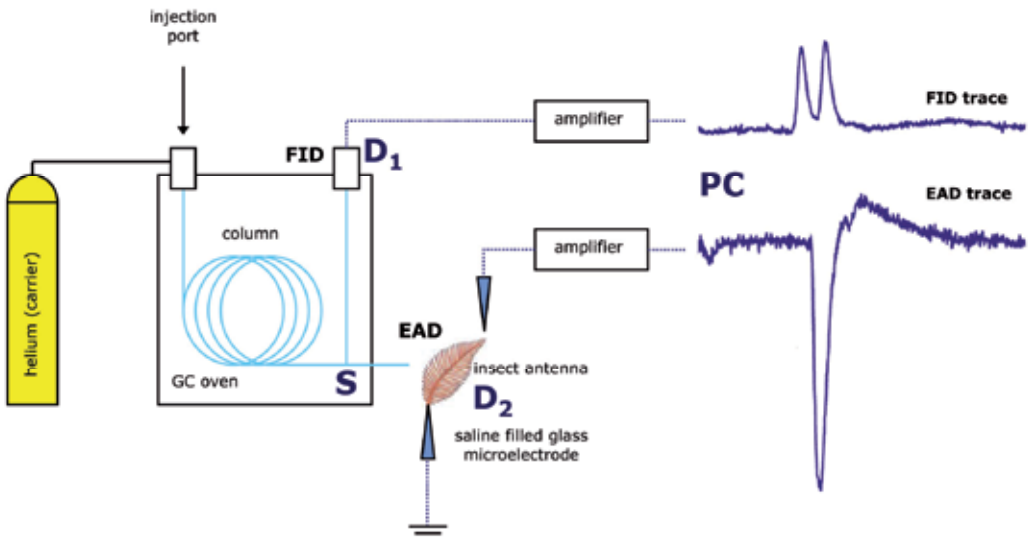
The standard GC×GC-TOFMS experiment involves MS analysis of hundreds "cut-outs" of the primary column chromatogram. Any analysis thus can be seen as a series of 2D-chromatograms eluting one after another. Depending on modulation parameters and peak widths, each compound eluting from the primary column shows up as several consecutive narrow peaks. These complex and voluminous data are further converted into a contour plot with the primary column retention plotted along the X-axis, and the secondary column retention plotted along the Y-axis (2D-visualization of GC×GC-TOFMS analysis), and eventually detector response on the Z-axis (3D-visualization of GC×GC-TOFMS analysis). On such chromatograms, compounds characterised by the same mass spectrum appear as one spot. As the quantity of compounds can be colour coded, the colour of individual spots continuously changes from peak base to its top. Such coloured contour plots make searching for possible active compounds much easier, especially when a display based on characteristic ion current is selected instead of a total ion current (TIC). The pattern analysis of 2D-chromatogram allows fast and effective comparison of many sets of obtained analytical data as well as a quick search of any differences. Thanks to set of two retention parameters, GC×GC-MS also makes identification more reliable in comparison with a standard one dimensional GC. The technique provides identification capabilities similar to performing two separate 1DGC separations on different columns, as required by some analytical methods. The identification by two dimensional retention times and mass spectral match between an analyte and a spectrum, offered either by a library or obtained by an injection of available standard, is very robust and the possibility of misidentification is low.

### 2.3 GC-EAD

GC-EAD is gas chromatography method in which a unique biological detector, based on living insect antenna, is used as one of two detection systems. GC-EAD is an analytical method which offers very fast and reliable identification of compounds in complex natural mixtures that stimulate the olfactory sensilla of insect antennae (Struble & Arn, 1984). In other words, the GC-EAD helps to discover what specific chemicals in tested mixture an insect can smell, respective, which compound elicits the electric antennal response.

It has been known for more than 50 years that the electric potential difference (voltage) measured by two tiny microelectrodes between the tip and the base of an insect's antenna changes measurably when the antenna is exposed to compounds of biological significance for the insect. This voltage represents the sum of potentials of all responding olfactory neurons around the recording electrode, and the voltage's amplitude approximately corresponds to an insect's sensitivity to a particular compound. The voltage produced by an antenna can be easily amplified, measured and recorded together with corresponding data delivered by a classical FID detector. The GC-EAD system is in principle very simple apparatus based on a standard chromatograph with FID detector. The only important change is a post-column splitter followed by doubled detection system. After the separation is the column effluent split in two parts, with the first half transported to a FID detector and the other one transmitted to the EAD detector. The effluent is cooled by mixing with purified air and blown over a live insect antenna attached to a high impendence amplifier.

The amplified electrical signals of both detectors are subsequently digitized, processed and recorded simultaneously against the time (Scheme 2).




(S) Splitter - column effluent is split in two halves; ($D_1$) FID produces a signal proportional to the amount of volatile in the column effluent; ($D_2$) EAD detection – with air mixed column effluent is blown over an insect's antenna; (PC) combined FID/EAD chromatogram.

Scheme 2. A standard GC-EAD system

## 3. Experimental part

### 3.1 Insects

#### 3.1.1 Determination of target bark beetle species

Bark beetles colonizing *Picea crassifolia* were collected randomly on several locations within the territory of two villages of Maixiu and Sibosha in Tongren and Beishan counties of Qinghai province (NW China). The specimens were collected in May 2007 and May 2008 directly from freshly infested spruce logs. The morphological characters of individual beetles were inspected and determined based on available taxonomic bark beetles literature and when possible also by comparison with the holotypes of particular species located in different natural history museum's collections (Naturhistorisches Museum Wien - Austria, National Museum Prague - Czech Republic, etc.). Parental and larval galleries of each respective species were investigated on both freshly infested and dead trees and their characteristics were associated with the identified species.

#### 3.1.2 Sex determination of target bark beetle species

For physiological experiments sex determination of living specimens was necessary since males and females often produce different sets of semiochemicals. In general, sex determination of living individuals of some bark beetles species is morphologically quite

straightforward (*Polygraphus*, *Pityogenes* etc.). In other species however, it can be rather difficult or impossible without the destructive method of dissection of male aedeagus.

In *Ips nitidus*, sex of beetles was determined under stereomicroscope by means of sex specific differences of hair density on head and pronotum as described for *Ips typographus* by Schlyter & Cederholm (1981). *Ips nitidus* females had more densely haired frontal part of pronotum and the frons from forehead view then males. In *I. nitidus*, other morphological sexual differences exist in the shape and size of the frontal and lateral tubercles on lateral margins of the elytral declivity, but these characters are more difficult for practical use.

In *Ips shangrila*, sex can be determined only under stereomicroscope based on sex-specific differences in the size of the frontal tubercle from lateral view. In males, this tubercle is remarkably large and its base is wider. Cognato & Sun (2007) reported other sex-specific differences, but the morphological features described by these authors were quite difficult to follow.

After sex determination, beetles were removed from infested treed during initial infestation phase, when males construct nuptial gallery and emit aggregation pheromone that serves as sex pheromone for females and synchronizes mass attack of bark beetles aiming to kill the tree. The beetles were then dissected and gut extracted the same day.

