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Pesticides in the Modern World

Trends in Pesticides Analysis

Edited by Margarita Stoytcheva

PESTICIDES IN THE MODERN WORLD – TRENDS IN PESTICIDES ANALYSIS

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Notice

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Meet the editor

Professor Margarita Stoytcheva graduated from the University of Chemical Technology and Metallurgy of Sofia, Bulgaria, with titles of Chemical Engineer and Master of Electrochemical Technologies. She has a Ph.D. and DSc. degrees in chemistry and technical sciences. She has acted in research and teaching in several Universities in Bulgaria, Algeria and France. From 2006. to the present she has participated in activities of scientific research, technological development and teaching in Mexico at the University of Baja California, Institute of Engineering, Mexicali, as a full time researcher. Since 2008. she has been a member of the National System of Researchers of Mexico. Her interests and areas of research are analytical chemistry and biotechnology.

Contents

Preface

Volume 1 of the unique book series "Pesticides in the Modern World" is a collection of 20 chapters addressing issues associated with pesticides detection, identification, characterisation, and determination, organized in three sections.

Chapters 1-3 included in the first book section provide on overview on the recognized principles for pesticides classification, and supply detailed data on pesticides physical properties (vapour pressure, solubility, soil adsorption coefficient, specific characteristics), and chemical interactions including oxidation, reduction, hydrolysis, and photolysis. The thermodynamic properties of imidacloprid in particular are investigated in order to solve the problem of its separation and purification. Environmental risks and safe pesticides management topics are discussed in Chapter 4. The electrochemical oxidation as a method of detoxification of obsolete pesticides stocks is suggested in Chapter 5.

Chapters 6 and 7 offer an exhaustive revision of the advanced chromatographic and alternative sensors and biosensors-based methods for organophosphorus pesticides determination. Chapter 8 comments on the improvement of the sensitivity and selectivity of some chromatographic techniques for pesticides analysis, and on the utility of the environmental proteomic approach in environmental pollutants assessment.

The second book section is devoted to the chromatographic pesticides quantification. Chapters 9-11 discuss various strategies for sample preparation, with emphasis on the processes of extraction. The basic principles of the modern extraction techniques, such as: accelerated solvent extraction, supercritical fluid extraction, microwave assisted extraction, solid phase extraction, solid phase microextraction, matrix solid phase dispersion extraction, cloud point extraction, and QuEChERS are comprehensively described and critically evaluated. The recent advances in the multidimensional chromatographic separation techniques are highlighted in Chapter 12. Chapters 13 and 14 cover topics on pesticides residues analysis in natural products, including sample preparation procedures. In Chapter 15 it is presented the deconvolution approach in the non-targeted pesticide analysis, allowing trace level pesticides determination. The application of the Hadamard transformation to gas chromatography/mass spectrometry for the sensitive detection of pesticides in rice is

commented in Chapter 16. The efficiency of gas chromatography/mass spectrometry is discussed in Chapter 17.

The third book section (Chapters 18-20) describes some alternative analytical approaches to the conventional methods of pesticides determination. These include voltammetric techniques making use of electrochemical sensors and biosensors, and solid-phase spectrometry combined with flow-injection analysis applying flow-based optosensors. Their remarkable analytical features are associated with the simple operation procedure without or with a minimum sample pretreatment, and the high sensitivity of the determinations.

Overall, the book offers a professional look on the recent achievements and emerging trends in pesticides analysis. It could act as an excellent reference for the specialists, involved in pesticides detection, identification, and determination.

All the contributing authors are gratefully acknowledged for their time and efforts in preparing the different chapters, and for their interest in the present project.

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Part 1

Pesticides: Overview

1

Identity, Physical and Chemical Properties of Pesticides

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1. Introduction

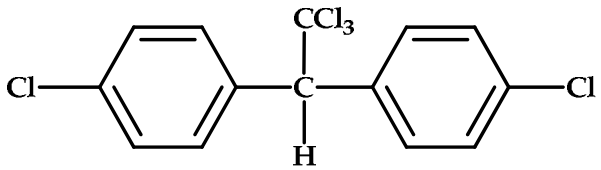
1.1 Definition of pesticide

According to FAO (1989) a pesticide is any substance or mixture of substances intended for preventing, destroying, or controlling any pest including vectors of human or animal diseases, unwanted species of plants or animals causing harm during, or otherwise interfering with, the production, processing, storage, or marketing of food, agricultural commodities, wood and wood products, or animal feedstuffs, or which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes chemicals used as growth regulators, defoliants, desiccants, fruit thinning agents, or agents for preventing the premature fall of fruits, and substances applied to crops either before or after harvest to prevent deterioration during storage or transport. The term, however excludes such chemicals used as fertilizers, plant and animal nutrients, food additives and animal drugs. The term pesticide is also defined by FAO in collaboration with UNEP (1990) as chemicals designed to combat the attacks of various pests and vectors on agricultural crops, domestic animals and human beings. The definitions above imply that, pesticides are toxic chemical agents (mainly organic compounds) that are deliberately released into the environment to combat crop pests and disease vectors.

1.2 Historical background of pesticides use in agriculture and public health

The historical background of pesticides use in agriculture is dated back to the beginning of agriculture itself and it became more pronounced with time due to increased pest population paralleled with decreasing soil fertility (Muir, 2002). However, the use of modern pesticides in agriculture and public health is dated back to the 19th century. The first generation of pesticides involved the use of highly toxic compounds, arsenic (calcium arsenate and lead arsenate) and a fumigant hydrogen cyanide in 1860's for the control of such pests like fungi, insects and bacteria. Other compounds included Bordeaux mixture (copper sulphate, lime and water) and sulphur. Their use was abandoned because of their toxicity and ineffectiveness. The second generation involved the use of synthetic organic compounds. The first important synthetic organic pesticide was dichlorodiphenyltrichloroethane (DDT) first synthesized by a German scientist Ziedler in 1873 (Othmer, 1996) and its insecticidal effect discovered by a Swiss chemist Paul Muller in

1939. In its early days DDT was hailed as a miracle because of its broad-spectrum activity, persistence, insolubility, inexpensive and ease to apply (Keneth, 1992).



***p, p`*-DDT**

P, p'-DDT in particular was so effective at killing pests and thus boosting crop yields and was so inexpensive to make its use quickly spread over the globe. DDT was also used for many non-agricultural applications as well. For example, it was used to delouse soldiers in the World War II and in the public health for the control of mosquitoes which are the vectors for malaria. Following the success of DDT, such other chemicals were synthesized to make this era what Rachel Carson (1962) in her book "The Silent Spring" described as the era of "rain of chemicals".

The intensive use of pesticides in agriculture is also well known to be coupled with the "green revolution". Green revolution was a worldwide agricultural movement that began in Mexico in 1944 with a primary goal of boosting grain yields in the world that was already in trouble with food supply to meet the demand of the then rapidly growing human population. The green revolution involved three major aspects of agricultural practices, among which the use of pesticides was an integral part. Following its success in Mexico, green revolution spread over the world. Pest control has always been important in agriculture, but green revolution in particular needed more pesticide inputs than did traditional agricultural systems because, most of the high yielding varieties were not widely resistant to pests and diseases and partly due to monoculture system (Vocke, 1986). Each year pests destroy about 30-48% of world's food production. For example, in 1987 it was reported that, one third of the potential world crop harvest was lost to pests. A further illustration to the pest problem in the world is shown in table 1.1 (Hellar, 2002).

Insect pests and rodents also account for a big loss in stored agricultural products. Internally feeding insects feed on grain endosperm and the germ the result of which is the loss in grain weight, reduction in nutritive value of the grain and deterioration in the end use quality of the grain. Externally feeding insects damage grain by physical mystification and by excrement contamination with empty eggs, larval moults and empty cacoons. A common means of pest control in stored agricultural products has always been the use of insecticides such as malathion, chlorpyrifos-methyl or deltamethrin impregnated on the surfaces of the storage containers (McFarlane, 1989).

On the other hand malaria remains the major vector-borne infectious disease in many parts of the tropics. It is estimated that over 300 to 500 million clinical cases occur each year, with cases in tropical Africa accounting for more than 90% of these figures (WHO, 1995). Other vector-borne diseases that present a serious problem especially in the tropics include trypanosomiasis, onchocerciasis and filariasis. It is therefore quite apparent that, the discovery of pesticides was not a luxury of a technical civilization but rather was a necessity for the well being of mankind.

Crop	Estimated % Losses			
	Insects	Diseases	Weeds	Total
Rice	26.7	8.9	10.8	46.4
Maize	12.4	9.4	13.0	34.8
Wheat	5.0	9.1	9.8	23.9
Millet	9.6	10.6	17.8	38.0
Potatoes	6.5	21.8	4.0	32.3
Cassava	7.7	16.6	9.2	33.5
Soybeans	4.5	11.1	13.5	29.1
Peanuts	17.1	11.3	11.8	40.4
Sugarcane	9.2	10.7	25.1	45.0

Table 1.1 Estimated % losses caused by pests in some world's major crops per year

1.3 Impacts of pesticides use in agriculture and public health

The use of pesticides in agriculture has led to a significant improvement in crop yield per hectare of land. Studies have established a possible correlation relationship between the quantity of pesticides used per hectare and the amount of crop yields per hectare (Hellar, 2002); table 1. 2. Pesticides like DDT and others proved their usefulness in agriculture and public health. Economies were boosted, crop yields were tremendously increased, and so were the decreases in fatalities from insect-borne diseases. Insecticides have saved the lives of countless millions of people from insect-borne diseases (Youdeowei, 1983).

1.4 Side effects of pesticides use to the environment and public health

Despite the good results of using pesticides in agriculture and public health described above, their use is usually accompanied with deleterious environmental and public health effects. Pesticides hold a unique position among environmental contaminants due to their high biological activity and toxicity (acute and chronic). Although some pesticides are described to be selective in their modes of action, their selectivity is only limited to test animals. Thus pesticides can be best described as biocides (capable of harming all forms of life other than the target pest). Further details on the side effects of pesticides are discussed inthe following chapter (ecological effects of pesticides).

Country/Area	Pesticide Use (kg/Ha)	Crop Yield (Ton/Ha)
Japan	10.8	5.5
Europe	1.9	3.4
USA	1.5	2.6
Latin America	0.2	2.0
Oceania	0.2	1.6
Africa	0.1	1.2

Table 1.2 Pesticides use and the corresponding crop yield in some countries/areas

2. Identity of pesticides

2.1 How can pesticides be identified?

Many of the pesticides that we use in our crops, gardens or domestic animals, are often a mixture of several chemicals mixed together in desired proportions suspended in appropriate carrier or diluent materials. These chemicals are called active ingredients that are responsible for killing or otherwise affecting the pests. Apart from the active ingredients, there are other chemicals that are formulated together with the active ingredients that usually do not kill pests. These are called inert ingredients that serve as carriers, diluents, binders, dispersants, prolong the shelf life of active ingredients or make the pesticide smell better. It is often the case that active ingredients on the container labels are named using common names. However, common names are not the only way to identify pesticides and in fact common names do not give complete information on the chemical nature of the pesticides. When chemists want to give a specific and unambiguous name to a chemical, they use what is called "systematic name". These names are usually long and complicated, but they are necessary for naming the millions of known chemicals. There are two main systems for deriving the systematic names of chemicals, one from the International Union of Pure and Applied Chemistry (IUPAC) and the other from the Chemical Abstracts Service (CAS). As an example of the two systematic naming described above, the following insecticide is names respective as;

IUPAC systematic name: (*E*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine

The same insecticide has the following CAS systematic name:

(2*E*)-1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine

In addition to a systematic name, CAS assigns a registry number to each chemical which is different from one chemical to another. For example the insecticide just described above has a CAS registry number of 138261-41-3

As pointed out earlier, systematic names are long and complicated for a mere user of pesticides (layman). For that matter, systematic names are more used by experts in the field of pesticides who pursue specific researches in which a proper identification of the chemical is needed. For many purposes, a relatively short and simple name would be helpful than a systematic name or registry number, and that is the role of common names.

2.2 How are the common names of pesticides derived?

What most people need when reading, writing or talking about a pesticides is a short, fairly simple and reasonably memorable name. Common names are approved by the International Organization for Standardization (ISO) based on given guidelines. For example the common name for the insecticide (E)-1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine is given as "imidacloprid" derived from parts of the systematic name. The process of registering common names usually starts with the pesticides manufacturers submitting proposals for names to ISO and the ISO committee checks that the proposed names comply with the rules, not misleading, and are not likely to be confused with the existing names of pesticides or drugs. Once common names are approved by ISO, they no longer belong to the company, but rather they can be used in other countries.

2.3 Classification of pesticides

The word "pesticide" is an umbrella term for all insecticides, herbicides, fungicides, rodenticides, wood preservatives, garden chemicals and household disinfectants that may

be used to kill some pests. Since pesticides varies in identity, physical and chemical properties, it`s therefore logical to have them classified and their properties studied under their respective groups. Synthetic pesticides are classified based on various ways depending on the needs. However, there are three most popular ways of classifying pesticides which are; classification based on the mode of action, classification based on the targeted pest species and classification based on the chemical composition of the pesticide (Drum, 1980).

2.3.1 Classification of pesticides based on the mode of action

Under this type of classification, pesticides are classified based on the way in which they act to bring about the desired effect. In this way pesticides are classified as contact (non-systemic) and systemic pesticides. The non-systemic pesticides are those that do not appreciably penetrate plant tissues and consequently not transported within the plant vascular system. The non systemic pesticides will only bring about the desired effect when they come in contact with the targeted pest, hence the name contact pesticides. Examples of contact pesticides are paraquat and diquat dibromide. On the other hand, the systemic pesticides are those which effectively penetrate the plant tissues and move through the plant vascular system in order to bring about the desired effect. Examples of systemic pesticides include 2, 4-D and glyphosate (Buchel, 1983). Under this classification, aslo are stomach poisons that bring about the desired effect after being eaten eg. Rodenticides. Fumigants are those pesticides that produce vapour which kills the pests.

2.3.2 Classification of pesticides based on the targeted pest species

In this type of classification, pesticides are named after the name of the corresponding pest in target as shown in table 2.1

Type of pesticide	**Target organism/pest**
Insecticides	Insects
Herbicides	Weeds
Rodenticides	Rodents
Fungicides	Fungi
Acaricides and Miticides	Arachnids of the order Acarina such as ticks and Mites
Molluscicides	Mollusks
Bactericides	Bacteria
Avicides	Bird pests
Virucides	Virus
Algicides	Algae

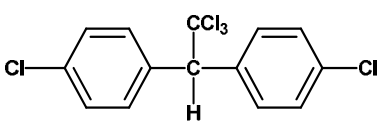
Table 2.1 Classification of pesticides based on the target organisms

2.3.3 Classification of pesticides based on the chemical composition

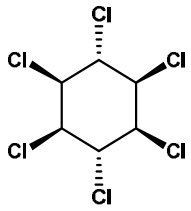
Under chemical classification, pesticides are categorized according to the chemical nature of the active ingredients. The chemical classification of pesticides is by far the most useful classification to reaserchers in the field of pesticides and environment and to those who search for details. This is because, it is from this kind of classification that gives the clue of the effficacy, physical and chemical properties of the respective pesticides, the knowledge of which is very important in the mode of application, precautions that need to be taken

during application and the application rates. Based on chemical classification, pesticides are classified into four main groups namely; organochlorines, organophosphorous, carbamates and pyrethrin and pyrethroids (Buchel, 1983).

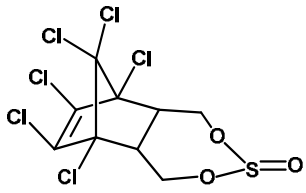
Organochlorines pesticides are organic compounds with five or more chlorine atoms. Organochlorines were the first synthetic organic pesticides to be used in agriculture and in public health. Most of them were widely used as insecticides for the control of a wide range of insects, and they have a long-term residual effect in the environment since they are resistant to most chemical and microbial degradations. Organochlorine insecticides act as nervous system disruptors leading to convulsions and paralysis of the insect and its eventual death. Some of the commonly used representative examples of organochlorine pesticides are DDT, lindane, endosulfan, aldrin, dieldrin and chlordane and their chemical structures are presented hereunder.



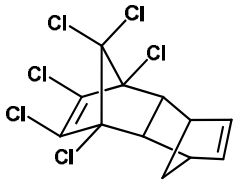
p,p`-DDT



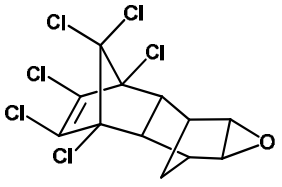
lindane



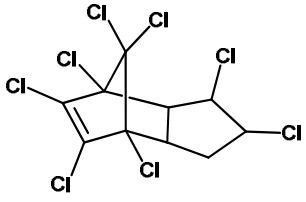
endosulfan



aldrin

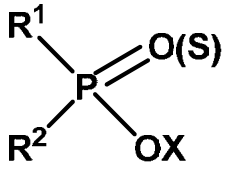


dieldrin



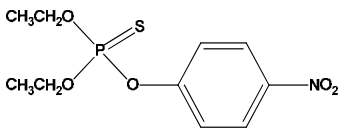
chlordane

Organophosphorous insecticides on the other hand contain a phosphate group as their basic structural framework as defined by Schrader's formula:

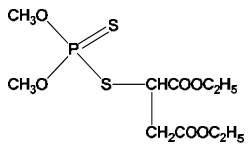


Where, R^1 and R^2 are usually methyl or ethyl groups, the O in the OX group can be replaced with S in some compounds, whereas the X group can take a wide diversity of forms. Organophosphorous insecticides are generally more toxic to vertebrates and invertebrates as cholinesterase inhibitors leading to a permanent overlay of acetylcholine neurotransmitter across a synapse. As a result, nervous impulses fail to move across the synapse causing a rapid twitching of voluntary muscles and hence paralysis and death. Unlike organochlorines, organophosphorous insecticides are easily decomposed in the

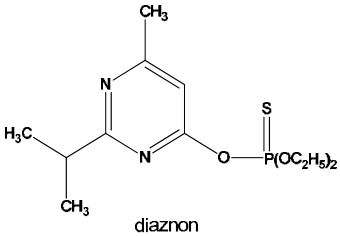
environment by various chemical and biological reactions, thus organophosphorous insecticides are not persistent in the environment (Martin, 1968). Some of the widely used organophosphorous insecticides include parathion, malathion, diaznon and glyphosate.



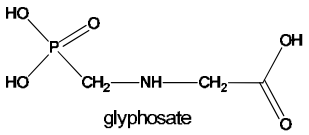
parathion



malathion

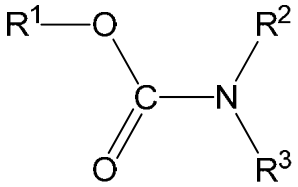


diaznon

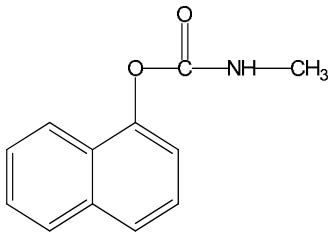


glyphosate

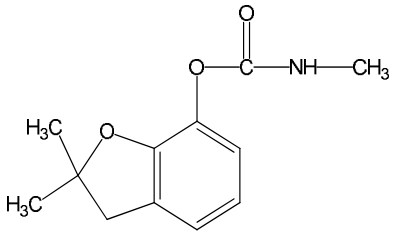
Carbamates are organic pesticides derived from carbamic acid with the general formula



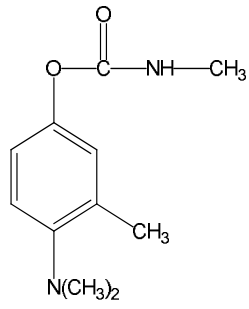
Where, R^1 is an alcohol group, R^2 is a methyl group and R^3 is usually hydrogen. Both oxime and aryl carbamates have fairly high insect and mammalian toxicities as cholinesterase inhibitors. The cholinesterase inhibitions of carbamates differ from that of organophosphorous in that, it is species specific and it is reversible (Drum, 1980). Some of the widely used insecticides under this group include carbaryl, carbofuran and aminocarb.



carbaryl

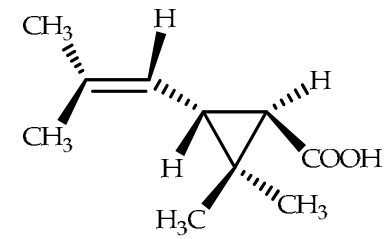


carbofuran

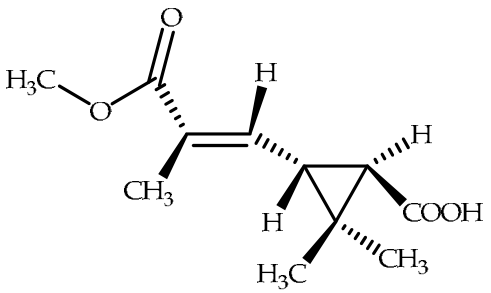


aminocarb

Pyrethroids are synthetic analogues of the naturally occurring pyrethrins; a product of flowers from pyrethrum plant (*Chrysanthemum cinerariaefolium*). The insecticidal components of pyrethrum flowers are the optically active esters derived from (+)-*trans*-chrysanthemic acid and (+)-*trans*-pyrethroic acid.

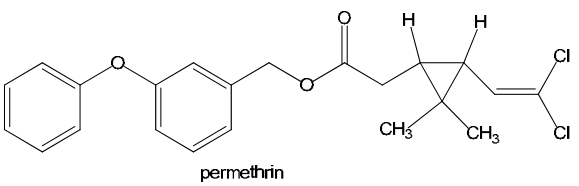


(+)-*trans*-chrysanthemic acid

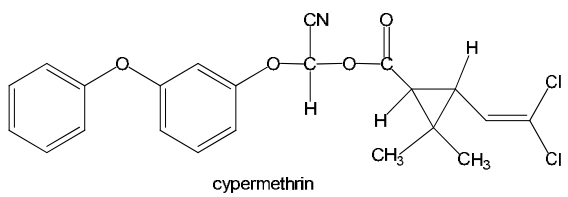


(+)-*trans*-pyrethroic acid

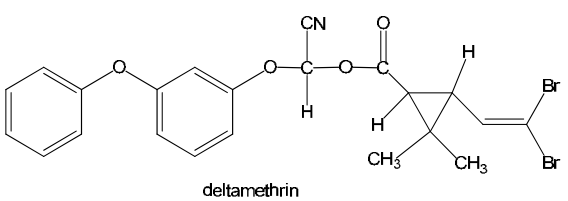
Pyrethroids are acknowledged of their fast nocking down effect against insect pests, low mammalian toxicity and facile biodegradation. Although the naturally occurring pyrethrins are effective insecticides, their photochemical degradation is so rapid that their uses as agricultural insecticides become impractical. The synthetic analogues of the naturally occurring pyrethrins (pyrethroids) were developed by the modification of pyrethrin structure by introducing a biphenoxy moiety and substituting some hydrogens with halogens in order to confer stability at the same time retaining the basic properties of pyrethrins. The most widely used synthetic pyrethroids include permethrin, cypermethrin and deltamethrin.



permethrin

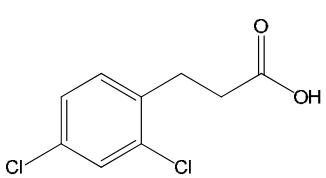


cypermethrin

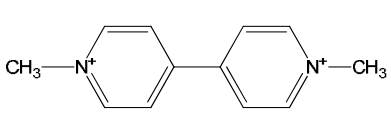


deltamethrin

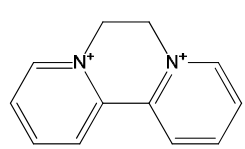
Other miscelenious groups of pesticides that are worth mentioning particularly in this book include among others phenoxyacetic acid under which the herbicide 2,4-D belongs and bipyridyls under which the herbicides paraquat and diquat belong.



2,4-D

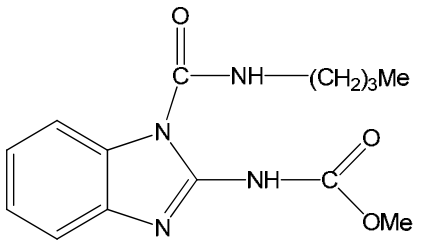


paraguat

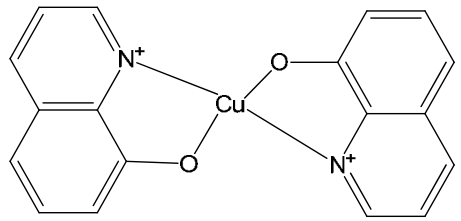


diquat

Fungicides are pesticides that are used for the control of fungal infections in crops. There are inorganic and organic fungicides. Inorganic fungicides include Bordeaux mixture, $Cu(OH)_2.CaSO_4$ and malachite, $Cu(HO)_2.CuCO_3$. Organic fungicides on the other hand include among others, benomyl and oxine copper (Manahan, 2001).



benomyl



oxime copper

2.3.4 Other minor classes of pesticides

2.3.4.1 Activity spectrum of the pesticide

In this system of classification, pesticides are classified into two groups as broad spectrum pesticides and selective pesticides. Broad spectrum pesticides are those pesticides that are designed to kill a wide range of pests and other non target organisms. They are non-selective and are often lethal to reptiles, fish, pets and birds. Some examples of broad spectrum pesticides are chlorpyrifos and chlordane. Selective pesticides on the other hand are those pesticides which kill only a specific or group of pests leaving other organisms with a little or no effect at all. A good example in this case is a herbicide 2,4-D which affects broad-leaved plants leaving the grassy crops unaffected.

2.3.4.2 Mode of formulation

Emulifiable concentrates (EC) are fine suspensions of oil droplets in water and appears milky in colour. They do not require constant agitation prior to each application.
Wettable Powders (WP) are suspensions of fine particles suspended in water. These suspension require constant agitation prior to each application.
Granules (G). Granules are obtained by mixing the active ingredient with clay for outdoor applications.
Baits. These are obtained by mixing the active ingredient with food base especially used for the control of rodents.
Dusts (D). Dusts cannot be mixed with water and they must be applied dry. The common carriers for dusts are clay, talc, silica gel or diatomacious earth.
Fumigants. These are gaseous insecticides usually packaged under pressure and stored as liquids. Some are tablets or pellets that release gas when mixed with water.

2.3.4.3 Toxicity level

The World Health Organization (WHO) has developed a classification system that group pesticides according to the potential risks to human health caused by accidental contact to human being and they are grouped into the following classes;
Class Ia = extremely hazardous
Class Ib = highly hazardous
Class II = moderately hazardous

Class III = slightly hazrdous
Class IV = products unlikely to present acute hazard in normal use

3. Physical properties of pesticides

The biological activity of a pesticide to the target pest species is greately influenced by its physical and chemical properties. The physical properties of a pesticide in particular determine the pesticide mode of action, dosage, mode of application and the subsequent environmental chemodynamics. The physical properties of pesticides varies greately according to their chemical nature and formulation. For simplicity, here are discussed some general physical properties of pesticides without going to specifics and then in table 3.1 are discussed the specific physical properties of the named representative pesticides.

3.1 General physical properties of pesticides

3.1.1 Molecular weight and form

In some references such as pesticide manual, the molecular weight (MW) and the physical form (appearance and odour) of the active ingreadient (AI) is usually given. Molecular weight of a substance is a summation of individual atomic weights of all the atoms making up the molecule in question. The molecular weight of a pesticide is an inherent property that distinguish one pesticide from the other except for stereoisomeric pesticides which share similar molecular weights differing only on the group spatial orientations at given chiral centres. The common gas-phase pesticides for example have a molecular weight of about 103 or less. However, it become very difficult to predict the state and form of complex molecules with molecular weight that are substantially greater than 500.

3.1.2 Vapour pressure (VP)

The vapour pressure of a substance is the measure of how easy it can volatilise and turn into vapour (gas state). For pesticides, the easy with which a pesticide can volatilise may be considered advantagious with respect to a particular mode of action on one hand but it can be of negative influence on the other hand. For example, a pesticide with a fumigant mode of action can have a useful penetrative power and thus it is advantageous to have higher vapour pressure. However, a high vapour pressure can cause vapour drift and environmental pollution. Pesticides with high vapour pressure need to be handled in such a way so that the vapours do not escape into the atmosphere. A pesticide with low vapour pressure does not move into air, so there is a potential to accumulate in water if it is water soluble. If it is not water soluble, the pesticide may accumulate in soil or biota. The usually preffered SI-unit for vapour pressure is millipascal (Mpa = $g.m^{-1}.^{-2}$ or 0.001 $N.M^{-2}$).

3.1.3 Solubility

Solubility is a measure of how easily can a given substance dissolve in a given solvent. Unless stated otherwise, the unit for solubility in water are given in ppm (parts per-million) which is the same as milligrams per litre (mg/L). When the solubility is too low, the units are given in ppb (parts per-billion) which is the same as micrograms per liter (µg/L). Measurements of solubility are influenced by temperature, pH, polarity of the substance, hydrogen bonding, molecular size and the method used. The following is an expresion for ppm (Linde, 1994);

$$\text{1 part per million = 1 ppm} = \frac{1}{1\text{ million}} = \frac{1\text{ mg}}{1\text{x}10^6\text{ mg}} = \frac{1\text{ mg}}{1\text{ kg}} = \frac{1\text{ mg}}{1\text{ L}}$$

The significance in environment fate of solubility of pesticides is that, a pesticide which is very soluble in water will tend not to accumulate in soil or biota because of its strong polar nature. This suggests that it will degrade via hydrolysis which is a favored reaction in water.

3.1.4 Octanol/Water partition coefficien- K_{ow} (Log K_{ow})

Partition coefficient is a measured ratio (at equilibrium) of the dissolved mass of the substance between equal layers of *n*-octanol and water.

$$K_{ow} = \frac{\text{Concentration in } n\text{-Octanol Phase}}{\text{Concentration in Water Phase}}$$

K_{ow} is a unitless parameter which provides a useful predictor of the other physical properties for most pesticides and other organic substances with molecular weight less than 500. Values of K_{ow} for organic chemicals can be quite large, and therefore for convinience it is often expressed as Log K_{ow} (which is log to the base 10 of K_{ow}) and the values range from -3 to 7. K_{ow} is considered to be a good indicator of bioaccumulation of pesticides in organisms and food chains. Pesticides with a positive correlation to Log K_{ow} are more likely to have bioaccumulation effects to organisms and food chains. The paramter is also a good indicator of systemic mode of action of a pesticide. Pesticides with low K_{ow} values (generally ≤2) indicate the likely systemic translocation of such pesticides or their metabolites in the plants transvascular system. K_{ow} values are generally influenced by the polarity of the pesticide and the general physical factors. Polar pesticides tend to be more soluble in water and hence low values of K_{ow}. For the general physical factors, K_{ow} will increase when the following physical properties increase; molecular surface area, molar volume, molecular weight, and density (Mallhot & Peters, 1988).

3.1.5 Soil adsorption coefficient K_{oc}/K_d

Adsorption of pesticides on soils and sediments is a major factor that determines the destination of pesticides in the environment and their eventual degradtion processes. Most pesticides are non polar and hydrophobic meaning that they are not very soluble in water. The non polar pesticides tend to be pushed out of water onto soils and sediments which contain non polar organic matter. K_d is called the sorption coefficient and it measures the amount of pesticides adsorbed onto soil per amount of water without considering the organic matter content of the soil. The values for K_d varies greately because the organic matter content of the soil is not considered in the equation. The preffered parameter to determine the soil`s ability to adsorb pestcides is K_{oc} since it considers the organic matter content of the soil. K_{oc} is the ratio (at equilibrium) of the mass of a substance, adsorbed onto a unit mass of soil, relative to the mass of the substance remaining in water solution. K_{oc} is also a unitless parameter and its value is dependent on the organic matter content of the soil, polarity of the chemical and soil pH.

$$K_d = \frac{\text{Concentration of a chemical in soil}}{\text{Concentration of a chemical in water}}$$

$$K_{oc} = \frac{K_d \times 100}{\% \text{ organic carbon}}$$

3.1.6 Henry`s law constant-H`

Henry`s Law Constant (HLC) is a measure of the concentration of a chemical in air over its concentration in water. It expresses the tendency of a material to volatilise from aqueous solution to air. It is sometimes measured, but more usually calculated as the ratio of vapour pressure (in pascals) x molecular weight / solubility (mg/L).

$$H` = \frac{16.04 * P * M}{T * S}$$

Where P = Vapour pressure,
M = Molecular mass
T = Temperature
S = Solubility

The environmental significance of Henry`s law constant is that, a pesticide with a high HLC value will volatilize from water into air and distribute over a large area. Conversely, a pesticide with a low HLC value tend to persist in water and may be adsorbed into soil and sediment. The HLC value is also an integral part in calculating the volatility of a chemical.

3.2 Specific physical properties of selected representative pesticides

Pesticide Name	Synonym/trade name	Type	Physical properties	Health effects	Handling procedures	Route of entry
Chlordane $C_{10}H_6Cl_8$	Toxichlor, Niran, Octachlor, Synklor, Corodane	Organochlorine insecticide	Viscous amber to colourless liquid with a mild odour	Suspected carcinogen, affect central nervous system, gastrointestinal tract and liver.	Goggles,chemical/solvent resistant gloves, apron	Inhalation, ingestion,skin, eye
Chlorpyrifos $C_9H_{11}Cl_3NO_3$	Dowco179, Dursban, Lorsban, Pyrinex,Killmaster	insectecide	White or colourless granular crystals, gas like odour	May affect the central nervous system and liver	Gloves, dust proof goggles	Inalation, ingestion, skin
DDT $C_{14}H_9Cl_5$	Dicophane, Agritan, Gesapon, Gesapex, Citox, Detox, Anofex	Organochlorine insecticide	Colourless solid or white to slightly off-white powder with faint odour	Probable carcinogen, reproductive, liver, and kidney problems, eye, nose, skin, throat irritant	Respirator, gloves, goggles and face shield	Inhalation, ingestion and skin

Diaznon $C_{12}H_{21}N_2O_3PS$	Basudin, Dazzel, Gardentox, Royazol, Out, Nucidol	Organophosphate insecticide	Oliy colourless liquid	Eye and skin irritant, may cause gastrointestinal symptoms	Glove, long pants, sleeves, face shiled, goggles	Inhalation, ingestion,skin
Dichlorvos $C_4H_7Cl_2O_4P$	Unitox, Lindan, DDVP, Vapona, Nuvan, Cypona	Organophosphate insecticide	Clear, slightly yellow liquid with a mild odour,combustible	Suspected carcinogen, can affect the central nervous system	Nitrile gloves, Tyvek clothes, respirat, safety glasses	Inhalation, ingestion,skin
Ethion $C_9H_{22}O_4P_2S_4$	Ethanox, Hylmox, Nialate, Rhodocide	Organophosphate insecticide	Colourless or light brown to pale yellow liquid or dust	Affect central nervous system and gastrointestinal system, chest, nose	Dust masks, gloves and safety glasses	Inhalation, ingestion, skin
Lindane $C_6H_6Cl_6$	Aficide, Agrocide, Benzene hexachloride, Bexol, Celanex	Organochlorine insecticide	White or colourless crystalline solid with slight musty odour	Suspected carcinogen, affects central nervous system, respiratory, reproductive systems	Goggle, gloves and respitator	Inhalation, ingestion and skin
Malation $C_{10}H_{19}O_6PS_2$	Chemathion, Malacide, Detmol, o,o-dimethyl thiophosphate	Organophosphate insecticide	Clear brown to colourless liquid with mild skunk-like odour	Skin, eye, nose irritant, affects respiratory and central nervous system	Nitrile gloves, Tyvek clothing,respirator, splash-proof goggles	Inhalation, ingestion, skin
Pentachlorophenol C_6Cl_5OH	PCP, Dowside 7, Permacide, Permagard, Pentakil,	Organochlorine fungicide	Colourless to white crystalline solid with benzene-like odour	Possible carcinogen, eye, skin, nose, throat irritant, liver and kidney damage	Glove, safety glasses	Inhalation, ingestion, skin, eye
Permethrin $C_{21}H_{20}Cl_2O_3$	Ambush, Ectban, Pounce, Nix Dragnet, Spartan	Pyrethroid insectcide	Odourless colourless crystalline solid or pale brown viscous liquid	Eye, skin, respiratory irritant, affect central nervous system	Gloves, face shiled	Inhalation, ingestion,skin
Rozol (Chlorophacinone) $C_{23}H_{15}ClO_3$	Amvac, Romix special, Mouce seed®	rodenticide	Bluish green solid, odourless	Skin, eye irritant, may affect liver	gloves	ingestion
Thymol $C_{10}H_{14}O$	6-isopropyl-m-cresol	fumigant	White crystal, aromatic odour, combustible	Skin and eye irritant	Mask, respirator, rubble gloves, safety glasses	Inhalation, ingestion,skin

Table 3.1. Specific physical properties of selected representative pesticides

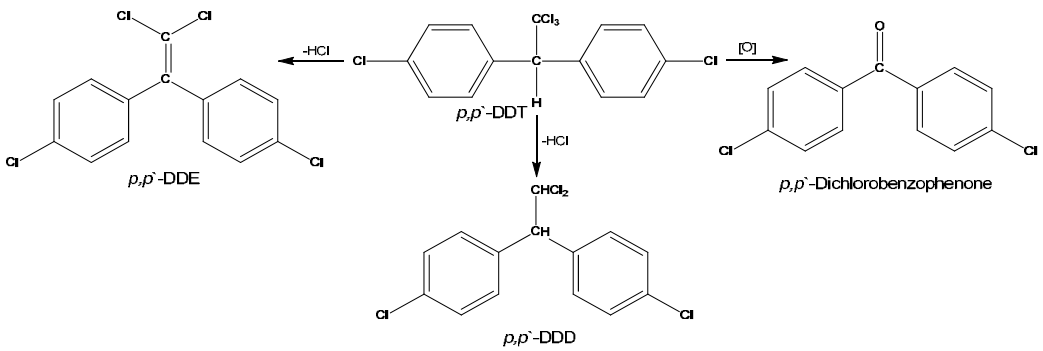
4. Chemical properties of pesticides

Following the release of pesticides in the environment, they undergo a complex series of interdependent processes that are collectively called chemodynamics of pesticides. The chemodynamic processes that a pesticide undergoes is essentially determined by its inherent physico-chemical properties and partly by environmental parameters such as pH, temperature, moisture, precipitation, salinity, light intensity and topography. The major chemodynamic processes that determine the pesticides persistence, distribution and their ultimate fate in the environment include transportation, retention, degradation and biota uptake. Among all these chemodynamic processes, degradation is of much relevance with regard to this section as it entails the chemical transformations of pesticides in the environment, hence chemical properties of pesticides.

Degradation of pesticides is the breakdown or chemical transformation of pesticide molecules into other forms that are not necessarily simpler and less toxic compared to the parent molecule. In some cases the degradation products are also toxic and have some pesticidal effects as well. A good example of this is the degradation of DDT to DDD, which is itself a pesticide. The rate of degradation of pesticides is usually measured in terms of half-life ($t_{1/2}$), which is the time required for the depletion of half (or 50%) of the amount of pesticide present initially. The degradation processes that bring about pesticides transformation can be categorized into two major groups; chemical degradation and biological degradation. Chemical degradation generally occur in water or atmosphere and it follows one of four reactions namely; oxidation, reduction, hydrolysis and photolysis. Biological degradation generally occurs in soil and in living organisms and it utilizes one of four reaction; oxidation, reduction, hydrolysis and conjugation. The type of the reaction in which a pesticide undergoes is largely determined by the pesticide inherent phyco-chemical properties and the environmental compartment (water, soil, air, biota) in which it is hosted.

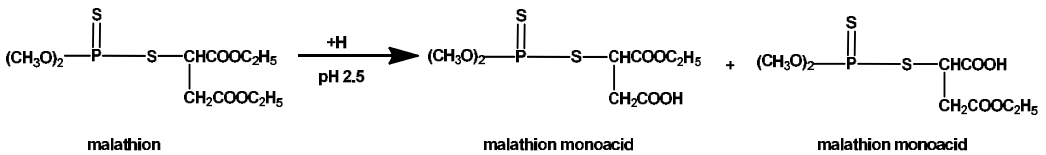
4.1 Oxidation reaction of pesticides

Oxidation of pesticides is a reaction process whereby the dissolved oxygen in the environment reacts with pesticides. This oxidation process can also be achieved by Singlet oxygen, ozone, hydrogen, peroxide, or other hydroxy radicals. Hydroxy radical (.OH) are the primary agents that bring about chemical oxidation of pesticides in water or atmosphere. The radical can be formed from either the pesticides or from other molecules in the environment. *P,p`*-DDT for example undergoes both reduction as well as oxidation reactions in the soil under the aid of *Enterobacter aerogenes* microorganisms in the presence of UV light and/or iron catalyst to form reduced products; *p,p`*-DDE and *p,p`*-DDD as well as oxidized derivative which ultmately form *p,p`*-dichlorobenzophenone.



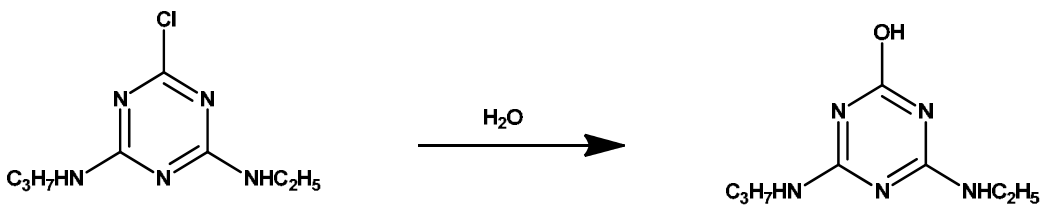
4.2 Reduction reaction of pesticides

Reduction of pesticides is a chemical reaction in which the substrate (pesticide) undergoes a reduction in oxidation state. The reducing agents in the environment are usually +H. For example, malathion undergoes a reduction reaction in acidic aquatic environment which proceed by the substitution of one of the ethyl group with +H resulting into the formation of two functional isomeric molecules of malathion monoacid at the end of one half life. However, malathion diacid would be the product at extended reaction time (Wolfe *et al*, 1977).