### 3.2 Guts and frass extraction

Guts of males and females of both species were dissected under stereomicroscopic control. Excised guts were placed in hexane (10 μL of hexane per one gut) and left in ambient temperature 24 hrs. Then, the extracts were filtered, transferred into glass capillaries, flame sealed and stored in a freezer until analysis. Except guts, we extracted also frass (a mixture of beetle-produced sawdust and excreta that accumulated at the entrance hole of the beetle gallery), collected from freshly colonized logs of *Picea crassifolia* using glass exhaustor. Obtained material was extracted by trace analysis grade hexane under ambient temperature for 1 hr. Extracts were filtered, transferred into glass break-seal ampoule and sealed until analyses.

### 3.3 SPME

SPME analyses of infested bark strips (males and females kept separately) were performed using CAR-PDMS fibre (Supelco, Sigma-Aldrich Group, St. Louis, MO, USA). For SPME experiments, sexed beetles were allowed to bore into 15×20 cm fresh strips of *Picea crassifolia* bark (separated sexes), placed in 20×25 cm containers. SPME fibres were exposed 60 minutes and then immediately analysed using for GC-EAD and/or GC×GC-TOFMS analysis, respectively.

### 3.4 GC-EAD

In GC-EAD experiments, isolated head-antennal preparations of bark beetles were used. Glass Ag/AgCl microelectrodes filled with insect saline solution were used (Roelofs, 1984). The reference electrode was inserted into the head, the recording one was brought into contact with antennal sensory epithelium on antennal club. Analysed samples (5-10 beetle equivalents) were injected splitless into a 5890A Hewlet-Packard gas chromatograph

equipped with an Agilent J&W DB-5 column (Agilent, Santa Clara, CA, USA; 30 m × 250 µm i.d. × 0.25 µm film) column. The column was splitted at the end by Graphpack 3D/2 four-arm splitter allowing divide the eluate to FID and EAD detectors. The GC was operated at an initial temperature 50 °C for 2 min then ramped at a rate 10 °C/min to 270 °C (with 10 min hold). The temperature of GC inlet and detector was set to 200 °C and 260 °C, respectively. A series of $C_8$-$C_{22}$ alkane standards was co-injected with analyzed samples to allow calculate Kovats' indices ($I_K$) of EAD active peaks and to check the correspondence of retention behaviour and antennal activity of authentic compounds with synthetic standards.

### 3.5 Achiral GC×GC-TOFMS analysis

The analyses were performed using a LECO Pegasus 4D Instrument (LECO Corp., St. Joseph, MI, USA). A slightly polar Agilent J&W DB-5 column (Agilent, Santa Clara, CA, USA; 30 m × 250 µm i.d. × 0.25 µm film) was used for GC in the first dimension. It was the same type of column used for GC-EAD. The second dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX, USA; 2 m × 100 µm i.d. × 0.1 µm film). Helium was used as a carrier gas at a constant flow of 1 mL/min. The temperature program for the primary GC oven was as follows: 50 °C for 2 min, then 50–280 °C at 7 °C/min, and finally 10 min hold at 280 °C. The program in the secondary oven was 7 °C higher than in the primary one and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration and the cool time between stages were set at 4.0, 0.8 and 1.2 s, respectively. The transfer line to TOFMS detector source was operated at 260 °C. The source temperature was 200 °C with a filament bias voltage of −70 eV. The data acquisition rate was 100 Hz (scans/s) for the mass range of 29–400 amu. The detector voltage was 1450 V. One microlitre samples were manually injected in the splitless mode. The inlet temperature was 220 °C. The purge time was 30 s at a flow of 80 mL/min. Data were processed and consecutively visualised on the 2D and 3D chromatograms (as TIC intensity) using the advanced LECO ChromaTOF™ software. As in GC-EAD experiments, $C_8$-$C_{22}$ alkane standards were analyzed under the same parameters to allow the comparison of Kovats' indices ($I_K$) with GC-EAD experiments. Two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST)V. 2.0- Mainlib and Replib) and our own-made database of terpenoids comprising 100 compounds were used. Mass spectral match factor, similarity >850, was used to decide whether a peak was correctly identified or not.

### 3.6 Chiral GC×GC-TOFMS analysis

The absolute configuration of antennaly active compounds was assigned using LECO Pegasus 4D Instrument (LECO Corp., St. Joseph, MI, USA). The chiral analysis was performed on a β-cyclodextrin HP-Chiral β column (Agilent, Santa Clara, CA, USA; 30 m × 250 µm × 0.25 µm film). The chiral column was used as the first dimension column. The second dimension column was used the same as described in non-chiral analysis section above. Helium was used as a carrier gas at a constant flow of 1 mL/min. The temperature program for the primary GC oven was as follows: 50 °C for 2 min, then 50–180 °C at 2 °C/min, then 180–210 °C at 15 °C/min and finally 35 min hold at 210 °C. The program in the secondary oven was 7 °C higher than in the primary one and was operated in an iso-ramping mode. The cryomodulator settings and all other GC×GC-TOFMS parameters were the same as described in non-chiral analysis section above.

### 3.7 Chemicals

Synthetic enantiomerically pure terpene standards (Contech Inc., Delta, British Columbia) were dissolved in hexane and analyzed or co-injected with extracts under the same conditions as authentic samples.

## 4. Results

### 4.1 GC-EAD and achiral GC×GC-TOFMS analysis

#### 4.1.1 *Ips nitidus*

The SPME GC-EAD analyses of volatiles emanating from male infested bark consistently showed two areas of EAD activities (EAD1 and EAD2) characterized by $I_{K(EAD1)}$ = 1099 and $I_{K(EAD2)}$ = 1150. These two active EAD areas were observed also in GC-EAD analyses of male and female guts and frass extracts. Except EAD1 and EAD2, GC-EAD analysis of guts extracts showed the 3rd area of EAD activity, EAD3, characterized by $I_{K(EAD3)}$ = 1175. Finally, GC-EAD analyses of male frass extract provided another two EAD peaks EAD4 and EAD5 with $I_{K(EAD4)}$ and $I_{K(EAD5)}$ calculated as 948.9 and 991.6, respectively. A typical section of the GC-EAD recording in area of EAD1-EAD3 is demonstrated in Figure 1.

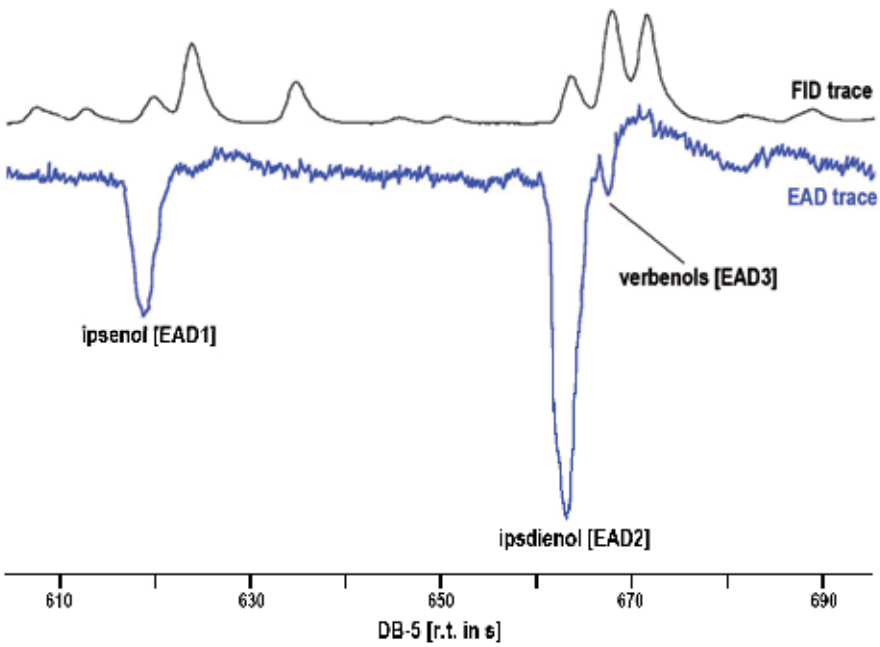
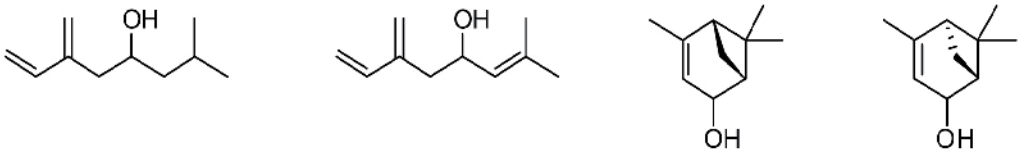



Fig. 1.