4.3 Hydrolysis reaction of pesticides

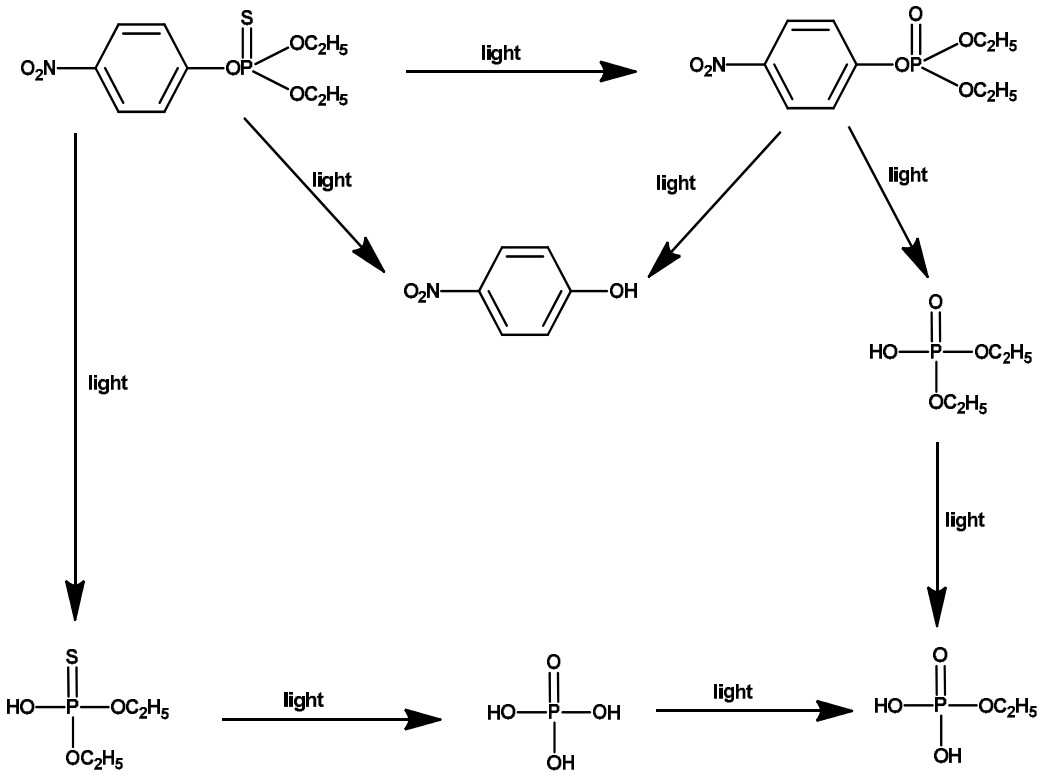
Hydrolysis is a pH dependent reaction in which pesticides react with water (i.e. Hydrogen ion and hydroxy ion). Hydrolysis is one of the most common reactions that most pesticides undergo in the environment. Most organophosphates and carbamates have particularly shown to be highly responsive to hydrolysis reaction under alkaline condition. A pesticide that is very soluble in water will tend not to accumulate in soil or biota because of its stronger polar nature. This suggest that it will degrade via hydrolysis which is the reaction that is favoured in water. The following example shows the hydrolysis of atrazine in water.



4.4 Photodegradation of pesticides

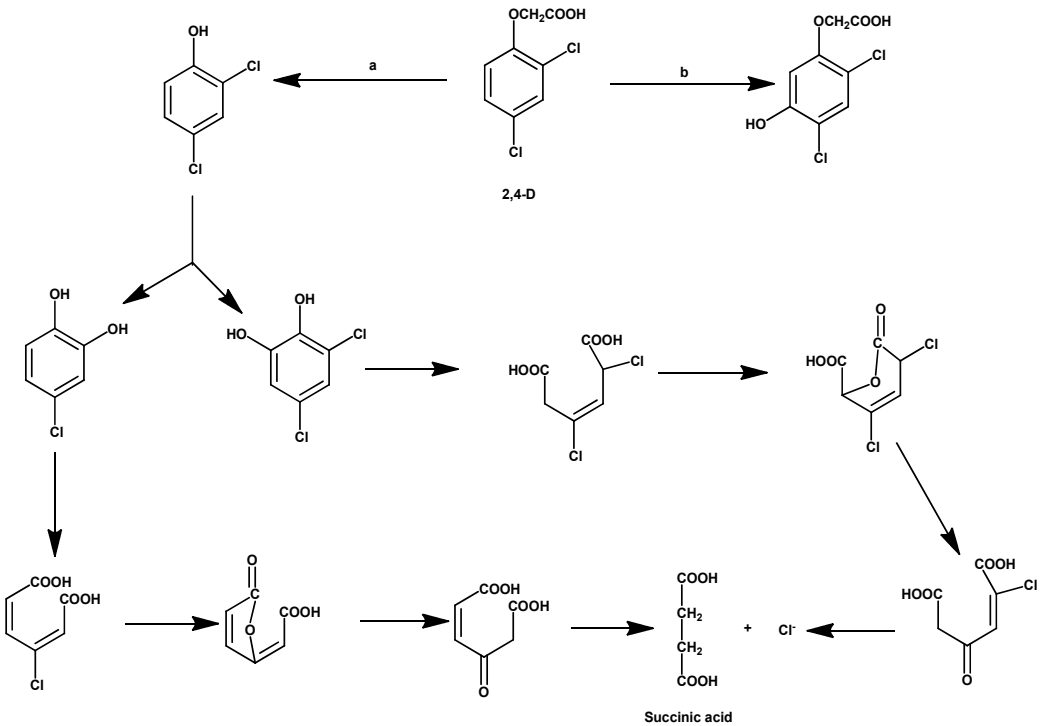
Photodegradation or photolysis is the breakdown or transformation of pesticides by sunlight that causes a rupture of chemical bonds. The organic molecule absorbs photons and become excited with the ensuing release of electron thus changing the molecule. Photolysis reactions are important for degrading organic molecules in the upper atmosphere, in shallow aquatic environment, on foliage and on the surface of soils. Pyrethroids are particularly susceptible to photolysis reactions. The total decomposition of a pesticide in the

air can take several steps which is illustrated by the following photo-decomposition of parathion (Linde 1994).

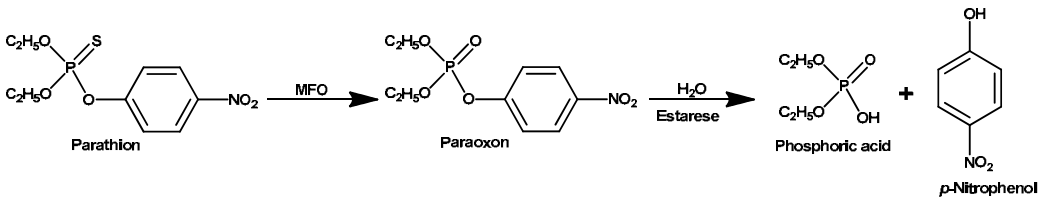


4.5 Biodegradation

Biodegradation is the breakdown or transformation of pesticides by microbial agents which normally occurs in water and soil. The rate of microbial degradation depends highly on the amount and nature of pesticides present in the soil, the microbial population in the soil and soil conditions that favours microbial activities, such as warm temperature, favourable pH, adequate soil moisture, aeration and high organic matter content. The microorganisms participating in biodegradation include fungi, bacteria and other microorganisms that use pesticides as their substrate. Pyrethroids, organophosphates and some carbamates have been found to be more susceptible to biodegradation. However, most organochlorines have shown to be formidable to biodegradation due to the strength of C-Cl bond. The following is a example of microbial degradation of 2,4-D. The microbial degradation of 2,4-D can follow different pathways depending on the types of microbes present. Path "a" occurs when the bacteria Flavobacterina and Arthrobacter sp are present. Path "b" occurs when the fungus Aspergillus Niger is present (Linde, 1994).



Furthermore, oxidation process in the environment is brought about by mixed function oxidases (MFO). MFO is a complex enzymatic system which contains an enzyme called cytochrome P-450 that is responsible for the oxidation of lipophilic compounds (Garvish, 1999). Enzymatic oxidation of parathion for example is achieved by mixed function oxidases (MFO) which involve conversion of P=S to P=O to form paraoxon which is further hydrolyzed to phosphoric acid and *p*-nitrophenol.



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Thermodynamic Properties and Crystallization Behavior of Pesticide Imidacloprid

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People's Republic of China

1. Introduction

A large quantity of pesticide is produced and consumed in all the world. Pesticide industry plays more and more important role in economy of agriculture. With the updating of pesticide products, traditional pesticide, due to its high toxic residue and poor performance, has been gradually replaced by new generation pesticide which is more friendly to environment and mankind. Imidacloprid has become a typical representative of the new generation pesticide for which gives due weight to its great efficiency, low toxicity and low residue, and has been developed since the 1990s. Imidacloprid (1-[(6-chloro-3- pyridinyl) methyl]-4,5–dihydro–N–nitro-1H-imidazol-2-amine, molecular formula $C_9H_{10}ClN_5O_2$, CAS Registry No.138261–4-3) ، acts on the diverse acetylcholine receptor (nAchR) of insect origin, is a new, selective, long-acting neonicotioid insecticide which can be used with reasonable environmental safety (PAN Y. M. et al., 2000; Kagabu, S. et al.,1997; Heijb roek W. et al., 1995). Imidacloprid is a commercial example of the chloronicotinyl insecticides acting at the nicotinyl acetylcholine receptor (Bai et al., 1991; Moriya et al., 1992; Leicht, 1993), is reported as highly active insecticide for homopteran pests (Iwaya and Tsuboi, 1992; Shiokawa et al., 1994; Gourment et al., 1996; Jian Zhong et al., 1996; Sannino, 1997; Ramaprasad et al., 1998; Kumar et al., 2000a) and for some speces of the order coleoptera, diptera and lepidoptera (Elbert et al., 1990, 1991). It has recently been registered in the world for plant protection practices. Its bioefficacy and persistence has been studied on few crops like wheat, barley, rice, cotton, chilli, okra, mustard and sugar beet (Dewar and Read 1990; Rike et al,. 1993; Ishii et al., 1994; Jarande and Dethe, 1994; Rouchaud et al., 1994; Iwaya et al., 1998; Kumar, 1999; Kumar et al., 2000a; Dikshit et al., 2000). So far, it has been one of the new competitive pesticides in the world market (A.W.M. Huiijbrgts et al., 1995; KONG et al., 2008). However, the original pesticide of imidacloprid contains huge amounts of 2-Nitroaminoimidazoline(4,5-Dihydro-N-nitro-1H-imidazol-2-amine, CAS Registry No. 5465-96-3, ab. NIM) and by-products (2-Nitriminoimidazolidine,1,1'- (2,5-pyridinediyl)bis-, ab. NMP) which affect the quality of imidacloprid in the producing process. Crystallization technology for the separation and purification of organic materials is used widely because of low energy consumption and higher purity. So the solid–liquid equilibriums are of interest for the development of theoretical models and in application of the chemical industry (Kojima et al.,1997; Matsuoka et al.1989; Dalmazzone et al., 2002; Shibuya et al., 1993; Khimeche et al., 2006; DING et al., 2000). As long as crystallization behavior is observed and

the data for the pure substances are known it is possible to use the data obtained in any solid-liquid equilibrium experiment to calculate the activity coefficient in the liquid phase. The study of solid-liquid equilibrium of binary/ternary mixtures provides information on both the intermolecular forces between solvent and solute and also on the nature of the intermolecular compounds in the solid phase(Yamamoto I., 1996). In the paper, the thermodynamic properties of the solid-liquid equilibrium on imidacloprid, 2-Nitroaminoimidazoline and NMP have been studied in order to solve the problem of the separation and purification of imidacloprid. In addition, the crystallization metastable zone width and crystallization behavior in some solvents and purification to the second powder concentrated of imidacloprid have been also determined.

2. Standard combustion enthalpy and thermal capacity of imidacloprid and some nitrogenous organisms

During the production process of imidacloprid, the yield and quality of product are influenced by 2-Nitroaminoimidazoline and accessory substance NMP. The structural formula of them are written in Fig.1.

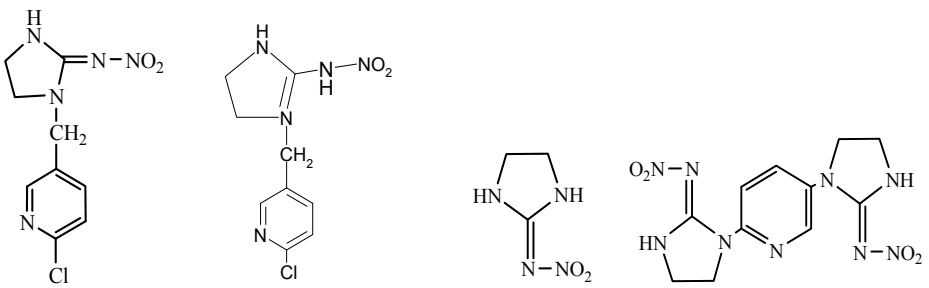

Fig. 1. The compound structure

The constant-volume combustion energies of three pure substance can be determined by a precision oxygen bomb calorimeter. The standard molar enthalpies of combustion and formation can be calculated on the basis of thermodynamic theory. Moreover, The relationship between the specific heat capacity and temperature could be discussed by mathematics according to the experimental data. The related studies can provide a thermodynamic basis for imidacloprid further application, and it will play an important role on gainning high-yield purification of imidacloprid and be available for the exploiting new synthesis method, engineering design and industry production of imidacloprid.

2.1 Medicines and experiment apparatus

The crystals both imidacloprid and NMP are purified by recrystallization with pure organic solvents, respectively, to obtain the purity of 99.9 %（mass fractions） by high performance liquid chromatography. The melting points of them are separately 416.80±0.05K and 423.85±0.05K by DSC-60 (Japan Shimadzu Co.), which agrees well with the literature value of (416.95±0.50 K) for imidacloprid (LIU, 2000). And NMP's melting point has been reported

(CHEN et al., 2009; ZHOU et al., 2010). 2-Nitroaminoimidazoline purchased from Jiangsu Tianze Chemical Industrial Co. Ltd, China (purity ≥ 99.00 %) has used as delivered without further purification, and the melting point of it is 495.13±0.05K by DSC-60. Benzoic acid used in the experiment is of AR grade with mass fraction purity of over 99.5% and purchased from Shanghai chemical reagent company, R.. P. China. α-Al_2O_2 is the powder for DSC Standard Material，provided by the Shimadzu company in Japan.
DSC-60 differential scanning calorimetry and DTG-60 thermogravimetric-differential scanning calorimetry are provided by the Shimadzu company in Japan. The error of electronia balance and microthermometer is, respectively, 0.1mg and ±0.01℃. SPN-500 nitrogen generator (the HP analysis technology research institute in Beijing, China.) is used to provides nitrogen atmosphere for the experiment of thermal analysis.

2.2 Principle of enthalpy of combustion determined

Based on the first law of thermodynamics, when a substance is burnt completely，the combustion energy of material at constant volume is shown in Eq.(1) (CHEN et al., 2009; Duane, 2000; Sergy, 1998; JIANG et al., 2005; ZHOU et al., 2002; AN et al., 2007).

$$Q_V = \Delta_C U_m \tag{1}$$

or if it is under constant pressure, the combustion enthalpy of material is shown in Eq.(2):

$$Q_p = \Delta_C H_m \tag{2}$$

Where,

$$\Delta_C H_m = Q_p = Q_V + \Delta nRT \tag{3}$$

Here Δn represents the change of mole number for gaseous materials when the combustion reaction occurs. R and T represent the universal gas constant 8.314 J·mol^{-1}·K^{-1} and the Kelvin temperature. When the sample is combusted completely, temperature of both the oxygen bomb calorimeter and surrounding medium (usually, that is water) rise because it give off quantity of heat. The temperature variation (ΔT) is determined by experiment. The combustion reaction heat Q_v can be obtained for the material at constant volume by Eq. (4) after the specific heat of Oxygen Bomb Calorimeter (ε_{calar}) is defined.

$$mQ_V = \left(3200C_{water} + \varepsilon_{calar}\right)\Delta T + Q_f \tag{4}$$

Where m is amounts of the sample, g; C_{water} is specific heat capacity of water, 4.18 J·g^{-1}·K^{-1}; ε_{calar} is the specific heat of calorimeter, J·K^{-1}; Q_f is an attach heat quantity generated by air of which is in the calorimeter, it is 0 J at the ideal state. Q_v is the energy of combustion. On the basis of the standard substance Q_v (26460 J·g^{-1}) of benzoic acid (Sergey, 1998; JIANG et al., 2005; ZHOU. et al., 2002; AN. et al, 2007) , the heat of calorimeter (ε_{calar}) can be obtained from the Eq. (4), and the standard molar energy of combustion ($\Delta_c U_m^\theta$) can calculaed by experimental data from Eq.(4). The standard moler enthalpy of the substance($\Delta_c H_m^\theta$)is referred to be the change of combustion enthalpy in the ideal combustiong reaction according to Eq.(3) at 298.15K and 101.325kPa.

Combustion calorimetry. The constant-volume combustion energy of sample can be determined by a precise thermal isolation oxygen bomb calorimeter (XRY-1C, shanghai changji geology apparatus Ltd., R. P. China), in which fitted with a stirred water bath. An amount of benzonic acid (BA) is taken and preformed by hand driven tablet machine. The preforming sample is placed in stainless steel pot, and a metal wire used as ignition is binded on a couple of electrode before oxygen is put into the oxygen bomb calorimeter, which is bured in oxygen at pressure 3.00 ± 0.50 MPa. Then the oxygen bomb is put into a bucket contained 3200mL water (at 298.15K). Stir is opened before the apparatus records automatically. After 5 min, the metal wire is ignited. Meanwhile, it is that the sample starts combustion when temperature quickly rised. Temperature readings are taken at 5s intervals before and after the ignition. After temperature reach at the most height point and continue 10min, the test could be stopped automatically.

2.3 Principle and procedure of specific heat capacity determined

To determine the specific heat capacity of sample by DSC, heat flow signal from the sample is compared to the DSC signal of a standard material of known specific heat (CHEN et al., 2009). Both curves are corrected by a zero line or base line to correction experiment. Where empty crucibles of both a reference and sample are separately placed in the furnace，the system signal drift is measured under identical experimental conditions.

The specific heat capacity of sample determined is accomplished according to three step technique progess at a linear heating rate by DSC-60:(i)assumes that the identical instrument settings and conditions are used for each experimental step. (ii) the same empty reference crucible is used for all steps and not removed from the DSC furnace. (iii) The three main steps defined as follows are done by DSC.

Step 1: empty sample crucible is scanned to obtain DSC sign of zero line determined.

Step 2: to scan sample crucible where contains zinc and indium used as the substance of calibration standard.

Step 3: to scan sample crucible in which contains the sample measured.

The experiments are done for each of them at least three times. Specific heat capacity C_p of the substance is then calculated by Eq.(5) as follows:

$$C_{p,sample} = \frac{M_{standard}}{M_{sample}} \times \frac{\varphi_{sample}(T) - \varphi_0(T)}{\varphi_{standard}(T) - \varphi_0(T)} \times C_{p,standard} \quad (5)$$

Where C_p, M, and φ are , respectively, specific heat capacity，mass of sample, and DSC output signal as heat flow rate of substance；subscript symbols, such as sample, standard and 0, are respectively, sample measured, standard chemical substance (e.g.zinc, indium), and zero line.

A small amount of powdery solid sample (3 to 5mg) is taken and sealed in an aluminum pan of DSC-60 for the analysis. The measurements are made under fixed conditions of which is the constant heating rate of 5 ℃ · min^{-1} and under nitrogen atmosphere (40 $mL \cdot min^{-1}$). α-Al_2O_3（standard material）is used as reference material in the process of the analysis. Before the samples are analyzed, it is necessary that the DSC-60 is calibrated with indium (purity=99.99%, T_m=429.78 K, $\Delta_m H$ =28.45 $J \cdot g^{-1}$) and zincum (purity=99.99%, T_m=419.58K, $\Delta_m H$=100.50$J \cdot g^{-1}$) (Japan Shimadzu Co.). Data acquisition and online processing could be done with TA-60WS Collection Monitor software.

2.4 Standard molar enthalpy combustion and formation of imidacloprid and some nitrogenous organisms

The detailed procedure has been described above-mentioned method. Freshly circular sheet solid sample is prepared for determining the combustion heat of substance. Smooth curves are fitted to the pre and after-period temperatures and the corrected temperature rise is calculated by means of data processing soft-ware in the oxygen bomb calorimeter working station using the Dickinson method (JIANG et al., 2005; ZHOU et al., 2002; AN et al, 2007), in which ΔT is the ordinate that encloses equal areas above and below the reaction curve. The energy equivalent of the calorimenter ε_{calar} could be determined with a standard reference sample of benzoic acid. From three experimental dada ε_{calar} is measured to be 15245 $J \cdot K^{-1}$. On the basis of thermodynamics principle，the standard molar enthalpy of combustion is

$$\Delta_C H_m^{\theta}(298.15\mathrm{K}) = \Delta H_1 + \Delta_C H_m(T) + \Delta H_2 = \Delta H_1 + Q_V + \Delta n R T + \Delta H_2 \tag{6}$$

Which

$$\Delta H_1 = \int_{298.15}^{T} \sum \left(a_j c_{p,j}\right)_{\text{reactant}} \mathrm{d}T \quad , \quad \Delta H_2 = \int_{T}^{298.15} \sum \left(b_k c_{p,k}\right)_{\text{product}} \mathrm{d}T \tag{7a,b}$$

The individual values of both the standard molar energy and enthalpy of combustion are listed in Table 1 together with the mean. The combustion reaction equations of imidacloprid, NIM and NMP, can be written as Eq.(8) to Eq. (10):

$$C_9H_{10}ClN_5O_2\,(s) + 41/2\ O_2\ (g) = 9\,CO_2(g) + 9/2\,H_2O\ (l) + HCl(g) + 5/2\ N_2\,(g) \tag{8}$$

$$C_3H_6N_4O_2\ (s) + 7/2\ O_2(g) = 3\ CO_2(g) + 3\ H_2O\ (l) + 2\ N_2\ (g) \tag{9}$$

$$C_{11}H_{13}N_9O_4\ (s) + 49/4\ O_2\ (g) = 11CO_2(g) + 13/2\,H_2O(l) + 9/2\ N_2\ (g) \tag{10}$$

and, $\Delta_c H_m = Q_v + \Delta n R T$ where Δn = -6, 1.5, -9, accordingly. Hence, the standard molar enthalpy of combustion can be obtained for various samples by Eq. (3) , and the results are listed in Table1. The obtained values are, respectively, -5536.34 $kJ \cdot mol^{-1}$, -2017.64 $kJ \cdot mol^{-1}$, -7976.88 $kJ \cdot mol^{-1}$ for imidacloprid, NIM and NMP.

The standard molar enthalpy of formation, $\Delta_f H_m^{\theta}$, can be calculated by Hess's law(Sergey, 1998), according to the thermochemical equations (8) to (10) as follows:

$$\Delta_f H_m^{\theta}[C_9H_{10}ClN_5O_2,s] = \left[9\Delta_f H_m^{\theta}(CO_2,g) + 9/2\Delta_f H_m^{\theta}(H_2O,l) + \Delta_f H_m^{\theta}(HCl,g)\right] - \Delta_C H_m^{\theta}[C_9H_{10}ClN_5O_2,s] \tag{11}$$

$$\Delta_f H_m^{\theta}[C_3H_6N_4O_2,s] = \left[3\Delta_f H_m^{\theta}(CO_2,g) + 3\Delta_f H_m^{\theta}(H_2O,l)\right] - \Delta_C H_m^{\theta}[C_3H_6N_4O_2,s] \tag{12}$$

$$\Delta_f H_m^{\theta}[C_{11}H_{13}N_9O_4,s] = \left[11\Delta_f H_m^{\theta}(CO_2,g) + 6.5\Delta_f H_m^{\theta}(H_2O,l)\right] - \Delta_C H_m^{\theta}[C_{11}H_{13}N_9O_4,s] \tag{13}$$

Where

$$\Delta_f H_m^{\theta}(CO_2, g) = (-393.52 \pm 0.13)\,kJ \cdot mol^{-1},\quad \Delta_f H_m^{\theta}(H_2O, l) = (-285.83 \pm 0.04)\,kJ \cdot mol^{-1},$$

$$\Delta_f H_m^{\theta}(HCl_2, g) = (-92.307)\,kJ \cdot mol^{-1}$$

(CHEN et al., 2009; Duane, 2000; George et al., 2006; WANG et al., 2002; ZHOU et al., 2010).
The standard molar enthalpy of formation for imidacloprid, NIM and NMP are calculated to be –(616.12)kJ·mol^{-1}, –(20.41) kJ·mol^{-1} and 1789.94 kJ·mol^{-1}, respectively, based on the standard molar enthalpies of combustion.
In the literature (CHEN et al., 2009), that the standard molar enthalpy of combustion $\Delta_c H_m$ is (-5153.9 kJ·mol^{-1}) for naphthalene agrees very closely with experimentally derived value of (-5158.43 kJ·mol^{-1}) in the work, the relative error is 0.088%. The result shows that $\Delta_c H_m$ value of reliability prediction is superior.

2.5 The specific heat capacity of imidacloprid and some nitrogenous organisms

The specific heat capacity of imidacloprid could be measured by means of DSC-60 Differential Scanning Calorimeter in the hermetically sealed chamber. The conditions of scanning: α-Al_2O_3 is used as the reference material，scanned area is between room temperature and melting temperature of the sample measured，sample mass is about 5mg，heating rate is 5℃·min^{-1}. The specific heat capacity is measured at least three times. Fig.2 shows the relationship between the specific heat capacity with temperature for the substances measured. The results indicate that a sequence of the specific heat capacity for the substance determined at same temperature is NIM, imidacloprid, benzoic acid, NIM. And the higher temperature is, the bigger the specific heat capacity is for the substances of nitrogenous organisms measurated. At the same time, the bigger the relative molecular weight is for the nitrogenous organisms, the bigger the specific heat capacity is also. Relationship between the specific heat capacity and temperature can be obtained with the least square method at solid phase states, and represented by Eq.s (14) to (17) (CHEN et al., 2009).

$$C_p(\text{imidacloprid}) = 2.04708 - 0.01949T + 5.77744\times10^{-5}T^2 \quad (14)$$

(R^2=0.99559, SD=0.034)

$$C_p(\text{NIM}) = 2.56469 - 0.01224T + 2.26134\times10^{-5}T^2 \quad (15)$$

(R^2=0.98341, SD=0.01001)

$$C_p(\text{NMP}) = 92.03739 - 0.86937T + 0.00272T^2 - 2.7657\times10^{-6}T^3 \quad (16)$$

(R^2=0.9916, SD=0.02746)

$$C_p(\text{benzoic acid}) = 11.62525 - 0.07464T + 1.33277\times10^{-4}T^2 \quad (17)$$

(R^2=0.99495, SD=0.02768)

Where R is the multiple correlation coefficient, SD is the standard deviation. The abovemented logistic equations accord with the statistical precision in mathematics so that it is believable.

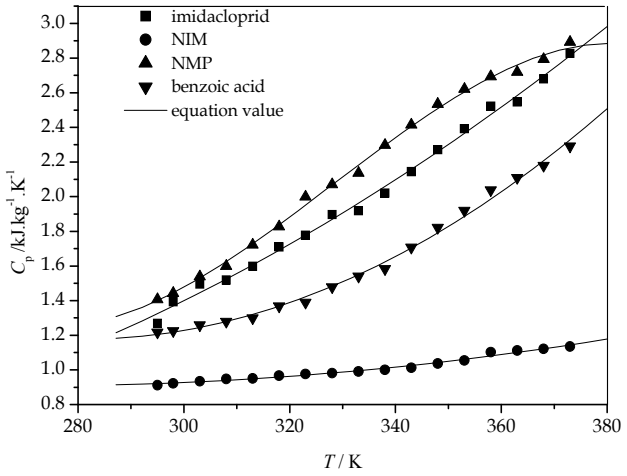

Fig. 2. Change curve specific heats as temperature for different matters

2.6 Melting temperature and the melting enthalpy of imidacloprid and some nitrogenous organisms

The melting temperatures and melting enthalpies of imidacloprid, NIM, NMP could be measured by DSC. The values of them are given to be 416.8K, 495.13K, 423.85K，and -109.93, -265.55, -102.22 J·g^{-1}, respectvely, in Fig.3. The thermal decomposition of the substances have been studied by thermogravimetric analysis. The results of thermal decomposition (in Fig.4~6) show that the heat stability of NIM and NMP is not good because they are decomposed as soon as melting, but imidacloprid has good heat stability. The decomposition temperature is, respectively, 525.10K, 495.13K, 450.25K for imidacloprid, NIM, NMP(CHEN et al., 2009) .

In conclusion, the thermodynamic properties of imidacloprid, NIM, NMP are listed in table 1.

Substances	T_m/K	ΔH_m J·g^{-1}	T_d/K	$\Delta_c H_m^\theta$ /kJ·mol^{-1}	$\Delta_f H_m^\theta$ /kJ·mol^{-1}	C_p=a+bT+cT^2+dT^3/kJ·kg^{-1}·K^{-1}			
						a	b	c×10^5	d×10^6
imidacloprid	416.80	109.93	525.10	5536.34	-616.12	2.04708	-0.01949	5.77744	0
NIM	495.13	265.53	495.13	2017.64	-20.41	2.56469	-0.01224	2.26134	0
NMP	423.85	102.22	450.25	7976.55	1789.94	92.03739	-0.86937	272	-2.7657

Table 1. The thermodynamic properties of imidacloprid, NIM and NMP

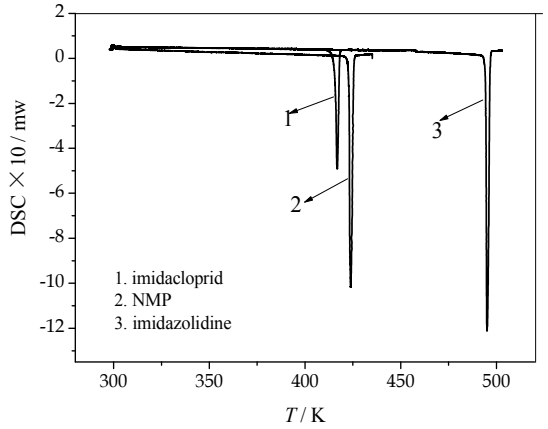

Fig. 3. The DSC melting curve of imidacloprid , NIM and NMP

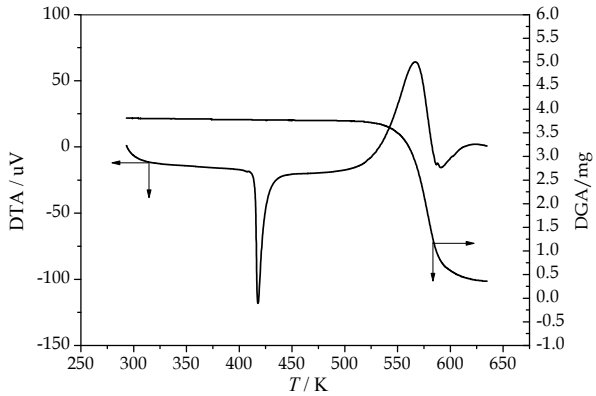

Fig. 4. The DTA-TGA curve of imidacloprid

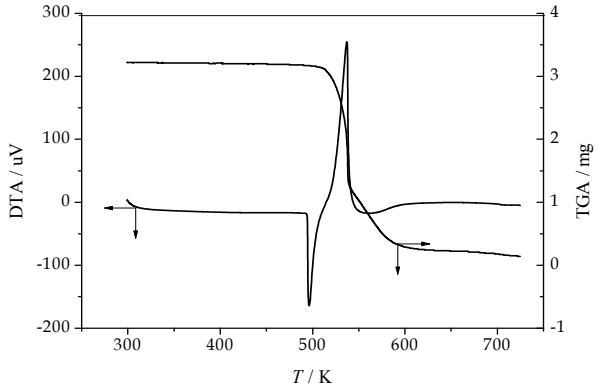

Fig. 5. The DTA-TGA curve of NIM

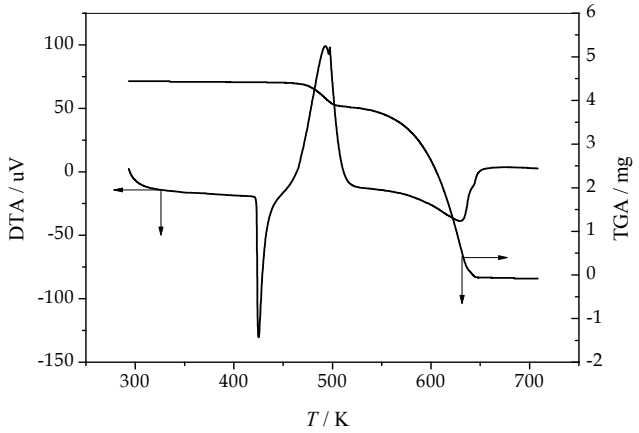

Fig. 6. The DTA-TGA curve of NMP

3. The mixed crystal of imidacloprid

The spatial disposition is different for the molecule or atom of substance in crystal lattice when they are crystallized. The reason is that the intermolecular bonding manner take place change and make different crystal structures formed by effect of various factors on crystallization precess, that is polymorphism. Only that the substance is dissolved or is melted make the lattice structure change can the polymorphism disappear. Therefore, this necessitated field experiments to study on pharmaceutical polymorphism is in favour of establish biological activity and bioavailability of chemical compound, can promote corresponding technology optimization, selection of the route of administration and dosage form, enhance the pharmaceutical quality and stability(WANG, 2005).

3.1 The method of polycrystallinity transformation

3.1.1 The recrystal method

Recrystallization can prepare corresponding crystal form by using different solvents, such as non-polar solvent aether, petroleum ether, chloroform, benzene, and polar solvent water, acetic acid, pyridine, as well as medium polar solvent methanol, alcohol, acetone and the mixture of organic solvent and water. To change and control the conditions of crystallization, such as concentration, temperature, and the rate of crystal, can obtain different crystal form (XIU, 1996). Only suitable solvent is used can the main crystal form be obtained.

3.1.2 The melting method

In general, the crystal form of low melting is able to translated into the crystal form of higt melting temperature at a suitable temperature. So we can use the melting method by heating the crystal form of low melting to reach an aim of crystal form transformed. For example, metoclopramine has a couple of endothermic peaks by using differential thermal analysis(DTA), which is 125 to 129℃ and 147 to 150℃, respectively. The first peak is solid – solid transforming endothermic peak of which metoclopramine (I) change crystal form (II) ,

another is solid –liquid endothermic peak of crystal form (II). If the substance is heated 15min at 135℃, the secongd peak will only appear when reusing differential thermal analysis(DTA). It shows that metoclopramine (I) could be quickly changed into crystal form (II) as long as above metoclopramine (I) melting temperature(XIU , 1996).

3.1.3 The sublimation method

Pharmaceutical is put into an evaporating dish, covered with the glass hopper, heated little and little, a large number sublimated crystal can be get in upper and border of the glass hopper. The method can make pyrimethaimine (crystal form A) transformed into crystal form (B) (ZHANG , 1995).

3.1.4 The smashing and grinding method

When using the smashing and grinding method，strong mechanical force made the surface crystal structure of crystal grain diminish and the local energy level of particle surface increase, so that lead to the misalignment and boundary deformation of crystal form. Commonly, the metastabletype crystal form could be changed into the stability type of crystal. There is also an exception to this rule, such as anhydrous caffieine is apt to make stability type transform into metastabletype when it is smashed and grinded (Pirttimaki Jukka and Laine Ensio, 1994).

3.2 Determine to imidacloprid polymorphism

To make clear a crystal substance is whether there is in polymorphism, the methods are commonly used as (1) melting pointmethod; (2) Thermal analysis; (3) X-ray diffraction; (4) infrared spectrometry; (5) nuclear magnetic resonance method. The polymorphism of imidacloprid has been determined by using thermal analysis method. The DSC melting curves of imidacloprid before and after crystal formation changed show in Fig. 7 and Fig. 8

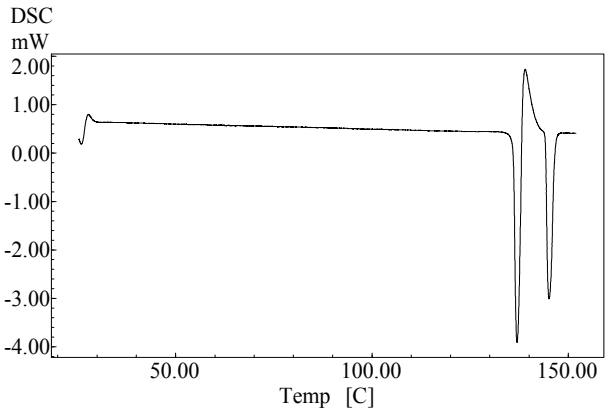

Fig. 7. The DSC melting curve of imidacloprid before changed crystal formation

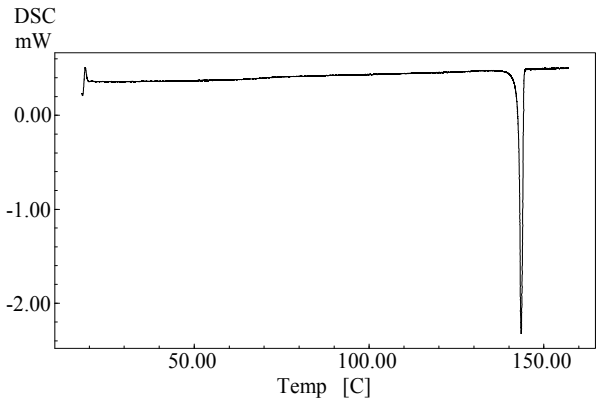

Fig. 8. The DSC melting curve of imidacloprid after changed crystal formation

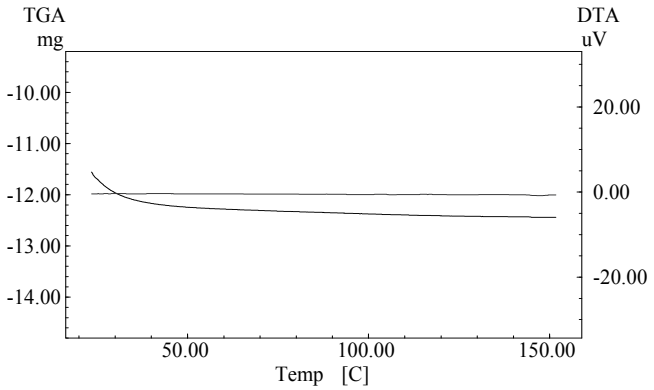

Fig. 9. The TGA melting curve of imidacloprid before and after recrystal

It is observed that there are two endothermic processes at 136.13℃ to 138.29℃ and 144.37℃ to 146.23℃ , and has a strong exothermic process in 138.29℃ to 144.37℃ according to Fig.7. Combining with Fig.9, there is no a significant change for TGA curve at 136.23℃ to 146.23 ℃, i.e. no weightlessness, so it show that the first peak is solid –solid transforming endothermic peak of which imidacloprid (I) change into crystal form (II) , another is solid – liquid endothermic peak of crystal form (II) in Fig.7.

By means of the recrystal method, imidacloprid could be dissolved in boiling water, then dissolved out crystal when it is cooled. This recrystal sample has been analyzed by DSC, in Fig.9, only there is a solid–liquid endothermic peak. And the TGA curves of imidacloprid are same before and after recrystal. The result indicates also that the crystal form of imidacloprid has been changed into a single form by solvent processing.

4. Crystallization metastable zone of imidacloprid in organic solvents

4.1 The solubility and supersolubility of imidacloprid

Materials. The process of recrystallization for imidacloprid is designed according to its the physical and chemical properties and obtained the solubility data in the laboratory

previously. The purity of imidaclaprid is analyzed by the melting point method, differential scanning calorimetry and high performance liquid chromtography. The simple physical method that is boiling water is used for transfering the mixed crystallization into a single crystal. Eventually, the purity of products meets the requirements of the factory for the first grade products(over 99%). Ethanol, acetone, 2-butanone, dichloromethane and 1,2-dichoroethane used for the experiments were of analytical reagent grade. Distilled deionized water of HPLC grade is used.

Apparatus and Procedure. The solubility and supersolubility of imidacloprid in different solvents is measured by the synthetic method (dynamic method). The laser monitoring observation technique is used to determine the dissolution temperature of the solid-liquid mixture of known composition. The laser monitoring system consists of a laser generator, a photoelectric transformer, and a light intensity display. The experiments are carried out in a jacketed glass vessel with a magnetic stirrer, a constant temperature (± 0.02 K) is maintained at the required temperature by circulating water through the outer jacket from a thermoelectric controller. The vessel has a perforated rubber cover plate to prevent the solvent from evaporating, through which a mercury glass thermometer is inserted into the inner chamber of the vessels for the measurement of the temperature. The uncertainty of temperature is ± 0.05 K at least.

Solvents for the solubility and supersolubility of measurement are prepared using an electronic balance with an accuracy of ± 0.0001 g. Predetermined amounts of imidacloprid and the solvents are weighed and transferred into the vessel. The contents of the vessel are heated very slowly at rates $1℃ \cdot h^{-1}$when the system would arrive balance. In the early stage of the experiment, the laser beam is blocked by the unsolved particles of imidacloprid in the solution, so the intensity of the laser beam penetrating the vessel is lower. The intensity increased gradually along with the increase of the amount of imidacloprid dissolved. When the last portion of imidacloprid just disappeared, the intensity of the laser beam penetrating the vessel reached the maximum, and the temperature is recorded, and the solubility expressed in mole fraction is calculated as follows

$$x_1 = \frac{\frac{m_1}{M_1}}{\frac{m_1}{M_2} + \frac{m_2}{M_2}} \tag{18}$$

In which m_1 and m_2 represent the masses of solute and solvent. M_1 and M_2 are the molecular weights of solute and solvent.

The contents of the vessel are heated continuely and temperature is rise 2 to 3℃ when the solid solute is dissolved completely. Then the contents of the vessel are coolled slowly at rates 4 to $5℃ \cdot h^{-1}$ by temperature of circulating water controlled through the outer jacket. The cooling rate is not too quickly toensure thermometer value in accord with them of actual system. When the last portion of imidacloprid just appeared, the intensity of the laser beam penetrating the vessel minished suddenly, and the temperature is recorded and obtain the supersolubility of the solute. The difference in temperature is expressed to be the crystallization metastable zone at the concentration.

4.2 The relationship of both the solubility and supersolubility with temperature for imidacloprid

Extraction-crystallization process is frequently used for the separation and purificaton of imidacloprid in industry. The relationships of both the solubility (w) and supersolubility

(w*) with temperature have been showed in Fig.10 and Fig.11 for imidacloprid in ethanol, acetone, 2-butanone, dichloromethane and 1,2-dichoroethane (ZHOU et al., 2010). They indicate that the solvent of having the most solubility is 2-butanone at same temperature, and in turn, acetone, dichloromethane, 1,2-dichoroethane and ethanol. So 2-butanone can used as extraction agent, and ethanol can used as washing agent to eliminate impurities of which are liable to dissolve in imidacloprid. In common with dissolution rule for a lot of crystal systems, it is found that the solubility and supersolubility of imidacloprid in solvents increase with temperature increase.