Reconstructed GC-EAD recording from isolated antennae of *Ips nitidus* in response to male guts extract. *I. nitidus* antennae responded to 3 compounds from the extract (EAD1-EAD3) corresponding to ipsenol (**1**), ipsdienol (**2**) and *cis*/*trans*-verbenol (**3**, **4**), respectively.




**1** **2** **3** **4**

Compound EAD1 subjected to GC×GC-TOFMS analysis gave the following diagnostic ion peaks; m/e 41, 53, 79, 85, 93, 121, 136, 154 ($M^+$), 68 (base ion peak). The $I_K$ was calculated 1111. This value, with counting the longer retention cause by the presence of the secondary column, matched well with $I_{K(EAD1)}$ = 1108 confirming the same identity in both GC-EAD and GC×GC-TOFMS analysis. Spectrum, retention and GC-EAD behaviour of compound EAD1 was identical to that obtained for synthetic ipsenol (2-methyl-6-methylene-7-octen-4-ol, **1**).

Compound EAD2 subjected to GC×GC-TOFMS analysis gave the following diagnostic ion peaks; m/e 41, 57, 67, 91, 109, 119, 134, 152 ($M^+$), 85 (base ion peak). The $I_K$ was calculated 1151. This value matched well with $I_{K(EAD2)}$ = 1140 confirming the same identity in both GC-EAD and GC×GC-TOFMS analysis. Spectrum, retention parameters and GC-EAD behaviour of compound EAD1 was identical to that obtained for synthetic ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol, **2**).

Compound EAD3 subjected to GC×GC-TOFMS analysis showed after spectra deconvolution process presence two close eluting geometrical isomers of verbenol (*cis* and *trans*), which were not separated well during the GC-EAD experiments. Were obtained the following diagnostic ion peaks; m/e 41, 55, 59, 67, 79, 91, 109, 119, 134, 137, 152 ($M^+$), 94 (base ion peak; for *cis*-isomer) and m/e 41, 55, 67, 81, 91, 109, 119, 137, 152 ($M^+$), 109 (base ion peak; for *trans*-isomer). The $I_K$ were calculated 1158 for *cis*-isomer, and 1164 for *trans*-isomer, respectively. These value matched well with $I_{K(EAD3)}$ = 1155 (unseparated *cis*/*trans*-isomers) confirming the same identity in both GC-EAD and GC×GC-TOFMS analysis. Spectra and retention behaviour of compounds hidden in peak EAD3 were identical to that obtained for synthetic *cis*-, respectively *trans*-verbenol (*cis*-4,6,6-trimethyl- bicyclo[3.1.1]hept-3-en-2-ol, **3** / *trans*-4,6,6-trimethylbicyclo[3.1.1]- hept-3-en-2-ol, **4**)

Compounds EAD4 and EAD5 subjected to GC×GC-TOFMS analysis gave the following diagnostic ion peaks; m/e 41, 53, 67, 77, 79, 105, 121, 136 ($M^+$), 93 (base ion peak; EAD4), and m/e 41, 53, 69, 77, 91, 107, 121, 136 ($M^+$), 93 (base ion peak; EAD5), respectively. The $I_K$ were calculated 947 and 989, respectively This value matched well with $I_{K(EAD4)}$ = 949 and $I_{K(EAD5)}$ = 992 confirming the same identities in both GC-EAD and GC×GC-TOFMS analysis. Spectrum, retention and GC-EAD behaviour of compound EAD4 and EAD5 were identical to that obtained for standards of α- and β-pinene.

### 4.1.2 *Ips shangrila*

The SPME GC-EAD analyses of male and female infested bark and male and female guts extracts consistently showed one area of EAD activities with retention time corresponding to EAD2 from analysis of *I. nitidus* extracts. $K_{I(EAD2)}$ in SPME GC-EAD experiments using *I.*

*shangrila* antennae was calculated as 1155 and subsequent GC×GC-TOFMS analyses gave identical results as in *I. nitidus* case – the active compound is ipsdienol (**2**). Activity in area of EAD1, also caused by identical compound as in *I. nitidus* – ipsenol (**1**), was observed only in few analyses.

The GC-EAD analyses of male frass extract showed in addition to EAD1/EAD2 one more EAD active area (EAD3) eluting later and characterized by $I_{K(EAD3)}$ = 1178. Compound EAD3 subjected to GC×GC-TOFMS analysis gave the same results as in the case of *I. nitidus* – under one GC-EAD peak were hidden both geometrical isomers of verbenol (**3**, **4**). Spectra and retention behaviour of compounds hidden in peak EAD3 were identical to that obtained for synthetic *cis*- (**3**), respectively *trans*-verbenol (**4**).

### 4.1.3 Frass extracts GC×GC-TOFMS analysis in both *Ips* species

GC×GC-TOFMS showed that frass extracts in both *Ips* species are more complex compared to guts ones, especially in the area of monoterpene hydrocarbons with $I_K$ ~ 900-1100, where analyses in both species gave practically identical results (Table 1). This finding is not surprising. While guts extracts contain putative pheromone together with the food, frass represents sawdust produced while beetles excavate their galleries (containing mainly host volatiles released from damaged bark tissue) plus beetles excreta enriched with pheromone. A typical section of GC×GC-TOFMS chromatogram in area $I_K$ ~ 900-1100 is showed in Figure 2.

| Name of Compound | Peak No. | Ret. Time [s] 1st/2nd dimension | $I_{K\text{-}EXP}$ | $I_{K\text{-}LIT}$ * | Identification ** |
|---|---|---|---|---|---|
| Tricyclene | **5** | 684 / 1.970 | 935 | 926 | L, A, O |
| α-Pinene | **6** | 700 / 2.190 | 647 | 939 | S, L, A |
| Camphene | **7** | 724 / 2.070 | 963 | 954 | S, L, A |
| Sabinene | **8** | 749 / 2.060 | 981 | 975 | L, A, O |
| β-Pinene | **9** | 763 / 2.070 | 989 | 979 | S, L, A |
| Δ3-Carene | **10** | 800 / 2.050 | 1018 | 1011 | S, L, A |
| α-Terpinene | **11** | 808 / 2.060 | 1024 | 1029 | L, A, O |
| *p*-Cymene | **12** | 820 / 2.170 | 1033 | 1024 | S, L, A |
| Limonene | **13** | 828 / 2.130 | 1039 | 1029 | S, L, A |
| γ-Terpinene | **14** | 864 / 2.130 | 1066 | 1059 | L, A, O |
| Sabinene hydrate | **15** | 884 / 2.230 | 1080 | 1070 | L, A |
| Terpinolene | **16** | 904 / 2.190 | 1095 | 1088 | L, A, O |
| Nonanal | **17** | 920 / 2.190 | 1108 | 1100 | S, L, A |
| Ipsenol | **1** | 924 / 2.170 | 1111 | 1100 | S, L, A |
| Pinene oxide | **18** | 928 / 2.330 | 1114 | 1099 | L, A |
| Thujol | **19** | 940 / 2.360 | 1123 | 1109 | L, A |

* Adams (2007);
** L – ChromaTOF™ software with NIST / Wiley MS spectra libraries installed on LECO Pegasus 4D data station, S – standard, A - Adams (2007), O – own MS spectra library measured on LECO Pegasus 4D

Table 1. Identified compounds ($I_K$ ~ 900-1100) typical for frass of *I. nitidus* and *I. shangrila*.

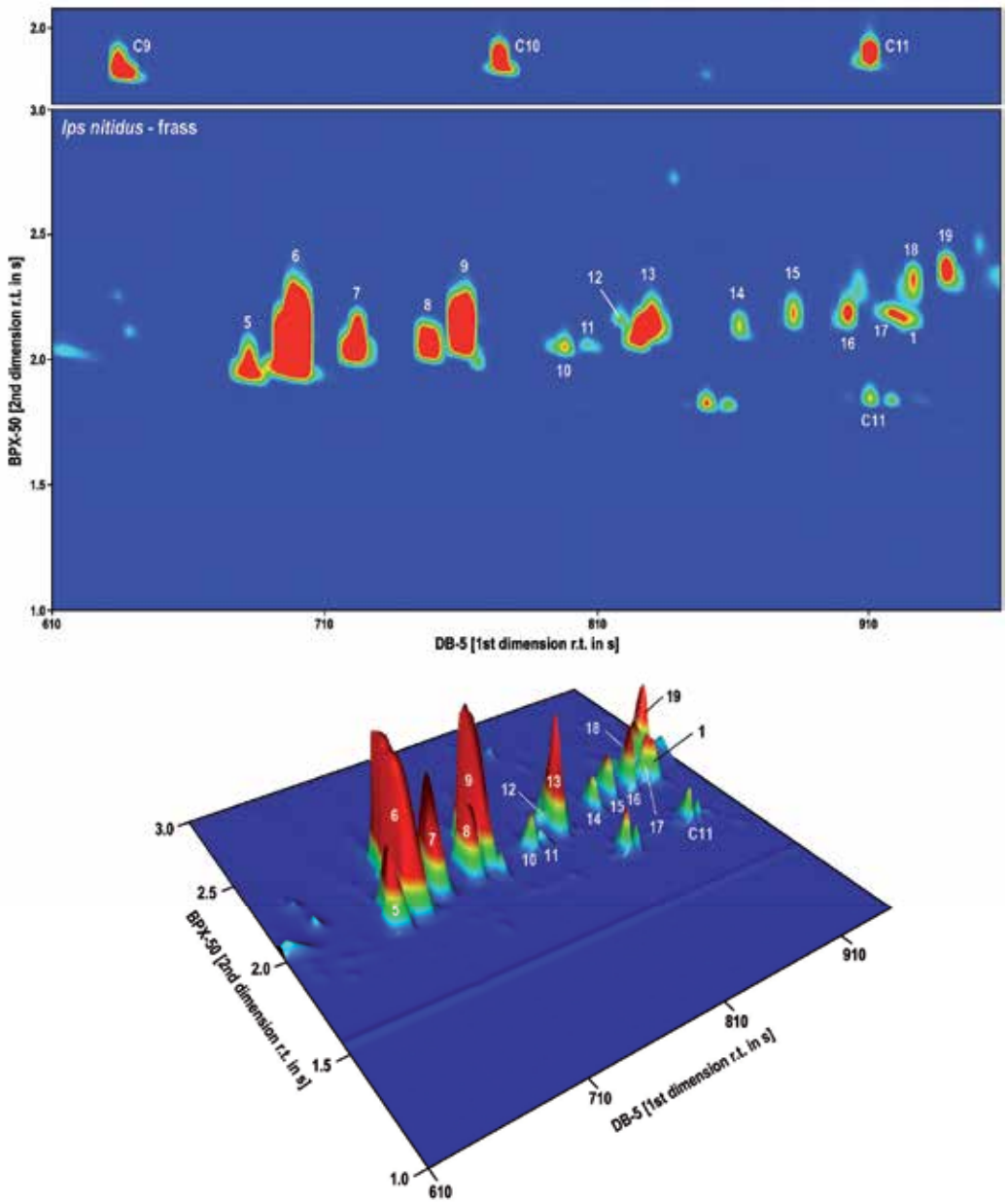



Fig. 2. Section of GC×GC-TOFMS TIC-chromatogram of *Ips nitidus* frass extract $I_K$ range ~ 900-1100 visualized as contour plot (with *n*-alkane standards in the upper part) and as 3D-view, respectively. Each spot in the upper 2D-chromatographic plane represents a single compound, the intensity of which is colour coded. White, yellow and red represent low, middle and high intensity, respectively. On the plane, the individual compounds are distributed based on their volatilities (X-axis) and polarities (Y-axis). The more polar and less volatile compounds elute at later retention times. The numbers indicate the identity of the compounds as listed in Table 1.

### 4.1.4 Species and sex specific differences

GC×GC-TOFMS analyses show that ipsenol (**1**), ipsdienol (**2**) and verbenols (**3**, **4**) were present in both *I. nitidus* and *I. shangrila*. GC-EAD tests aimed to test the cross reactivity of *I. nitidus* extracts on *I. shangrila* antennae (example in Fig. 3) and vice versa supported the assumption.

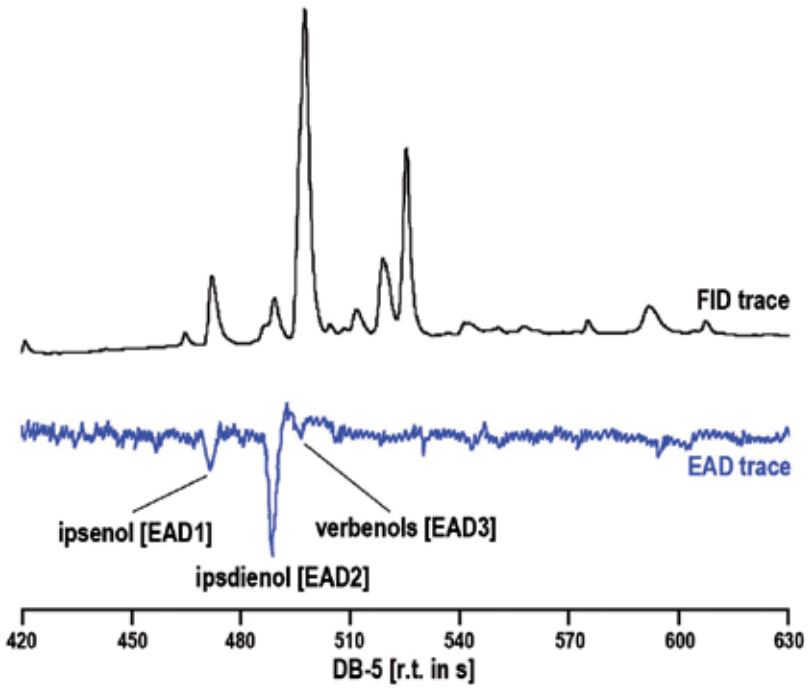



Fig. 3. Reconstructed GC-EAD recording from isolated antennae of *Ips shangrila* in response to *Ips nitidus* male guts extract. *I. shangrila* antennae responded to 3 compounds from the extract (EAD1-EAD3) corresponding to ipsenol (**1**), ipsdienol (**2**) and *cis*/*trans*-verbenol (**3**, **4**), respectively.