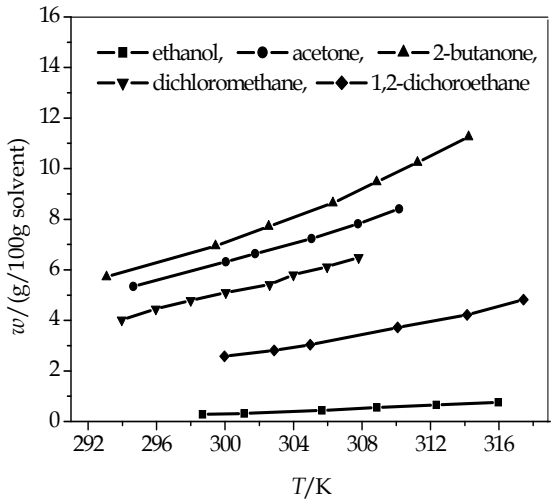

Fig. 10. The solubility of imidaclprid in organic solvents

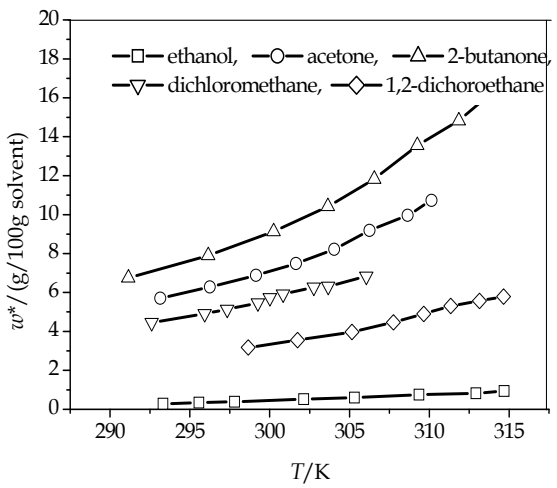

Fig. 11. The supersolubility of imidaclprid in organic solvents

4.3 Crystallization metastable zone width of imidacloprid

In order to produce crystals with a specified purity and crystal size distribution at minimum cost, it is necessary to operate the crystallizer at the optimum supersaturation within the metastable zone. In generally, only the actual metastable zone of substance is known can the nucleation and the rate of crystallizing process be controlled in industry production (ZHOU et al., 2002; AN et al., 2007). The operational condition of crystallization must be controlled within metastable zone to ensure the quality of the crystal.

The metastable zone width is an experimentally measurable quantity and it depends mainly on the cooling rate, temperature, concentration, presence of impurities and mechanical effects. The metastable zone width is expressed as a maximum undercooling $\Delta T_{\max}$, which is given by

$$\Delta T_{\max} = T_{\mathrm{s}} - T_{\mathrm{n}} \tag{19}$$

In which T_{n} and T_{s} are respectively, the temperatures corresponding to the onset of nucleation and the saturation. The relation of maximum supersaturation to the maximum undercooling can be expressed by

$$\Delta w_{\max} = \int_{T_{\mathrm{p}}}^{T_{\mathrm{s}}} \left(\frac{\mathrm{d}w^*}{\mathrm{d}T} \right) \mathrm{d}T \tag{20}$$

Since the maximum undercooling is generally not very large, the temperature dependence of $\mathrm{d}w^*/\mathrm{d}T$ can be assumed constant over the temperature range examined. Thus, the maximum supersaturation can be siven by the following equation (Soloway et al., 1978):

$$\Delta w_{\max} = \left(\mathrm{d}w^* \big/ \mathrm{d}T \right) \Delta T_{\max} \tag{21}$$

Where w^* is the equilibrium concentration of substance in soild-liquid phase，$\mathrm{d}w^*/\mathrm{d}T$ is the slope factor of some temperature point in the curve of relation between solubility and temperature.

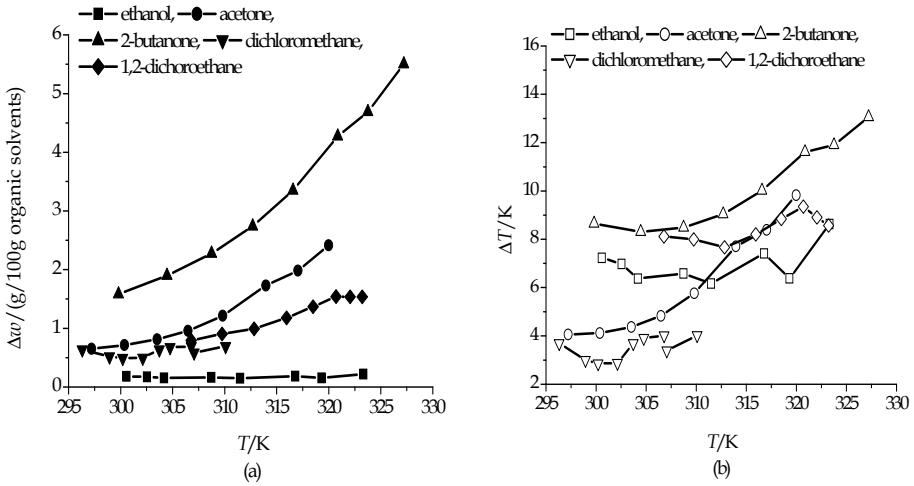

Fig. 12. The metastable zone width of imidacloprid in organic solvents

The saturate and supersaturate characteristics of imidacloprid in ethanol, acetone, 2-butanone,dichloromethane and 1,2-dichoroethane at different temperature are investigated by using laser detection technique. The crystallization metastable zone width of imidacloprid in mentioned above solvents are also determined. The solubility, super-solubility and crystallization metastable zone width (Δw) of imidacloprid in mentioned above solvents increase with temperature rising, and those properties of imidacloprid in 2-butanone are high compared with other solvents. The crystallization metastable zone width (ΔT) of imidacloprid is about 4.4℃，5.8℃，1.2℃，1.8℃ and 2.4℃ in 2-butanone , acetone , dichloromethane, 1,2-dichoroethane and ethanol at the same agitation rate, respectively, in Fig.s 12(a), (b). (ZHOU et al., 2010)

4.4 The solid-liquid equilibrium data of binary and ternary organic systems for imidacloprid+2-nitroaminoimidazoline +NMP by DSC

A complete data obtained by DSC of solid-liquid equilibriums(SLE) for (MIP+ NIM), (IMIP + NMP), (NIM + NMP) and (NIM + NMP+ IMIP) systems are reported to solve the problems of separation and purification in the production of imidacloprid. (ZHOU et al., 2011)

Procedure. Products (purity ≥99 .00%) were used directly. A series of binary systems of IMIP+ NIM, IMIP + NMP, NIM + NMP were prepared in the following method. The samples were weighed by electronic analytical balance (type BS110S, Germany) with the accuracy of ±0.1mg. Usually, the sample is prepared as follows: the compounds at some molar ratio are heated very slowly inside a glass cell above the melting temperature of the major component. Then the liquefied sample with continuous stirring was solidified. The solvent-soluble method was selected for obtained an intimate mixture sample to avoid the substances sublimation or decomposition above the melting temperature of the major component. The sample at some molar ratio was put into amount of organic solvent. Then the solution was stirred continuously, heated very slowly to ensure crystals dissolved. And the glass cell was put into vacuum dryer at constant temperature 45℃ until the solvent was evaporated completely. The solidified sample was grinded to be powdery in a glass mortar box with mortar pestle before the sample was kept in a desiccator with silica gel. Then a small amount of powdery solid (3 to 4mg) is taken and sealed in an aluminum pan of DSC-60 for the analysis.

Solid-liquid phase diagrams (SLPD) for binary systems were measured by DSC. Fig. 13 to 15 show the relationship for three binary systems between the experimental temperatures of solid–liquid equilibrium and molar fraction of components. There were eutectic point for the binary systems of (IMIP+NIM), (NIM+NMP) and (IMIP+NMP) because that there were the characteristics of which first peak appeared at the constant temperature(Teu), and the second peak temperature(Tsl) was change with molar ratio of components. The area of the eutectic peak in a DSC curve was affected by both the amount of the sample and heat of fusion of the melting component. The corresponding eutectic molar composition (xeu, 1) was 0.4962, 0.4229, 0.5506 , and the eutectic temperatures (Teu) was 402.41 K, 407.46 K and 383.13K, respectively.

Moreover, solid-liquid phase diagram (SLPD) was investigated for the ternary system of (NMP (1) +NIM (2) +IMIP (3)) mixtures with the above-mentioned method. The results showed that it was also the eutectic type, the molar eutectic composition (xeu, 1) and eutectic temperatures(Teu) is 0.3507 and 373.77K at x3/x2=1.857 , respectively, and are presented in Fig. 16.

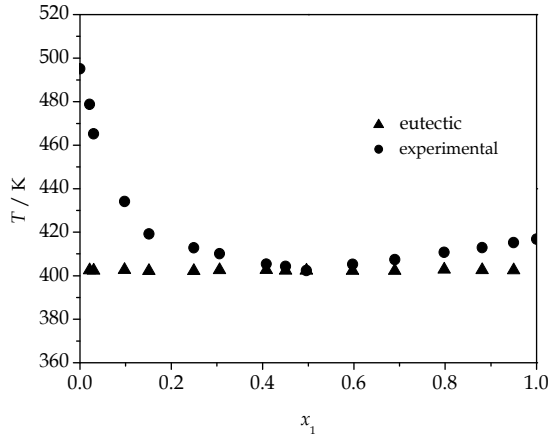

Fig. 13. Determined SLE for IMIP (1) + NIM (2)

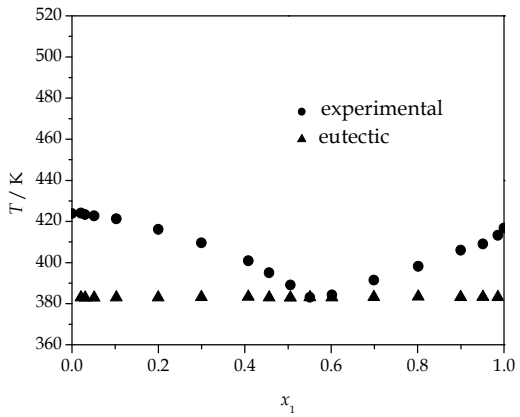

Fig. 14. Determined SLE for IMIP (1) + NMP (2)

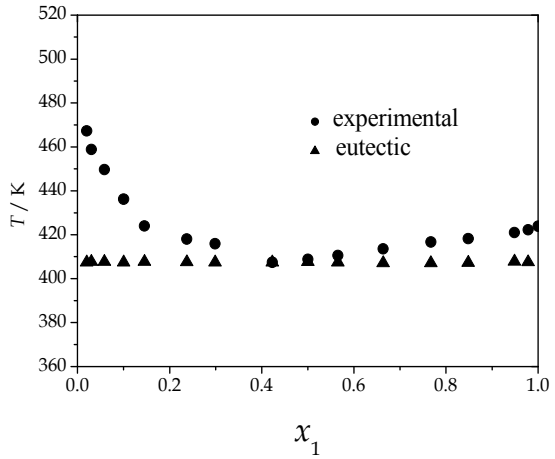

Fig. 15. Determined SLE for NMP (1) + NIM (2)

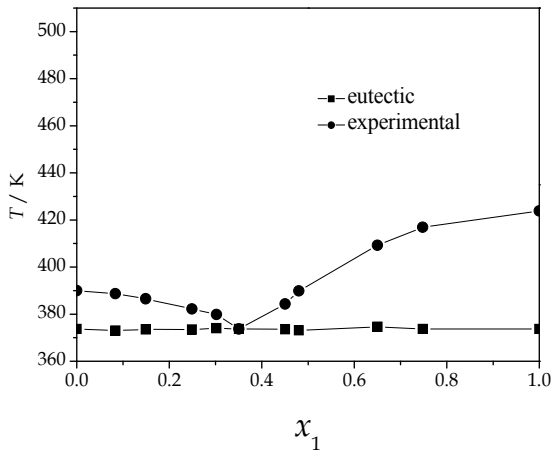

Fig. 16. Phase diagram of SLE for NMP (1) + IMIP(2) + NIM (3)

5. Purification to the second powder concentrated of imidacloprid

So far, studies on to purifiy the second powder concentrated of imidacloprid have not been conducted. Because imidacloprid has the solid state with by-products, filtering method cannot reach an aim of it purified. For distillation, due to (i) imidacloprid has two kinds of crystal form and the boiling point of them is much the same; (ii) imidacloprid has high boiling point, long time and high temperature distillation process could make material easily coked and breaked down in the kettle bottom, so also cannot be used. If separation purification technology of crystallization is employed, not only it can simplify the process flow, is apt to do mass production, but also it improve the quality of crystal, can effectively reduce the production cost. Combined with the features of raw material of imidacloprid, this experiment method of solvent extraction - recrystallization could be used to separate and purify imidacloprid from the mixture of the second powder.

5.1 Experimental process flow

The experimental procedure is designed as follows on the basis of the preliminary experiments:

Experimental operation steps: (i)take a certain amount of raw material (second powder of imidacloprid) and put into a certain proportion of material and organic solvent A , stired at room temperature for 20 minutes to remove impurities and decoloring part; (ii)after filtrated, liquid is placed in bottle, filter cake dried is put in ing boiling water for 30 minutes; (iii) filtrated in heating state, cleaning filter cake dried with organic solvent B to remove the impurities of some soluble in B; (iv) according to steps (ii) after filter cake dried is put in organic solvent C and heat to 60℃, natural cooling until crystallization dissolved out. The crystal dried is used continuely recrystalled at least three times by the organic solvent C; (v) after filtrated, liquid is evaporated by totating evaporator to recover solvent, filter cake is imidacloprid.

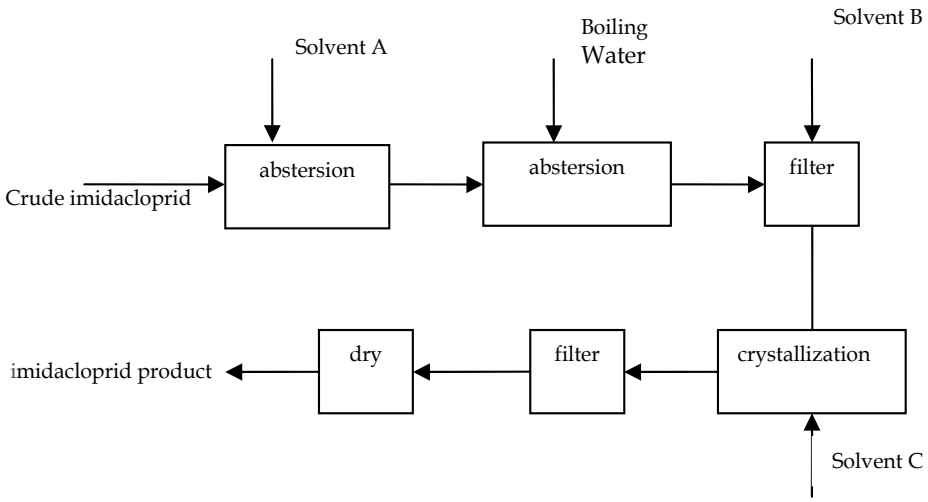

Fig. 17. The process chart of purification technology

5.2 Imidacloprid analysis

Imidacloprid used as standard substance (99.3%) 0.2 g (accurate to 0.1 mg) is put in a volumetric flash (50mL), add methanol to scale shake and make it completely dissolved . They are used as reserve. Using a pipette take 1mL to put in a volumetric flash (25mL) from above prepared reserve liquid, add a mobile phase make it diluted to scale, shake well, filtrate it with 45 μm filter membrane to prepare for analysis by HPLC. And this is identical for the sample of product imidacloprid.

Agilent1100 high-performance liquid chromatography, and 4.6×250mm，C18 DOS Chromatographic column, Chemical Station chromatographic data workstations, are used to analyzed product imidacloprid. Chromatographic analysis conditions are as follow: column temperature 25℃; methanol (gc) : pure water (double steamed water) = 40:60 (mL/mL) as mobile phase; velocity 1.0mL·min^{-1}; detected wavelength 260nm; sampling volume 20 0μL .

The external standard method is used to calculate an amount of imidacloprid, and can be expressed by

$$x = \frac{A_2 \times C_1}{A_1 \times C_2} \times 100\% \tag{22}$$

Where C_1, C_2 is , respectively, concentration of standard substance and sample product for imidacloprid（mg·L^{-1}）; A_1, A_2 is , respectively, chromatographic peak area of them. The analysis results are listed in table 2. Sensitivity experiments showed that the minimum amount of detecting substance found to be 5×10^{-14}g (SNR ≥ 3) in HPLC for standard imidacloprid solution in the above conditions, so that it is a method of high sensitivity, cando with the analysis of residues.

To determine the accuracy of the above methods, were analyzed at least five for a sample. The results showed the standard deviation is 0.28, and the coefficient of variation is 0.32%.

sample	Peak area ofHPLC	Product purity ,%
Standard sample of imidacloprid	1811.66	99.70
The first batch of imidacloprid products	1790.00	99.69
The second batch of imidacloprid products	1750.00	99.24
The third batch of imidacloprid products	1730.00	99.73

Table 2. The results of analytical reagent for the purity of the Imidacloprid and NMP

5.3 Polycrystal transformation

The separation extracted of imidacloprid can get the crystals mixed, but not satisfy a single effective crystal, so we could use recrystallization method to change its polycrystal. At present, it is still not seen have to imidacloprid more crystal phenomenon reported.

We can take the experimental procedure: (i) take a quantitative products to join in a glass container of which quantitative of cold water has been accommodated, heat to a nearly boiling in a boiling water bath, then take the container to make natural cooling at room temperature before filtrated and dried; (ii) take quantitative products to join separately in glass container where a quantitative of solvent B, methylene dichloride, 1,2-dichloroethane, chloroform, acetone, butanone, make solid dissolved completely after natural cooling to room temperature, recrystallization, and filtrated and dried ; (iii) analyze the product respectively with melting point instrument, thermoanalysis and HPLC on the qualitative and quantitative analysis.

According to the above experiment steps, the test results see table 3.

solvents	Crystal form	Content of imidacloprid ,%
boiling water	II	95.3
Solvent B	I	96.5
methylene dichloride	I	97.4
1,2-dichloroethane	I	96.4
chloroform	I	96.4
acetone	I	97.2
butanone	I	97.5

Table 3. Crystallization of mixed polymorph in deferent solvents

6. Conclusion

The enthalpy of combustion is a measure of the energy available from a substance. An appropriate knowledge of this value is essential for providing the basis of a commercial conttact between producer and user. Physical chemistry texts provide the necesssary tools needed to calculate the combustion enthalpy of pure substances. Using DSC differential scanning calorimetric, the thermodynamic basic data such as the melting point, melting enthalpie of taurine and specific heat capacity, were examined. Relationship between the specific heat capacity of taurine and temperature was accomplished with DSC. So these thermodynamic basic data are available for the exploiting new synthesis method, engineering design and industry production of imidacloprid. The results obtained in this work indicate that solubility of imidacloprid can be determined accurately by laser

monitoring technique. From the experimental data, it can be found that the solubility data of imidacloprid in the organic solvents over the temperature range from 293.00 K to 323.0 K increased with increasing temperature. And 2-butanone was a better solvent for the purification of imidacloprid. The solid-liquid equilibrium data of both three binary systems and one ternary system had been determined for imidacloprid, 2-Nitroaminoimidazoline, NMP by DSC. All of them are identified as eutectic type. The SLE data can be successfully applied on separation and purification processes of the substances.

There are two kinds of crystal forms for imidacloprid. Using the boiling water, the crystal form(I) can be transformed crstal form(II) for the imidacloprid product mixed crystal. The separation technology of extraction-crystallization employed the purity of imidacloprid could reached over 99% for the second powder concentrated of which is produced in industry process.

7. Acknowledgment

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List of symbols

εcalar	—the specific heat of calorimeter
C	—heat capacity，J·g^{-1}·K-1 or J·mol^{-1}·K^{-1}
DS C	—DSC signal for sample curve at temperature, mV
H	—enthalpy，J·mol^{-1} or kJ·mol^{-1}
m	—mass of sample, mg
M	—relative molecular mass of substance, or an amount of sample
n	—mole number
Q	—quantity of heat，J·mol^{-1} or kJ·mol^{-1}
R	—the universal gas constant，8.314J·mol^{-1}·K^{-1}
t	—time，min
T	—Kelvin temperature，K
U	—energe of thermodynamics，J·mol^{-1} or kJ·mol^{-1}
w	—mass fration, or mass number
x	—molar fration
Greek symbol	
φ	— DSC output signal
Δ	—variable quantity
superscript	
θ	—standard state
*	—phase equilibrium state
Subscript	
c	—combustion heat
f	—reaction heat
lit	—literature
m	—material, or melting enthalpy
max	—the maximum value
n	—the onset of nucleation.
p	—constant pressure

s	—saturation
sample	—substance measured
standard	— substance used as the standard
V	—constant volume
0	— zero line of DSC

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3

Photolysis of Some Benzimidazole Based Pesticides

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1. Introduction

The need for increased food production and control of disease vectors brought about a major development in the area of pest control (Martin, H., 1971). Public concern about pesticide contamination only became apparent after the publication of the biologist Rachel Carson (Johnson, D. 1968). This concern has generated a large number of studies in all aspects of pesticide research, including the possible effects and extent of environmental pollution (Tweedy, B. *et al*, 1968; Johnson, D. 1968).