Thus, both GC×GC-TOFMS and GC-EAD analysis show that pheromones of the two species share common compounds. GC×GC-TOFMS and GC-EAD analysis also showed that identified EAD active compounds were present in guts extracts of both sexes. Specifically, ipsenol, ipsenol and *cis*/*trans*-verbenol were found both in *I. nitidus* male and female guts extracts. Available GC×GC-TOFMS analyses for *I. nitidus* guts extracts (N = 5) showed that male extracts contained more ipsenol and ipsdienol then female ones. On the other hand in *I. shangrila* guts extracts, ipsenol was equally present in traces in both sexes, while the content of ipsdienol was higher in males than in females. Due to limited number of analysis available, more data is needed to confirm the observed trends in pheromone sex-specific differences.

Following two figures (Fig. 4 and Fig. 5) are depicting complete achiral GC×GC-TOFMS separations in the area of EAD activity of both studied *Ips* species displayed as total ion chromatograms (TIC). The series of *n*-alkane standards (A), male frass (B), male guts extract

(C) and female guts extract (D) were analysed. The figures show differences in composition of frass and guts extracts of both sexes. EAD active compounds ipsenol (**1**), ipsdienol (**2**) and *cis*/*trans*-verbenols (**3**, **4**) are present in all analyzed samples of both *Ips* species.

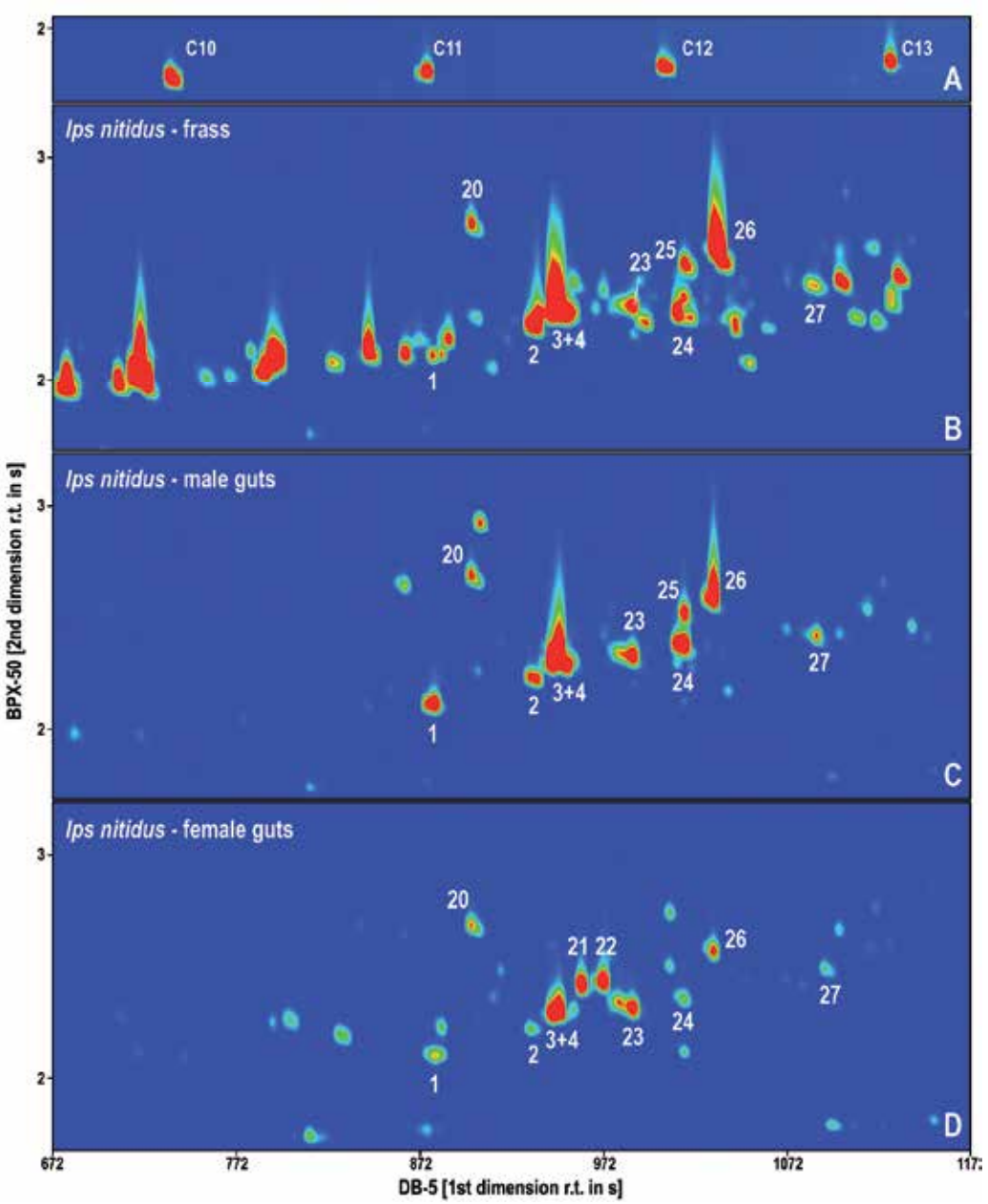



Fig. 4. Section of GC×GC-TOFMS TIC-chromatograms of *n*-alkane standards (A), *I. nitidus* male frass (B), *I. nitidus* male guts extract (C) and *I. nitidus* female guts extract (D) in $I_K$ range ~ 1000-1300.

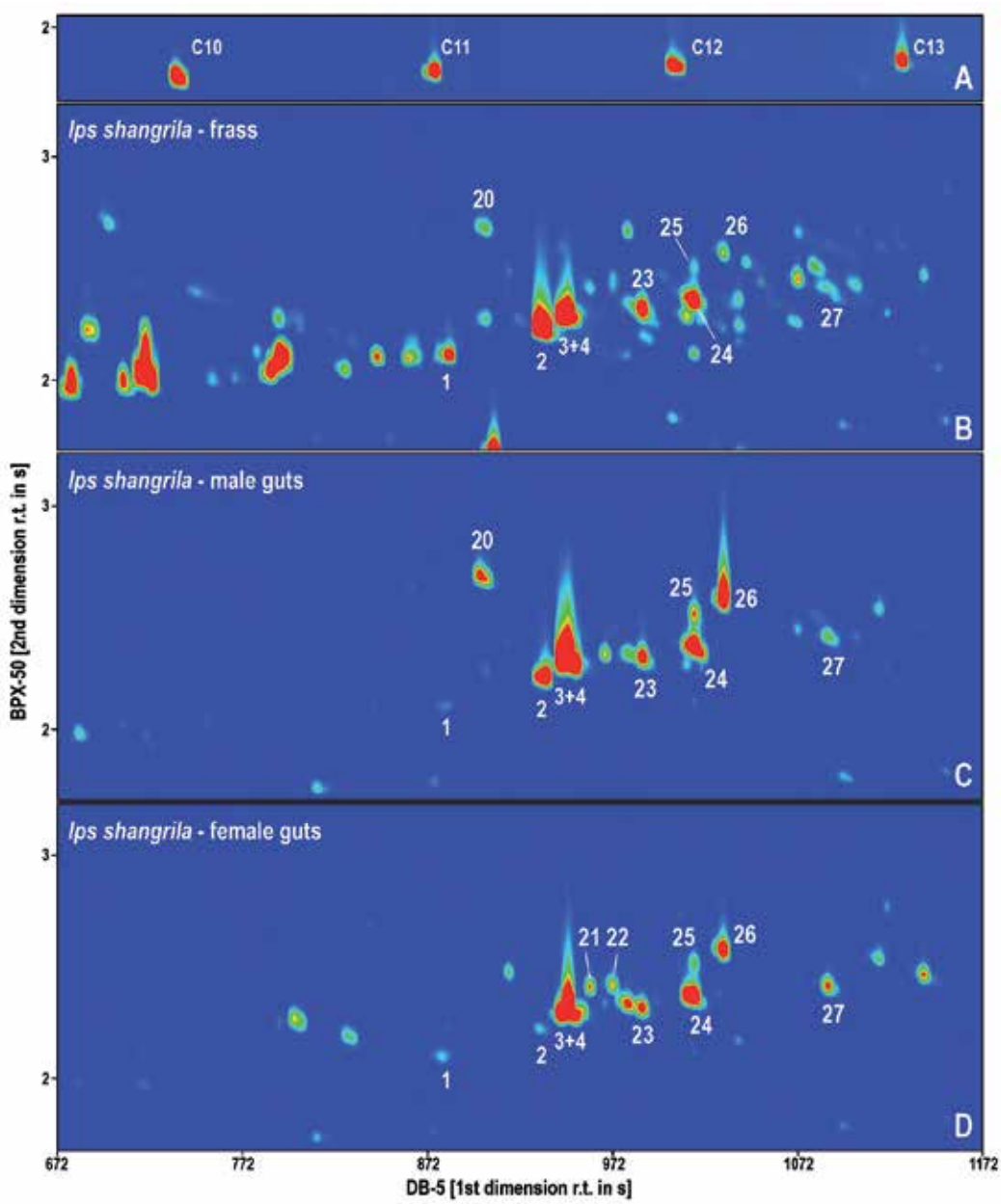



Fig. 5. Section of GC×GC-TOFMS TIC-chromatograms of *n*-alkane standards (A), *I. shangrila* male frass (B), *I. shangrila* male guts extract (C) and *I. shangrila* female guts extract (D) $I_K$ range ~ 1000-1300.

Except discussed possible pheromonal components **1 - 4** we identified in this $I_K$ range of all samples also several, mostly oxygenated terpenoic compounds, originated from host tree: phenylethanol (**20**), borneol (**23**), myrtenol (**24**), myrtenal (**25**), verbenon (**26**) and myrtanol (**27**).

In *I. nitidus* samples, chromatograms of examined extracts partially overlap, EAD active compounds ipsenol, ipsdienol and *cis*/*trans*-verbenols are present in all three sample groups with relatively lowest concentration in female guts extracts. However, two still unidentified female specific compounds (**21** and **22**; $M^+$ 154) from group of monooxygnated monoterpenes were found. In *I. shangrila* samples was the situation practically the same, except generally low (almost trace) amounts of ipsenol (**1**) in all three sample groups.

### 4.2 Chiral GC×GC-TOFMS analysis

Chiral GC×GC-TOFMS analysis focused to the antennally active compounds **1 - 4** in *I. nitidus* frass and guts extracts revealed the presence of (*S*)-ipsenol (**1**), racemic ipsdienol (**2**), (*S*)-*cis*-verbenol (**3**) and racemic *trans*-verbenol (**4**) in all analyzed samples (Figure 6). The same analysis of *I. shangrila* frass and guts extracts gave different results, especially in the case of ipsdienol (2), where we found high excess of its (*R*)-enantiomer (Figure 7B-D). (*S*)-Ipsdienol (**2**) was present only in low amounts in guts extracts and in *R*:*S* ratio circa 10:1 in frass (Figure 7B). Also the amount of components **1** and **2** was substantially lower in female guts extracts in comparison with frass and male gut extracts (Figure 7D). The obtained results of all semiquantitative chiral analyses of compounds **1 - 4** in both *Ips* species samples are given in Table 2.

| | **ipsenol [1]** | | **ipsdienol [2]** | | ***cis*-verbenol [3]** | | ***trans*-verbenol [4]** | |
|---|---|---|---|---|---|---|---|---|
| | (*R*)- | (*S*)- | (*R*)- | (*S*)- | (1*R*)- | (1*S*)- | (1*R*)- | (1*S*)- |
| ***Ips nitidus*** | | | | | | | | |
| frass | 0 | +++ | ++++ (racemic) | | + | +++++ | +++++ (racemic) | |
| male guts | traces | +++ | ++++ (racemic) | | traces | +++++ | +++++ (racemic) | |
| female guts | 0 | ++ | ++++ (racemic) | | 0 | +++++ | +++++ (racemic) | |
| ***Ips shangrila*** | | | | | | | | |
| frass | 0 | ++ | +++ * | + | + | ++++ | +++++ (racemic) | |
| male guts | 0 | traces | ++ | traces | + | ++++ | +++++ (racemic) | |
| female guts | 0 | + | + | traces | traces | ++++ | ++ | ++++ |

+: 1 – 3%, ++: 3 – 10%, +++: 10 – 15%, ++++: 15 – 30%, +++++: >30% (percentage counts from sum of **1**-**4** TIC peak area); **R*:*S* ca 10:1; SD ~ 5-24%; N = 5

Table 2. Results of chiral GC×GC-TOFMS analyses of compounds **1 – 4** in frass and male / female guts extracts of *Ips nitidus* and *Ips shangrila*.