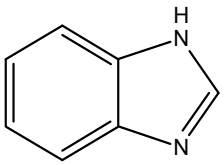
Gaetano Rango… *et al*, studied the photo and thermal degradation of Albendazole, Fenbendazole and Mebendazole. They found that all drugs showed high photosensitivity in solution but a reliable stability in solid form and when exposed to a temperature up to 50 °C. Photolysis was found to cause demethylation or decarboxylation of the parent compounds (Gaetano R., *et al*., 2006).

Abdou M., … *et al*, studied the effect of singlet oxygen on the photostability of Carbendazim in aqueous hydrochloric acid. They could identify some of the photoproducts such as the brownish colored insoluble dimer of benzimidazole (Abdou M. *et al*., 1986). Boudina A. *et al*, investigated the quantum efficiency of carbendazim in aqueous solution and the effect of pH value on the photostability of the pesticide. They suggested a possible phtodegradation mechanism and proposed a final photoproduct using HPLC and GC-MS analytical techniques. Monocarbomethoxy-guanidine was the most stable photoproduct (Boudina A. *et al*., 2003).

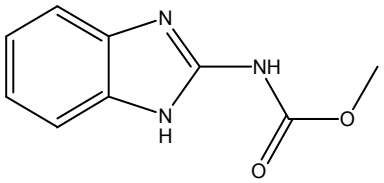
Saien J. and Khezrianjoo studied the kinetic photodegradation of carbendazim in the presence of TiO_2 catalyst (Saien J., and Khezrianjoo S. 2008). Fluorescence quantum yield of Carbendazim in aqueous solution at 254 nm was determined by Mazellier *et al*, (Mazellier P. *et al*. 2002).

The benzimidazole fungicides have been shown to be outstanding agents in disease control (Mazellier P. *et al*. 2002; Fleeker J. and Lacy H., 1977). They are used in the Jordanian agricultural valley where harsh weather conditions are known to exist. Although, such molecules are known to be stable towards solar radiation, the solar spectral distribution in the Jordanian valley extends down to 280 nm where photons in this range of wavelength can be absorbed by such molecules (Yousef A. *et al.,* (2007). Therefore, focusing on the effect of UV light on these molecules can be considered an important agricultural and environmental aspect to that area.

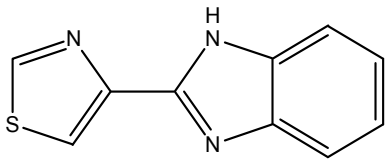
Important members of this group are Methyl 2-Benzimidazolecarbamate [Carbendazime] and 1H-Benzimidazole, 2(-4-thiazolyl) [Thiabendazole]. The molecular structure of benzimidazole and its two derivatives is shown in the figure below,



1H-Benzimidazole



Methyl 2-Benzimidazolecarbamate



1H-Benzimidazole, 2(-4-thiazolyl)

Our interest in the photophysical properties of the benzimidazole analogues arose from earlier work utilizing the photodegradation of Carbaryl and Benomyl in acetonitrile and aqueous solutions (Yousef A. *et al.,* (2007).

In neutral solutions the absorption and fluorescence maxima of benzimidazole and its two derivatives occurs in the UV range between 280 and 350 nm (Boudina A., *et al.*, ; Yousef Y.A, *et al.,* 2001). The main objectives of the project are:

1. To investigate the photodegradation kinetics of above molecules using spectroscopic techniques such as fluorescence and sub nanosecond fluorescence lifetime systems.
2. To characterize the possible photodegradation products using GC/mass spectrometry.

2. Experimental

2.1 Chemicals

Spectroscopic grade acetonitrile purchased from Aldrich Company was used as received. No emission was detected when solvent is excited between (280 –500 nm). Solid samples of benzimidazole, carbendazime and thiabendazole were a gift by a local pesticides production company MOBEDCO. Excitation spectra were used for determining the purity of the fluorescent compounds.

2.2 Instrumentation

Rayonet Photochemical reactor model RPR- 100 manufactured by The Southern New England Ultraviolet Company was used for the photolysis. The unit contains a set of 15 lamps emitting at a maximum wavelength of 300nm. A cooling fan is used to prevent thermal effects on the sample.

Shimadzu Model 1600 was used to record the UV/VIS spectra operating at a medium speed of scanning. All fluorescence measurements were preformed using a home-assembled fluorometer. The system is a modified version of the system mentioned in a previous work (Yousef Y. *et al*, 2007). A block diagram for its different components is shown in figure 1. It is

similar to the previous system except for the air cooled detector model ICCD. The ICCD consists of 512x376 photodide elements with higher sensitivity in the UV region. Fluorescence lifetime measurements were performed using time correlated single photon counting system (TCSPC) from Edinburgh Instruments model- Lifespec-II. Pico-second light emitting diode (LED) light sources and laser diodes were used as the source of excitation. High resolution monochromator of 30cm focal length was used for emission spectral lines separation. The photodetector was a multichannel plate (MCP) with a fast rise time of several picosecends. Special software supplied by Edinburgh Instruments was used for manipulating the decay curves to extract the fluorescence lifetimes.
GC-MS measurements were performed by using a GC model 7100 coupled to VG-7070E E/B sector field mass spectrometer. The system control and acquisition data station, originally supplied with the instrument, was upgraded with new hardware and PC software from MSS Company. The software includes a mass spectral data library from NIST for automatic comparison of sample spectra with a standard. The following conditions were used: Sample volume 3µl, column 10m capillary 0.5mm i.d and 0.25mm stationary phase film thickness, injector temperature 250^{o}C, initial oven temperature 50^{o}C, waiting time 2min, rate 15^{o}C/min, final temperature 300^{o}C, mass range (20- 400 amu), scan rate 15amu/sec, ionization method EI, ion source temperature 250^{o}C, ionization energy 70eV, trap current 50µA. NIST mass data library was used for the identification of the mass spectral results.

3. Results and discussion

3.1 Characterization of fresh samples

The main component in the mobile phase mixture used for the analysis of pesticides by HPLC analytical technique is acetonitrile. Therefore focusing on the photochemical properties and photostability of these molecules in acetonitrile solvent can have important practical applications. The ground state UV absorption spectra of the three pesticides in acetonitrile solvent are shown in figure-1. Benzimidazole absorption spectrum is characterized by two sharp bands at 272 and 280 nm. Carbendazime exhibits two lower resolution and red shifted bands peaking at around 285 and 290 nm. Thiabendazole shows a broad band at around 302 nm with an unresolved tail shoulder at around 315nm. The bands are mainly attributed to n-π^* and π- π^*. Changes in amplitude and position maxima will be used for monitoring the photodegradation of above molecule. A second important technique used to follow the photodegradation is UV fluorescence. Figure-2 shows the emission spectra of the three molecules in acetonitrile solvent. The continuous increase in the width of the emission band and the peak red shift in going from benzimidazole to thiabendazole is clear. Figures 1&2 are used as fingerprints for the molecules in acetonitrile solvent. Knowing the values of absorption and emission of peaks maxima for these molecules are also important for the sensitive detection of molecules by HPLC using UV absorption and fluorescence detection techniques. A complementary technique to fluorescence is fluorescence lifetime. Figure 3, shows the fluorescence decay lifetimes of the molecules in acetonitrile solvent. The decrease in fluorescence lifetime in going from benzimidazole to thiabendazole is clear. Tway et al, were unable to determine the lifetime of benzimidazole due to instrumental limitations (Tway P. C., and Love L. 1992). In this work we report the fluorescence lifetime in acetonitrile solvent to be 5.4 nsec, ± 0.01 nsec. Carbendazim shows a lifetime of 0.7 nsec while thiabendazole has the

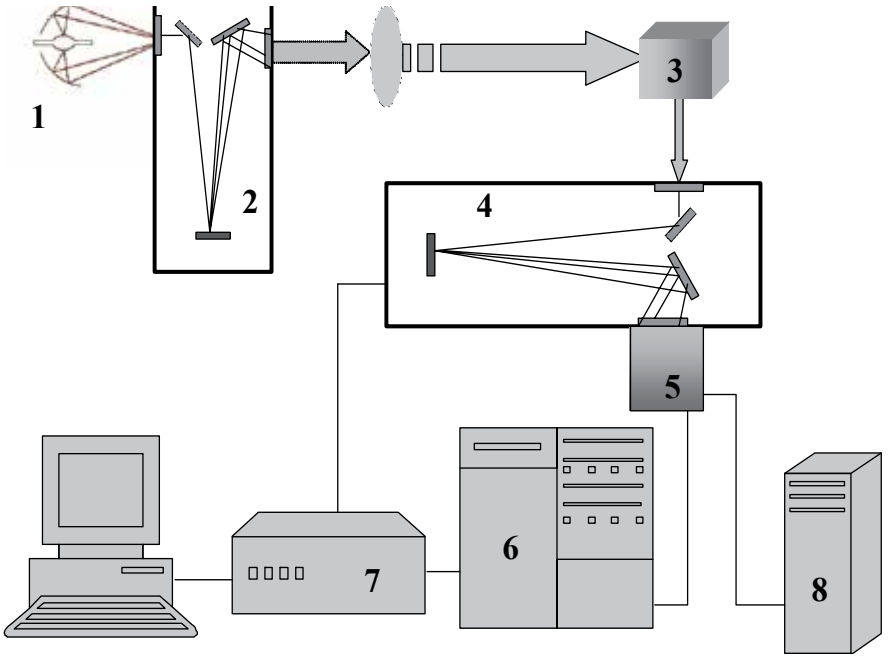


Fig. 1. A block diagram for the home assembled diode array fluorometer. 1- Xe lamp, 2- exc. Monochromator, 3- sample holder, 4- multi-resolution spectrograph, 5- ICCD, 6- detector controller, 7- spectrograph controller, 8- ICCD temperature controller.

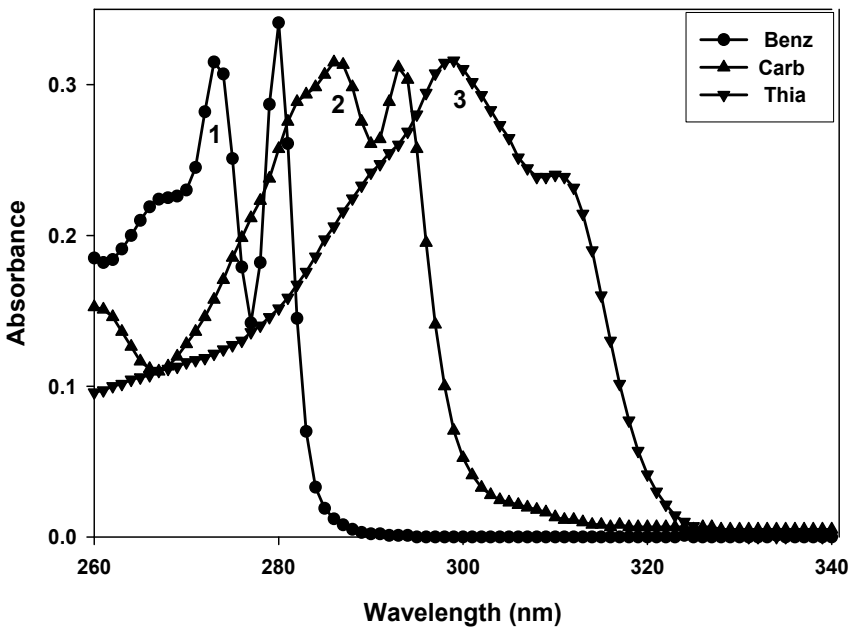


Fig. 2. UV absorption spectra of fresh samples of $1x10^{-4}$M solutions (1) benzimidazole, (2) carbendazime, (3) thiabendazole in acetonitrile solvent.

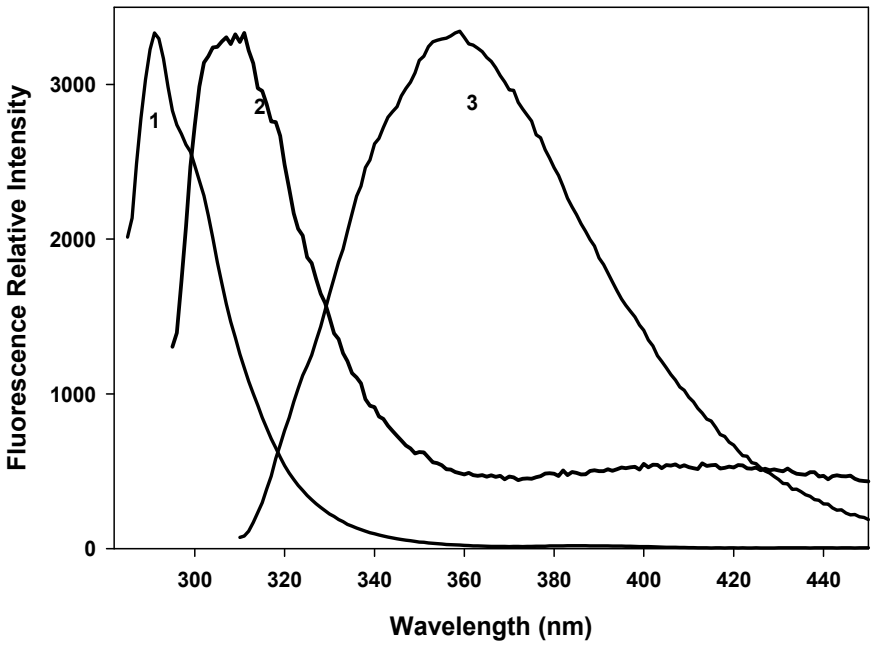


Fig. 3. Fluorescence spectra of fresh samples of $1x10^{-4}$ M solutions (1) benzimidazole, (2) carbendazime, (3) thiabendazole in acetonitrile solvent. Excitation bands correspond to those obtained from the absorption maxima.

shortest lifetime value of 0.3 nsec. Above values correlates well with those obtained by Tway... et al, for carbendazim and thiabedazole pesticides.

Mass spectral analysis of solid and acetonitrile solution samples of the compounds indicated the purity of the compounds. The mass spectrum of each compound was compared with that reported by NIST library data base and found to have exact characteristics. Benzimidazole showed high molecular ion stability. 118 amu was the base peak which corresponds to its molar mass. Carbendazim and thiabendazole showed base peak with a mass lower than the molecular ion peak with values of 191 and 201 amu respectively.

3.2 Photolysis of benzimidazole

Clear changes in the spectra of benzimidazole when UV photolyzed for different periods of time. Figure 4, shows the absorption spectra of fresh and photolyzed samples of benzimidazole in acetonirile solvent. A continuous decrease in the amplitude of the bands at 272 and 280nm accompanied by the appearance of a new broad band between 290 and 400 nm. The appearance of the new band indicates the formation of a new photoproduct. Absorption and fluorescence curve analysis of figures 4and 5, yielded a zero order chemical kinetics for the photodegradation process. Fluorescence lifetime analysis indicated the formation of a photoproduct having a fluorescence lifetime of 1.7 nsec. The decay curve for benzimidazole is of single exponential character where the photolyzed decy curves are of double exponential, as is shown in figure 6. Fluorescence lifetime data for a fresh sample solution of this compound in acetonitrile yielded a value of 1.7 nsec.

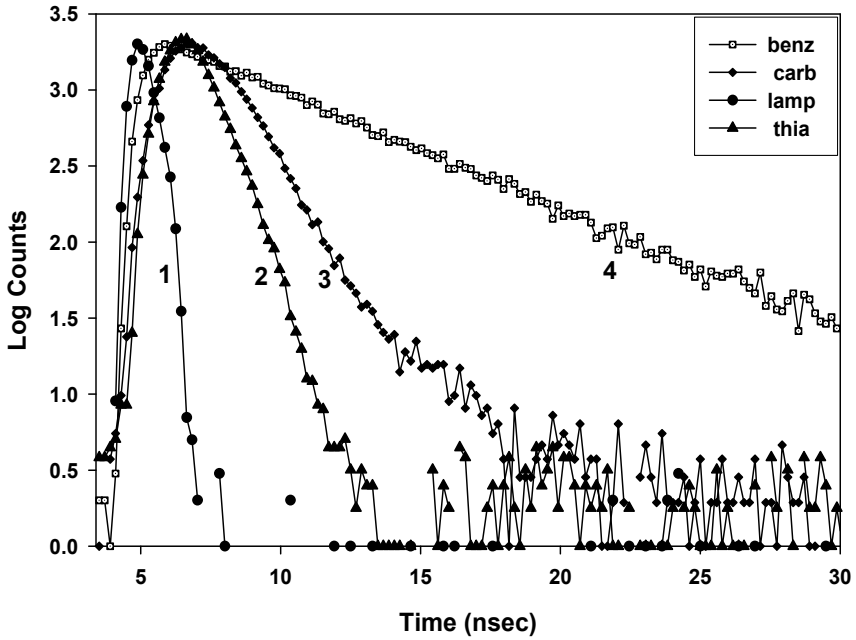


Fig. 4. Decay time curves for a fresh samples of ≈10^{-4} M solutions, (1) lamp pulse decay, (2) thiabendazole, (3) carbendazime, (4) benzimidazole in acetonitrile solvent. Excitation and fluorescence bands correspond to those obtained from the absorption and emission maxima.

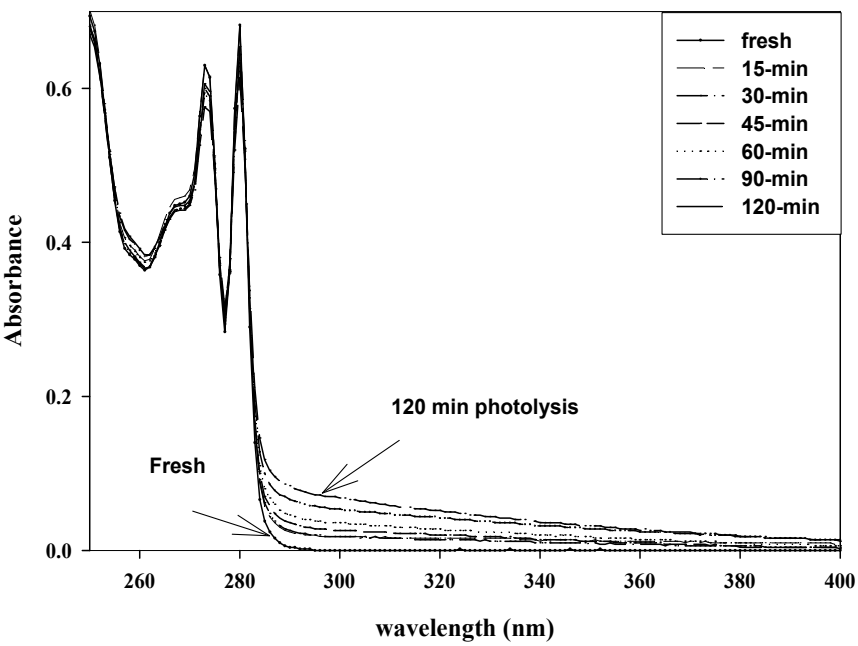


Fig. 5. UV absorption spectra of fresh and photolyzed samples of 1x10^{-4}M solutions of benzimidazole in acetonitrile solvent.

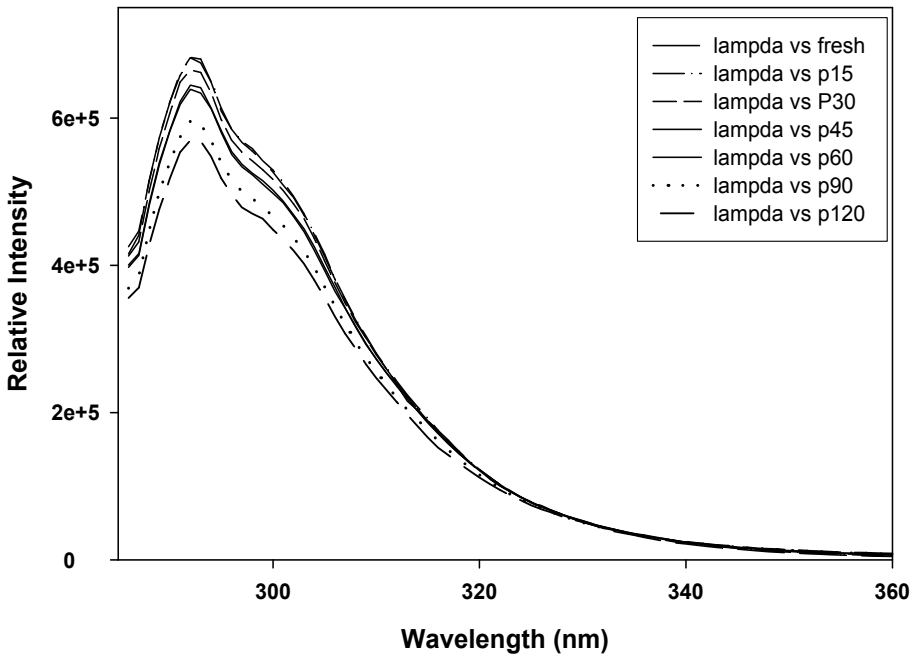


Fig. 6. Fluorescence spectra of fresh and photolyzed samples of $1x10^{-4}$M solutions of benzimidazole in acetonitrile solvent excitated at 280 nm.

GC/MS results reviled the possibility of 2-amino benzimidazole as a possible photoproduct. Mass spectrum was compared with the reported data in the NIST data base. Benzimidazole dimmer was also observed and confirmed by the presence of a molecular ion with an M/z value of 234 amu.