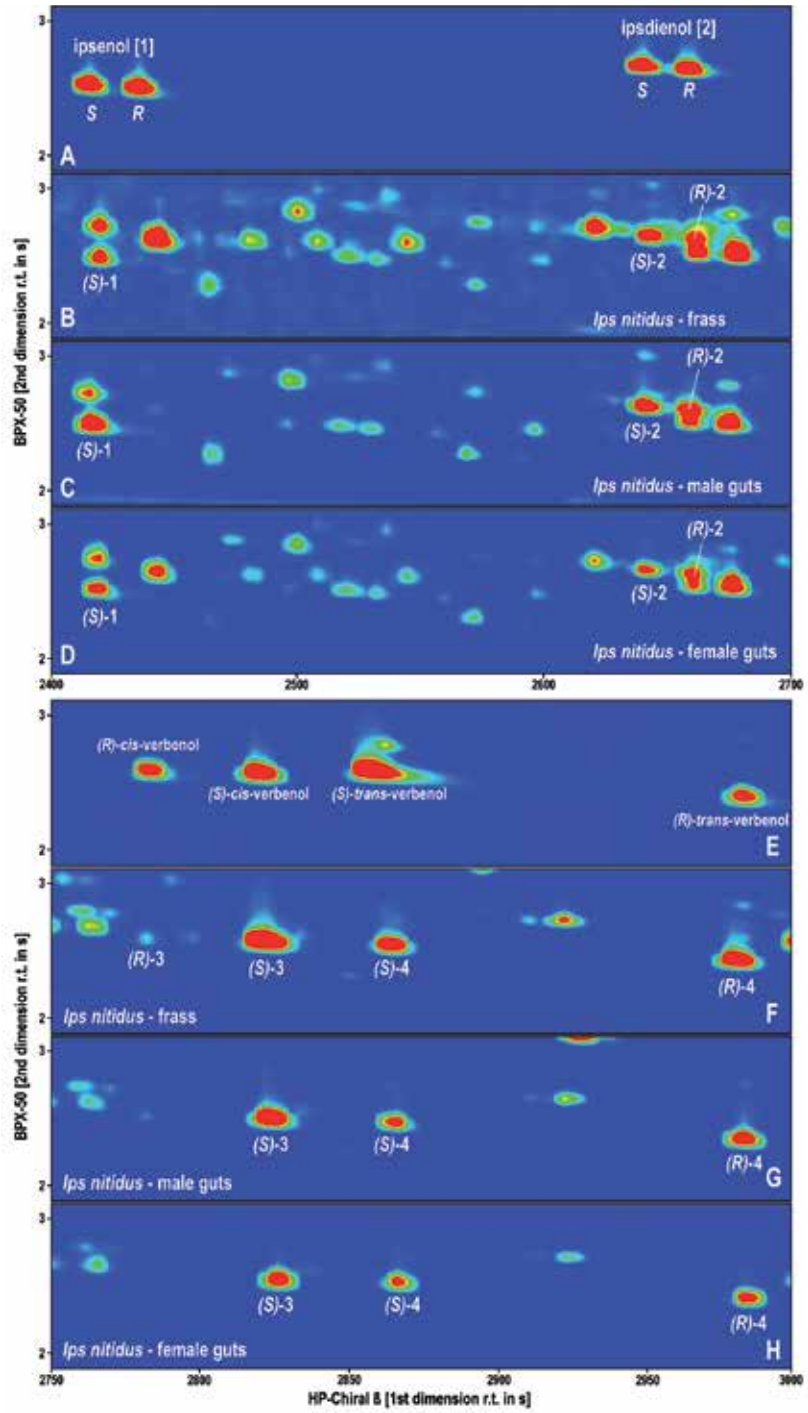



Fig. 6. Sections of GC×GC-TOFMS TIC-chromatograms (contour plots) depicting the chiral separation of compounds 1 - 4 in Ips nitidus samples (frass, males a females guts). Chromatograms with synthetic standards are assigned by letters A and E, respectively.

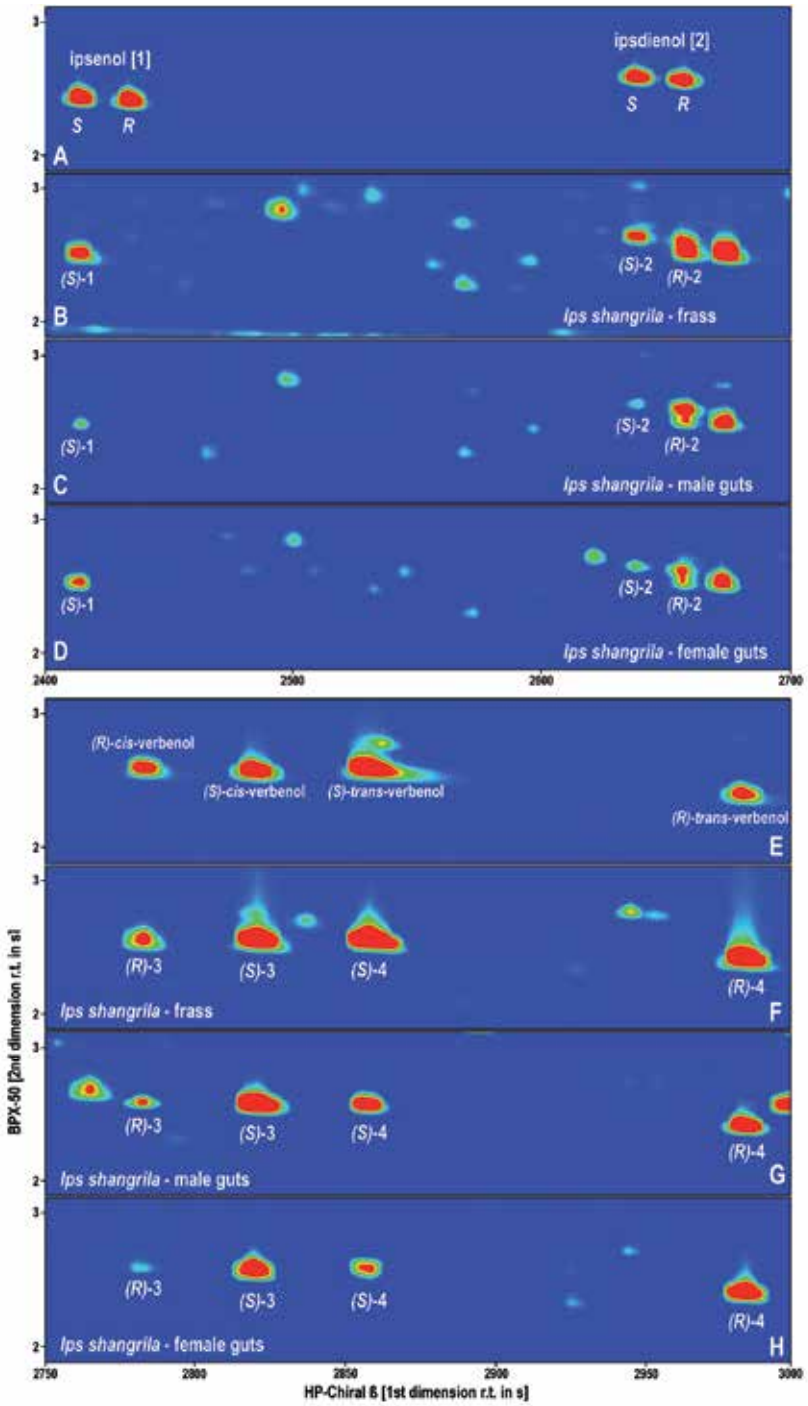



Fig. 7. Sections of GC×GC-TOFMS TIC-chromatograms (contour plots) depicting the chiral separation of compounds **1** - **4** in *Ips shangrila* samples (frass, males a females guts). Chromatograms with synthetic standards are assigned by letters A and E, respectively.

## 5. Conclusion

The aims and conclusions of the presented study can be divided into two parts. The first part, in a majority methodological, verified the usability of modern gas-chromatographic techniques, achiral and especially chiral GC×GC-TOFMS in combination with GC-EAD, in the area of insect semiochemical research. The second part, more chemico-ecological, was directly focused on finding the possible aggregation pheromone components of two important spruce pests in NW China – bark beetles *Ips nitidus* and *Ips shangrila*.

In last 15 years, multidimensional GC technique has been used for analysis of a broad spectrum of organic compounds in many applications and has shown to be superior to standard one dimensional GC. The three principal advantages of comprehensive GC×GC are: a) separation of coeluting compounds; b) improvement of detection sensitivity (Górecky et al., 2004) and c) the second dimension separation of the background chemical noise from the biologically active compound, already mentioned in chapter introduction (Kalinová et al., 2006). These three advantages have a real key importance in the area of insect semiochemicals research, since volatile physiologically active compounds are often in extremely low quantities occurring components of complex mixtures. The small amounts of semiochemicals produced by pheromone glands make the analysis of insect semiochemicals challenging. While using standard GC/MS, we quite often faced a situation at which a biologically active compound was present, however the MS spectrum remained hidden in the chromatographic background noise (Svatoš et al., 1999). Here, in similar cases, where we are limited by an amount of experimental material or the concentration of the target analytes is extremely low, the use of the GC×GC-TOFMS technique is extremely helpful in the semiochemical identification.

During analyzing the biological samples obtained from bark beetles *Ips nitidus* and *Ips shangrila,* we faced another problem - whether the quantity of electrophysiologically active target components was relative high and thus sufficient for standard 1D-GC experiments, the analytes, moreover chiral, were hidden in complex mixtures of often coeluting terpenes and other natural compounds. In this case, the valuable GC×GC's advantage of offering an elegant and robust solution of the coelution, baseline separation in the second dimension, was really critical. Coelution problems, hardly avoided in 1D-GC, here were solved by 2$^{nd}$ dimension separation in all analyses of bark beetle samples. Nice examples of resolved coelution can be demonstrated on baseline separation of (*S*)-ipsenol (*S*-**1**) and 2,3-epoxypinane, or on separation of (*R*)-ipsdienol (*R*-**2**) and *trans*-2-caren-4-ol, respectively – both in chiral analyses of *I. nitidus* samples (Figure 6B-D). These two cases in a standard 1D-GC analysis will cause great troubles not only in compound identification, but also in the their semiquantitative (or quantitative) analysis. We found numerous similar troublemaking coelutions in other achiral and chiral analyses of *Ips* samples (see Figures 4-7).

Both achiral and chiral separations of all studied samples can also serve as typical examples of another great feature of GC×GC-TOFMS analyses, the possibility of fast and useful pattern analysis of obtained data visualized in the form of contour plot 2D-chromatograms (see Figures 4-7). These coloured contour plots make searching for target components much easier, with possibility of another improvement when display based on characteristic ion current is selected instead of total ion current (TIC) (Kalinová et al., 2009). This feature is closely connected with time-of-flight MS detector key attribute, the fast recording (up to 500

spectra per second) of full mass spectra in relative broad range (1 – 1000 amu), which is not reachable by standard quadrupole detectors. The helpfulness of 2D-chromatograms pattern analysis itself is clearly visible in Figures 6 and 7, where you are able to interpret the enantiomeric composition of analyzed samples "in-one-sight", by their simple and fast visual comparison. The same interpretation process in case of standard 1D-chromatograms ("a forest of hundreds peaks...") is more time demanding and often complicated by coelutions.

If the great usability of achiral and chiral GC×GC-TOFMS technique in the semiochemical research was clearly verified, the problem of the aggregation pheromone composition in both *Ips* species still would not be completely resolved. Our GC-EAD and subsequent achiral GC×GC-TOFMS analyses of bark beetle samples revealed the same four EAD active compounds in both species – ipsenol (**1**), ipsdienol (**2**), *cis*-verbenol (**3**) and *trans*-verbenol (**4**). The presence of these possible pheromone components in studied samples is not surprising – all of them were reported as pheromone components of the taxonomically closest Eurasian species – *Ips typographus* and *Ips amitinus* (Bakke et al., 1977; Francke et al., 1980; Schlyter, 1987). The observed strong *Ips nitidus* antennal response to pinenes also is not unusual. The similar reaction was reported in sister species *Ips typographus* (Dickens, 1981; Erbilgin et al., 2007), although the real biological significance of this phenomenon (possible synergistic effect to the aggregation pheromone?) is still questionable and unclear. The observed differences in the amounts of target compounds **1**-**4** in both species and also in their sexes (we found only traces of ipsenol in *I. shangrila* guts extracts) needs further detail study. Surprisingly we did not found any trace of 2-methyl-3-buten-2-ol in SPME-GC×GC-TOFMS analyses of both species frass. This compound, important pheromone component in many bark beetles species (Schlyter, 1987), was reported in two recently published studies dealing with pheromones of both studied *Ips* species (Zhang et al., 2009a,b). Early eluting highly volatile 2-methyl-3-buten-2-ol was not detected during our GC×GC-TOFMS analyses of the extracts thanks to longer solvent delay time (400 s) protecting sensitive and expensive CCD-array of Pegasus IVD TOFMS detector. However, it is also interesting that 2-methyl-3-buten-2-ol did not elicit any electrophysiological responses on both species antennae. From our point of view the occurrence and real biological function of 2-methyl-3-buten-2-ol in both studied *Ips* species deserves further experiments, both analytical and behavioural.

The chiral GC×GC-TOFMS analyses of bark beetle samples showed clearly the advantage of the comprehensive two dimensional gas chromatography - all obtained data were easily interpreted and results showed the strong enantioselectivity in the pheromone component composition, especially in ipsenol (**1**) and *cis*-verbenol (**3**). We found practically only (*S*)-**1** and high excess (>1 : 10) of (1*S*)-**3** in both species, in contrast to *trans*-verbenol (**4**) which was in examined samples present mostly only in racemic form. Racemic ipsdienol (**2**) we found in *I. nitidus* and predominantly as (*R*)-isomer in *I. shangrila.*

The obtained results of achiral and chiral analyses of both species samples differ from data published in works of Chinese colleagues (Zhang et al., 2009a,b). Except above mentioned absence of 2-methyl-3-buten-2-ol in our SPME samples we found other notable differences in amount and enantiomeric composition of compounds **1** - **4**. In *I. nitidus* we found in all samples only racemic ipsdienol (**2**) in contrast to data published by Zhang et al. (2009a), reporting very high excess of (S)-**2** enantiomer. Also the concentration of racemic *trans*-verbenol (**4**) in our samples was substantially higher in comparison with Zhang's results

(small amounts of (1*S*)-**4** enantiomer only). In *I. shangrila* we found a small, but well measurable amount of (*S*)-ipsenol (*S*-**1**), especially in frass samples. Also the enantiomeric composition of ipsdienol (**2**) and occurrence of the racemic *trans*-verbenol (**4**) were different from published results (Zhang et al., 2009b). All these observed differences in obtained results need further experimental confirmation. In our opinion the field tests results published by Zhang et al. (2009a,b) clearly inform us about yet undiscovered pheromone components and/or their possible different ratios in both *Ips* species aggregation pheromone systems.

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The aim of this book is to describe the fundamental aspects and details of certain gas chromatography applications in Plant Science, Wine technology, Toxicology and the other specific disciplines that are currently being researched. The very best gas chromatography experts have been chosen as authors in each area. The individual chapter has been written to be self-contained so that readers may peruse particular topics but can pursue the other chapters in the each section to gain more insight about different gas chromatography applications in the same research field. This book will surely be useful to gas chromatography users who are desirous of perfecting themselves in one of the important branch of analytical chemistry.

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