3.3 Photolysis of carbendazime

Photolysis of 2-Benzimidazolecarbamic acid methyl ester (carbendazim) is shown in figures 7, 8 and 9. Photolysis of a fresh sample solution of carbendazim in acetonitrile for 5 minutes caused a large decrease in the absorption maxima specially the band at 290 nm. Further photolysis showed slower changes as is clearly indicated in figure 8. A new and broad band with a maximum around 330 nm is also shown in the figure which indicates the formation of a new photoproduct absorbing in this wavelength range. An isospestic point at around 300 nm is clearly shown in the UV spectra. Normalized fluorescence spectra of fresh and photolized samples of carbendazim solutions are shown in figure 9. The drastic increase in the fluorescence intensity in the range 330 to 400 nm indicates the formation of a photoproduct with a fluorescence maximum at 350 nm. Analysis of the absorption and fluorescence data in figures 8 and 9 reviled a second order kinetics. In comparing the spectra in figures 5and 6 with those in figures 8 and 9 we can conclude that carbendazime have much lower UV photostability than benzimidazole.

Fluorescence decay time results in figure 10, shows decay curves of double exponential nature for photolyzed carbendazim. One exponential corresponds to the parent molecule with a value of about 0.7 nsec, while the second exponential of about 5 nesec indicates the formation of 2-cyanomethyl benzimidazole. Further photolysis showed the degradation of

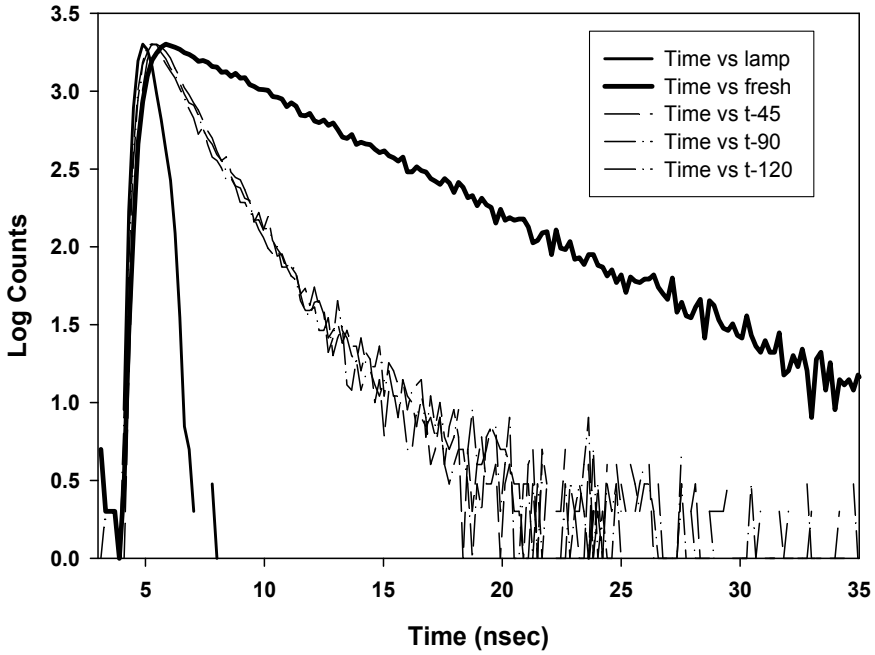


Fig. 7. Fluorescence decay time of fresh and photolyzed samples of $1x10^{-4}$M solutions of benzimidazole in acetonitrile solvent excitated at 280 nm.

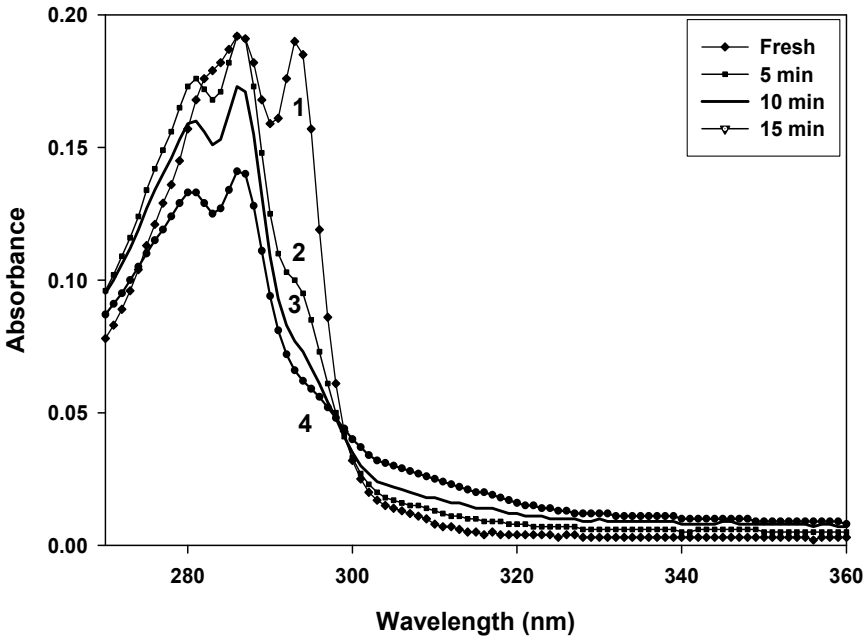


Fig. 8. UV absorption spectra of fresh and photolyzed samples of $0.5x10^{-4}$M solutions of carbendazim in acetonitrile solvent, (1) fresh, (2) 5 min, (3) 10 min, (4) 15 min photolysis periods.

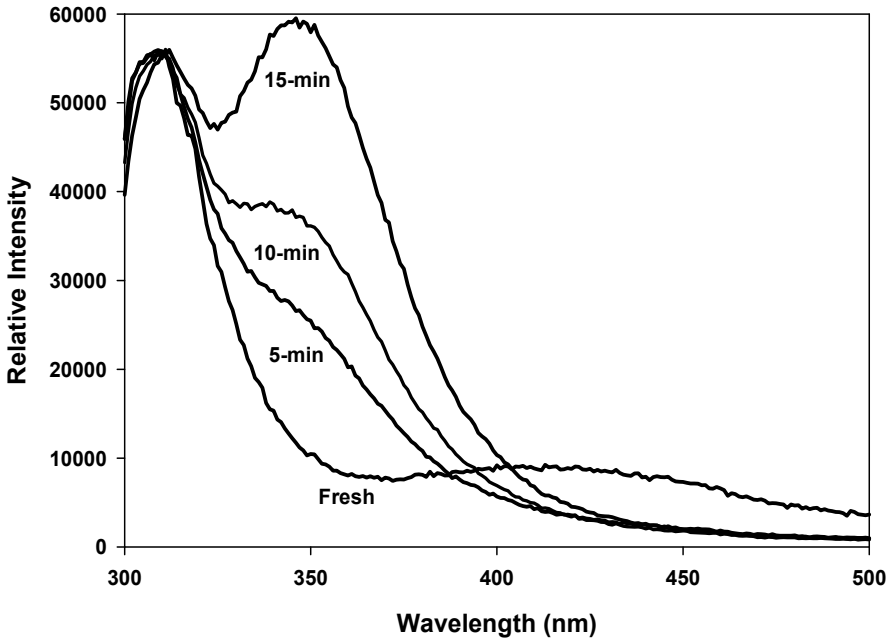


Fig. 9. Normalized fluorescence spectra of fresh and photolyzed samples of $0.5x10^{-4}$ M solutions of carbendazim in acetonitrile solvent excited at 300 nm.

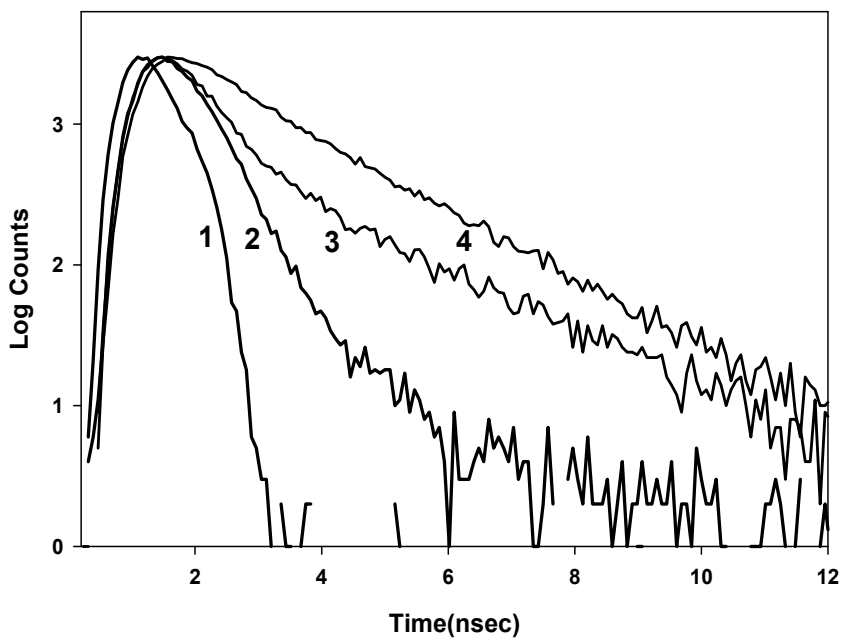


Fig. 10. Fluorescence decay time of fresh and photolyzed samples of $0.5x10^{-4}$ M solutions of carbendazim in acetonitrile solvent excited at 300 nm. (1) lamp, (2) Fresh, (3) 10 min, (4) 20 min photolysis time.

the primary potoproduct to those obtained by benzimidaole with a lifetime of about 1.7 nsec. Fluorescence lifetime technique proved to be a powerful system for monitoring photodegradation process. GC/MS analysis supported the above results obtained by the presence of a molecular ion with am M/z of 157amu during the first periods of photolysis.

3.4 Photolysis of thiabendazole

Benzimidazole 2- (4-thiazolyl) commonly named thiabendazole was irradiated for different periods of time. Photodegradation was monitored by UV absorption, fluorescence, and lifetime spectroscopic techniques. Thiabendazole shoed high sensitivity to UV radiation as is clearly appears in figures 11, 12 and 13. Figure 11 shows the absorption spectra for fresh and photolyzed sample solutions in acetonitrle. The band at 295nm almost disappeared within the first 5 minutes of irradiation. The new band between 300 and 350 nm characterize the new photoproduct. An isospestic point around 300 nm is clearly shown in the figure. Analysis for the data in figure 11 confirmed that photodegradation follows 2nd order kinetics. Emission spectra for fresh and photolyzed samples of the molecule for different irradiation periods. Continuous decrease in fluorescence intensity at around 360 nm accompanied by the formation of a new and increasing band at around 450 nm can noticed. An isospestic point at about 420 nm is shown. Data analysis indicated that photodegradation follows 2nd order kinetics. Fluorescence lifetime data in figure 13 can be used to follow the photodegradation at the end of each irradiation period. Fresh sample gave a short lifetime of 0.2 nsec. Photolysis resulted in the formation of a photoproduct with fluorescence lifetime of 5 nsec corresponding to the formation of benzimidazole as a photoproduct. GC/MS spectral analysis showed the appearance of a mass at 118 amu which is the mass of benzimidazole.

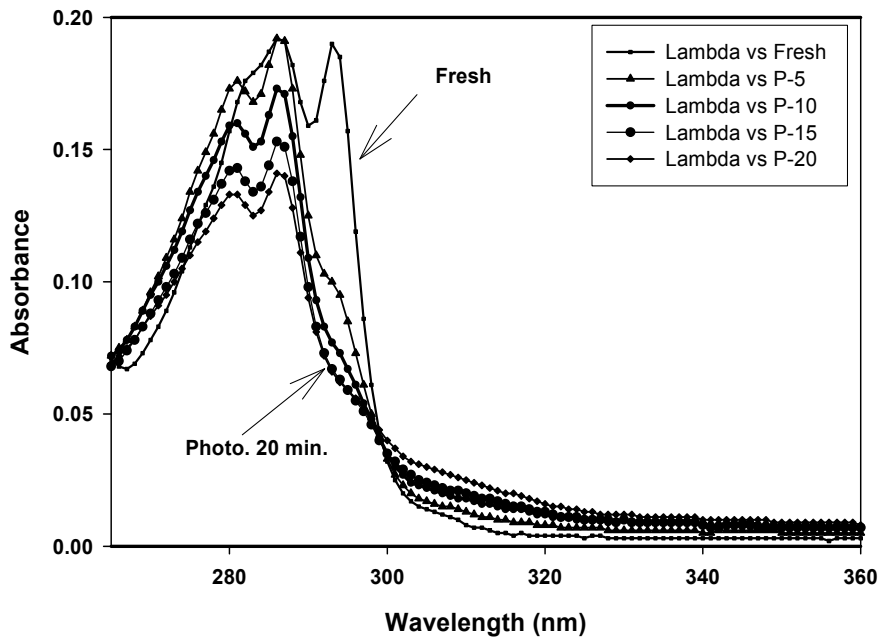


Fig. 11. UV absorption spectra of fresh and photolyzed samples of 1.5x10^{-4}M solutions of thiabendazole in acetonitrile solvent.

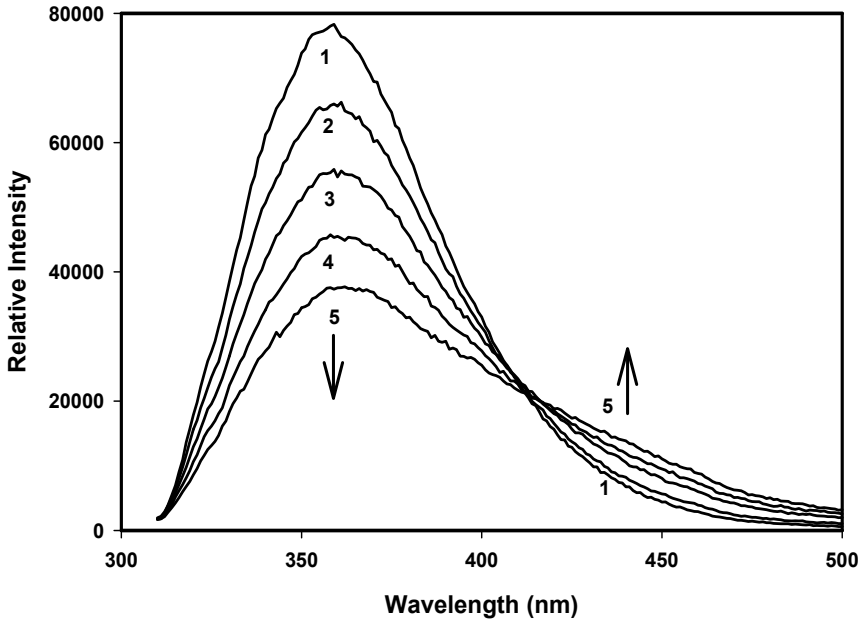


Fig. 12. Fluorescence spectra of fresh and photolyzed samples of $1.5x10^{-4}$M solutions of thiabendazole in acetonitrile solvent excited at 300 nm. (1) fresh, 2 5 min, (3) 10 min, (4) 15 min, (5) 20 min photolysis periods

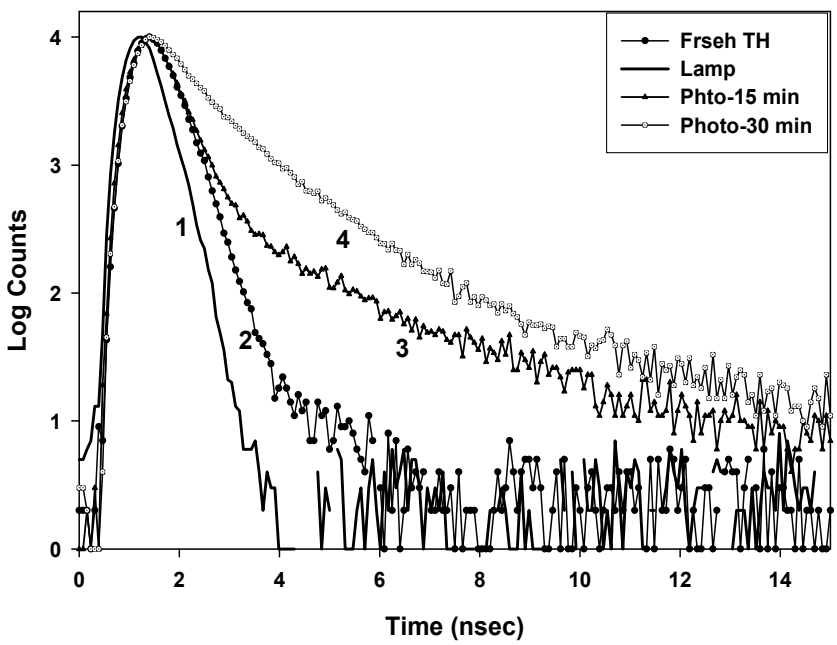


Fig. 13. Fluoresence decay time of fresh and photolyzed samples of $1.5x10^{-4}$M solutions of thiabendazole in acetonitrile solvent excited at 300 nm. (1) lamp, (2) Fresh, (3) 10 min, (4) 20 min photolysis time.

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4

Pesticides Empty Containers (EPC) in the Area of Ouagadougou: Actors, Risks and Prospects of Secure Management

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1. Introduction

The use of the pesticides knew since the middle of last century a considerable expansion in the developed countries but also in those of the Third World for the treatments improvement of the cultures of export and subsistence (Ramade, 2008). According to International Fertilizer Development Center (IFDC)(2004) Burkina Faso consumes chemical inputs more and more. Indeed, the concern of increasing the agricultural outputs leads the producers to the generalized use of the pesticides for the control of the parasitic attacks on the cultures and the weeding of the Also, the pesticides according to their chemical formulation have other uses in particular in maintenance of the gardens, conservation of the foodstuffs, the cleaning of swimming pools etc. However, this practice is not without detrimental consequence on the biophysics environment and populations (FAO, 1996; Traoré & Toé, 2008; Assogba-Komlan and al, 2007). The residues of the pesticides can be found in the cow's milk, in fish, in the ground and cashew nut (Ramesh & Vijayalakshmi, 2002). One of the important aspects in the process of dissemination of the pesticides and their residues in the ecosystems are empty pesticide containers (EPC). This aspect has a very little attention in our country, yet also a diagnosis of this activity reveals a number of risks on the environment and human populations are exposed.

Indeed, some studies have shown that empty containers including vials are commonly used by farmers for repackaging food (IFDC, 2004). According to several authors (Whitford and al. 2006; Nesheim & Fishel, 2005; Bliefert & Perraud, 2008), empty containers of pesticides are toxic and hazardous waste and must be collected as all other waste and eliminated in healthy and rational environment.

It appears that the management of empty pesticide containers must constitute a concern for governments but also for all actors of the development. The contamination of environmental matrices is related to the ability to rationally manage these packages. It is, therefore, appropriate to characterize these actors, the modes of management of empty containers, the ecological evaluation of the risks and to seek opportunities to manage safely these empty packages. It is the objective through this study in the area of Ouagadougou.

2. Methodological approach

2.1. Presentation of the study area

2.1.1 Location of study area

Ouagadougou (Map 1) is the capital of the province of Kadiogo which is located in the heart of Burkina Faso. Its area is 2826.28 km² . Ouagadougou also located in the Sudano-Sahelian area.

Map 1. Location of the area study

2.1.2 Biophysical setting

Climate: Ouagadougou, the capital of the Province of Kadiogo , is characterized by a tropical climate with two main seasons: the rainy season and the dry season. The first one (from May to October) is marked by the moist winds of the monsoon. The water depth rarely exceeds 700 mm per year. The month of August is the wettest. The second one, the dry season, is longer (from October to May) and is dominated by the Harmattan winds. Regarding temperatures, they vary between 22 ° and 35 ° minimum of maxima.

Soils: they are essentially tropical ferruginous laterite type and clay resting on a large mass of granite cracked. There are four classes of soil which are raw mineral soils or lithosols, the poorly evolved brown surface and greyish-brown or pale in depth, the soil leached ferruginous tropical and the soils leached tropical ferruginous brown-modal pale brown near the surface and brown brilliant in depth.

Vegetation: The canopy is the most dominant shrub savannah dotted with clear large trees and an herbaceous layer. At the level of the alluvial terraces and along the main drainage,

there is rock vegetation. This vegetation consists mainly of medium-sized trees (Shea, locust, and baobab) shrubs, especially thorny grass, part of which is widely used in the manufacture of straw (roof boxes, attics or sheds, etc.). This vegetation is sparse due to its intense exploitation for domestic needs, craft and construction. Along seasonal rivers developed savanna woodland.
Relief: it is low peneplain type (300 to 400 meters) from the sea level and characterized by plateaus emerged in places where battleships mounds or ridges armored dismantled and often convex shape (plate) ; axes that constitute the drainage Massili (a branch of Nakambé or White Volta) and its many offshoots; glazes battleships inserted between the plates and the relatively high drainage axes.).

2.2 Selection of actors

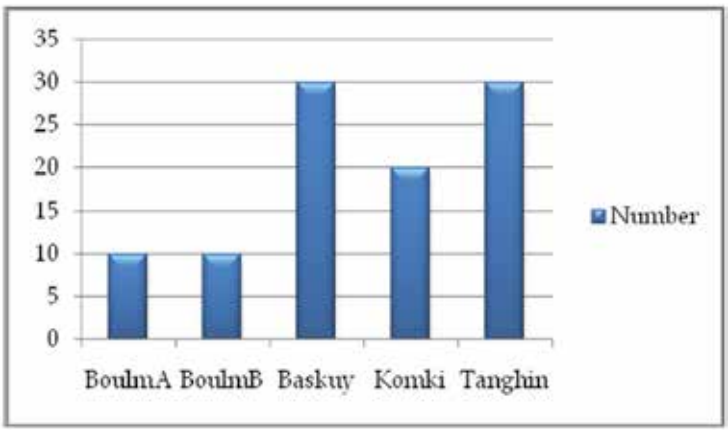
For data collection, a sample of actors was identified by taking into account all the links in the chain of pesticide management. This was:

i. Manufacturing companies and distribution of pesticides
ii. Traders and shopkeepers and sellers of pesticides (Table 1)
iii. Farmers (vegetables, rice, floriculture) using pesticides within a radius of 45 km from Ouagadougou (Figure 1)

For each type of actors a questionnaire was applied and the questions were related to supplies, the types of molecules sold, the management mode of empty packaging, the perception of risks, the prospects for secure management.

Zone	Market 1 (Sankariaré, Ouidi)	Market 2 (Baskuy)	Market 3 (Théâtre Populaire)	Peripheral zone (45km from Ouagadougou)	TOTAL
Number	**07**	**10**	**11**	**17**	**45**

Table 1. Distributors surveyed by zone



BoulmA: Gardeners of Boulmiougou zone A; sector 17
BoulmB : Gardeners of Boulmiougou zone B; sector 17
Baskuy : Gardeners of sector 19
Komki : Komki-ipala

Graph 1. Farmers surveyed per site

2.3 Parameters evaluation

In order to appreciate the level of ecological risk, a grid was elaborated. It gives the knowledge standard of the different actors (traders; distributors; farmers) about the risk linked to the management of the Empty Pesticides Containers (EPC).

Actors	Level of appreciation		
	satisfying	insufficient	very insufficient
-Distributors -Gardeners -Rice producers -Floriculters	- To protect itself with the Individual Protection Equipments (IPE) - To rinse the EPC - To apply flushing waters to the culture - To perforate and store far from the dwellings - To hide in an adequate pit - To burn in an adapted incinerator	- To protect the Individual Protection Equipments (IPE) - To burn in a vat or a pit	- To burn in the free air - To give up in the refuse vats - To throw in the absorbing wells or any pit.

Table 2. Matrix of the actor's knowledge assessment

3. Dynamic EPC of actors

3.1 Distributors

3.1.1 General characteristics

The findings on the profile of the distributors are recorded in the graph 2. They reveal that the distribution of the pesticides is done through wholesalers and retailers who have more or less shops. About 72% of the investigated distributors are organized either associations or cooperatives.

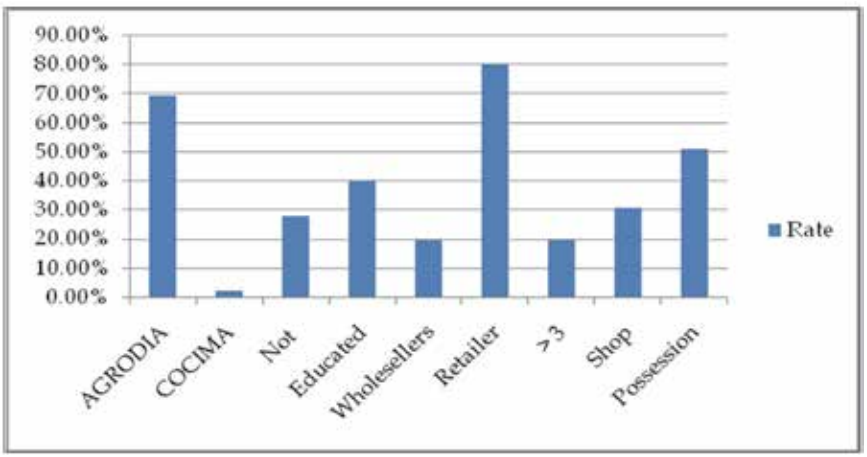
This organizational structure will help, in one hand, to have a hierarchy in the responsibilities and in the other hand, to organize very well the branch (department) of the integrated management of the EPC.

Few distributors were provided with schooling. It means that they have not even the primary school level (only 40% have utterly secondary school level). The diversity of the education standard will allow choosing well some approaches for a better comprehension of the possible formations that they have to receive for the development of their professional skills.

The findings on the size and the state of the warehouse (Wholesalers or retailers, number of the employee superior to 3; shops well structured in its different branches) allow to target very well the proportion of the shop which would possibly do the collect of the EPC and to transport them toward a possible centre of decontamination.

Moreover, 51.12% of distributors admit they have a professional approval. Basing our argument on the *decree, 98/481 about the consents to operators of the commerce, the industry and handcraft department (1998*) all distributors should possess consent in order to prove their

ability to advise the pesticides buyers about the agricultural good practices and especially about the risk linked to the empty containers.

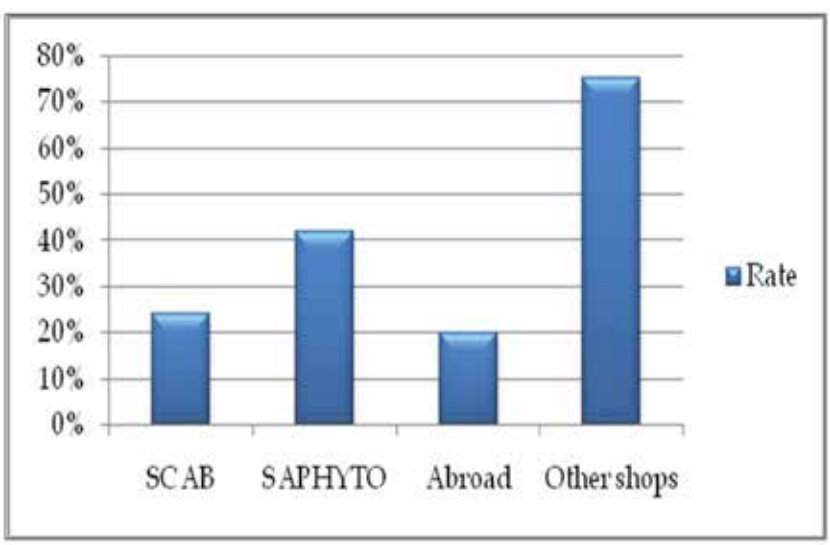


AGRODIA (Pesticides salesmen Association**)** = member of AGRODIA; **COCIMA** (Pesticides salesmen cooperative**)**= member of COCIM;, Non**=** Not adhering to an association or cooperative;. Whole seller**s** = who sells with retailers and OPA (Organization of Agricultural professionals); Shop= Structured different positions (administrative, inventory management, sales).; >3=Shops that employ more than 3 workers.

Graph 2. Profile of surveyed distributors

3.1.2 Pesticides supply channels

Generally speaking, the farmers' pesticides supplying sources are the manufacturing firm (SCAB, SAPHYTO); abroad industries and other shops of Ouagadougou. The graph 3 summarizes the contributive share of each supply channel.

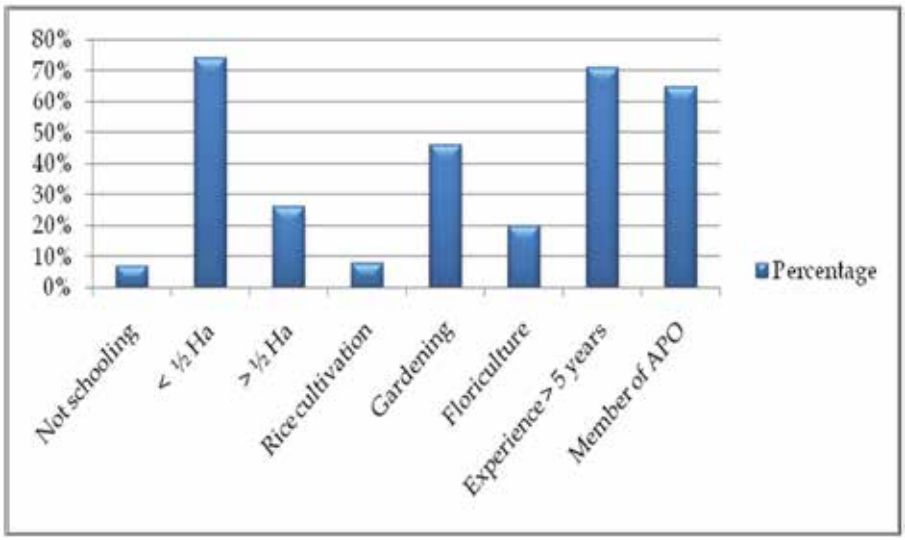


Graph 3. Supply channels of pesticides

The majority of wholesalers (Talata Agrochen ; King Agro; EKF; Ets Bonou etc.) stock up with the great pesticides societies of Burkina (SAPHYTO, SCAB) or abroad (Côte d'Ivoire; Ghana; Togo; Benin etc.).
Most of the wholesalers which stock up with Saphyto do so for the quality of the products and confidence reasons. The findings show that the channels are very dynamic. Indeed, the inputs are the subject of a particular attention as the bargain and the prices become autonomous. So, this entails a multiplication of the actors, the products; the supply sources and of the distribution channels what is more and more difficult to apprehend. The knowledge of these findings identifies the main suppliers and should make easier the relation supplier-distributor for the research of the collective solutions to manage the EPC.

3.2 Producers

The identified agricultural producers were rice-producing gardeners; flower-gardeners. Their profile is given in graph 4.



Graph 4. Famers surveyed profile

The graph 4 shows a very low schooling standard (7%) which is directly linked with the finding about the knowledge of the methods to lessen the risks. The knowledge about the schooling and the experience levels will allow choosing well the approaches to train those producers about the risks of the EPC in order to develop their competences and to professionalize the agricultural department. We must note that the need of a regular and adapted training is justified.
The finding got about the cultivable areas (few gardeners have more than ½ ha) gives information on the repackaging to offer a packing adapted to small areas.
It will allow fixing some other ambitions to extend the study to the regions where the use will be significant in order to see many details about the risks linked to EPC management method.
It emerges from this graph that the sample did not concern the cotton producers for time and logistic reasons. Moreover, we must note that during the observations of the containers on the agricultural areas, many pesticides containers authorized for cotton and not

authorized for market gardening were seen. These pesticides are essentially composed of ROCKY 500 EC and ROCKY 386 EC both having as active raw material endosulfan. They are also composed of FANGA 500 EC having as active raw material profenofos. That shows that the gardeners use some inappropriate and non-authorized pesticides in gardening. These risked practices are already bought to the fore by ODEPAB (2006). At this stage also, the training need, regulations enforcement and the adapted pesticides supplying for gardening are justified.

Therefore, 65% of the investigated producers are members of an APO. It represents an advantage for a good organization of the EPC management. Indeed, it will be easier to intervene with a producer who is member of an APO than with one who is not member. It will be also a trump to organize well the training sessions about the risks caused by the pesticides, generally speaking, and especially about the empty packing.

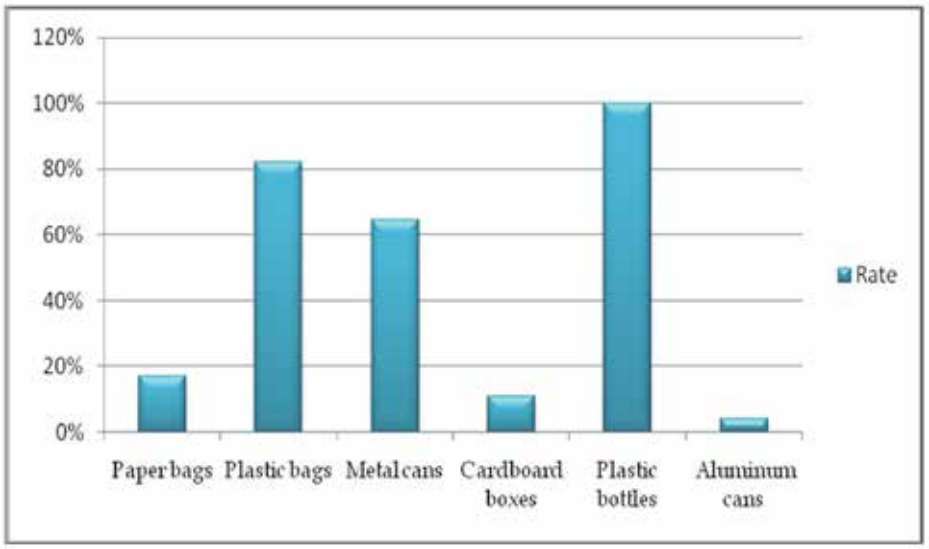
4. EPC management by the actors

4.1 To the suppliers

4.1.1 The types of packing which are in shops

The main wrappings in supplier's shops are plastic cans of which type is the one in PHD (polyethylene high density) in relation to PET (Polyethylene terphtalates); the plastic bags (of which the aluminum covers the internal part) and metallic boxes (graph 5). The proportions of these types of wrapping seen in the shops are liable to be found anarchically in the water and the soil after use.

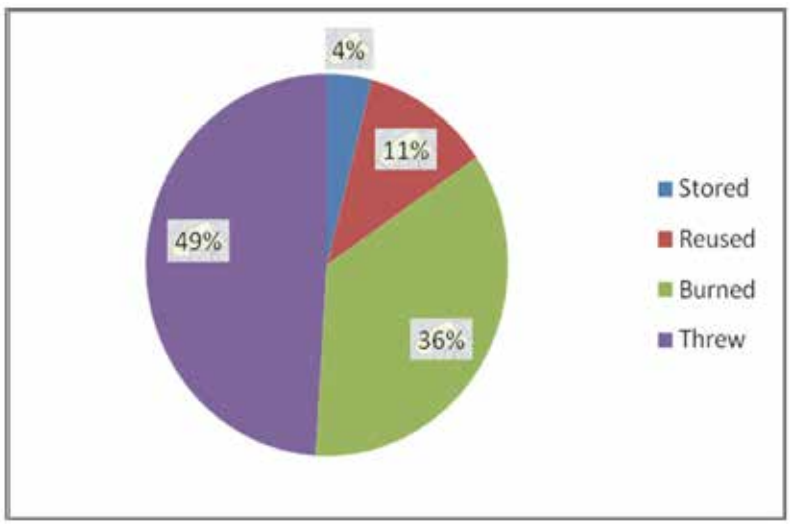
In one hand, the findings will allow to have an idea about the existing EPC and then to think about how to manage the wrapping once empty. We do not use the same way to manage a metallic box and PEHD can or PET; in the other hand, the findings will allow having a credibility of information about repackaging. We can say to exaggerate that the type of wrapping which in shops are the same ones than those seen to the customers.



Graph 5. Packaging type found in the shops

4.1.2 Suppliers way of managing the EPC

To respond to the expectations, many suppliers choose the repackaging of the pesticides. It emerges from the findings of the investigation that 52% of the suppliers repack pesticides in their own workshops. The repackaging is not very explicit in the national decree but it is against the international law of behavior about the supply and the use of pesticides. Nevertheless, the FAO code should be applied in that case. The Article 10 of that code clearly specifies that "the governments must take the measures conforming to the necessary regulation to forbid the repackaging etc."
Talking about the way to manage the EPC after repackaging, it is shown in graph 6.



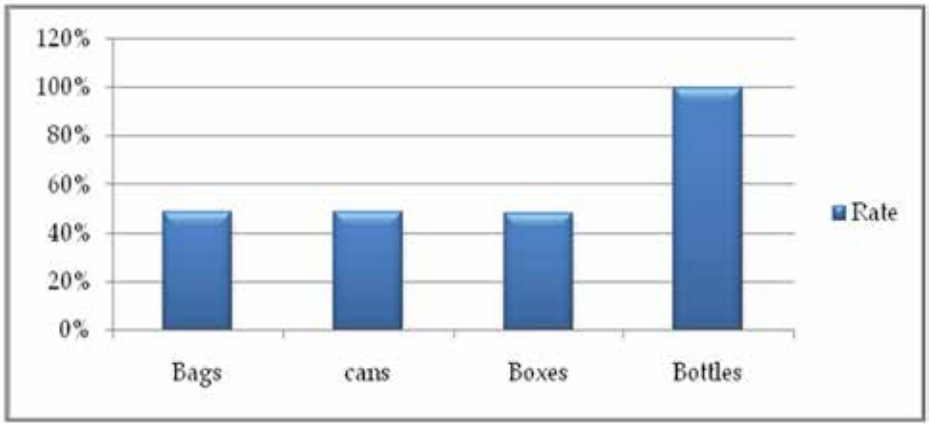
Graph 6. EPC management by distributors

It emerges from this graph that the abandoning seems to be a common practice. That behavior is practiced by about 49% of suppliers. The supplier's category (36%) has chosen to burn EPC.
-*Mode of repackaging the liquid products*: Generally speaking, the suppliers have some wrappings which initially were in one liter cans; half a liter cans and in cans of 200 ml to 250ml. The process of repackaging ends up at products contained in 10-100ml little bottles sold at 250 or 300frcs.This shows that the official wrappings are not adapted in cost and in volume to the producer. These two elements oblige both producer and supplier to repackage unlawfully to have benefit. This practice is adopted by all the sales assistants of the outlying areas so be it 38% of the 45 investigated suppliers.
-*Mode of repackaging powder products*: Initially, the suppliers possess powder products in ½ Kg or 1kg plastic bags packing and in 25-50kg bags. At the end of the repackaging, some 10-100g bags are sold from 50 to 500frcs. 80% of the retailers do this practice.

4.2 To the producers

4.2.1 Type of packing used by producers

Almost half of the interviewed producers use pesticide in plastic cans of 500ml to 1000ml(49%) and pesticide packing in metallic boxes of 200-250ml(48%),in addition to the little glass bottles of 10-100ml(100%) as showed in the graph 7.

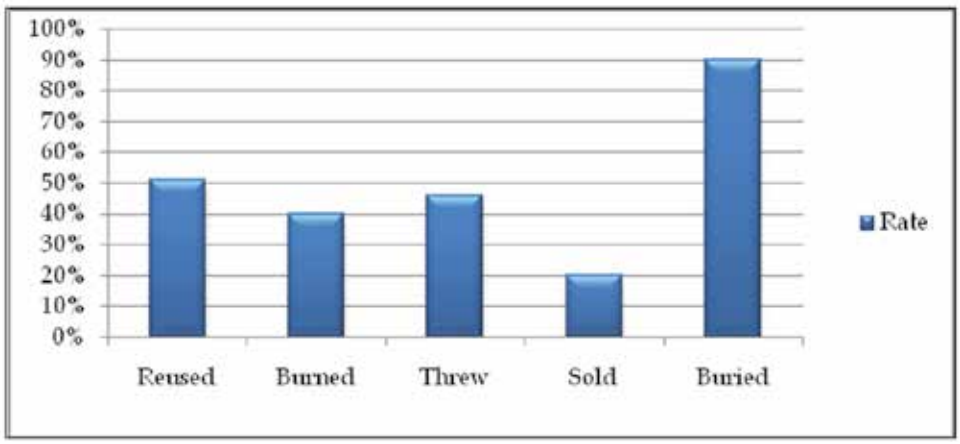


Graph 7. Packaging type found among famers

We noted an absence of glass bottles at the level of packing pesticide sold by suppliers. We can conclude that these bottles come from the practices of repackaging by non-investigated structures (suppliers or agricultural producers).

4.2.2 Mode of managing EPC by agricultural producers

The mode of the managing EPC by the producers follows five options according the graph 8.



Graph 8. Mode of managing EPC by the agricultural producers

We can read the graph 8 as follows:
51% of the agricultural producers say they use again wrapping for food purposes.
40% of producers opt systemically or occasionally for burning at the open air. This practice is totally different of a burning done in an appropriate system (such as a farm incinerator).
46% of the producers prefer to throw or to abandon EPC in the living areas or in lost empty wells.
20% of the producers resell systematically or occasionally the empty packing in order to favor repackaging.
90% of the producers partially bury the packing in the fields or in living areas.

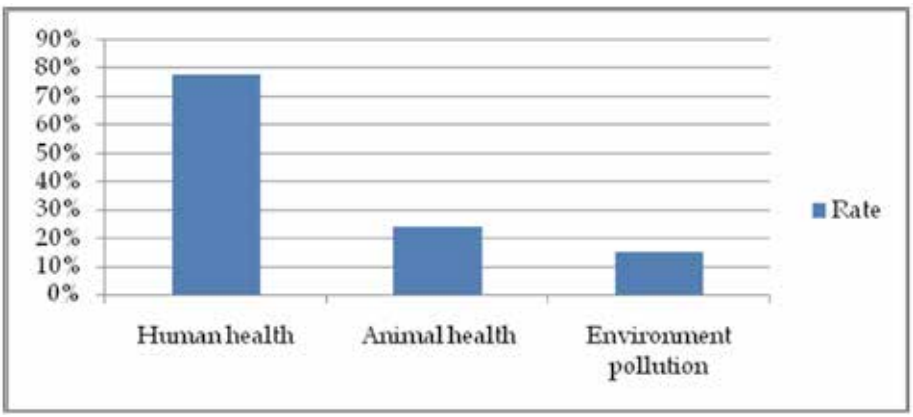
These pieces of information show that an ecological and safety management to give a sense of security is not reassured. The different modes of managing for a better elimination (re-use; burning; open air throwing; sales; anarchic burying) are non-rational methods. These mentioned forms of management are already emphasized (FAO, 1996; IFDC, 2004) especially in cotton agro systems and generally speaking in agricultural areas.

5. Knowledge of the environmental problems

5.1 How the suppliers perceive environmental problems

5.1.1 Knowledge of the risks sources

It is fundamental to perceive the risks caused by an irrational management of the EPC on the environmental matrices in order to establish some processes to reduce the risks. The standard knowledge is shown in graph 9.



Graph 9. Risk perception by distributors

A large majority of distributors (78%of the cases) know that EPC can have some risks on human health. The major part of this group gives a precision about a feasible poisoning by breathing or orally. But few distributors think about skin poisoning.

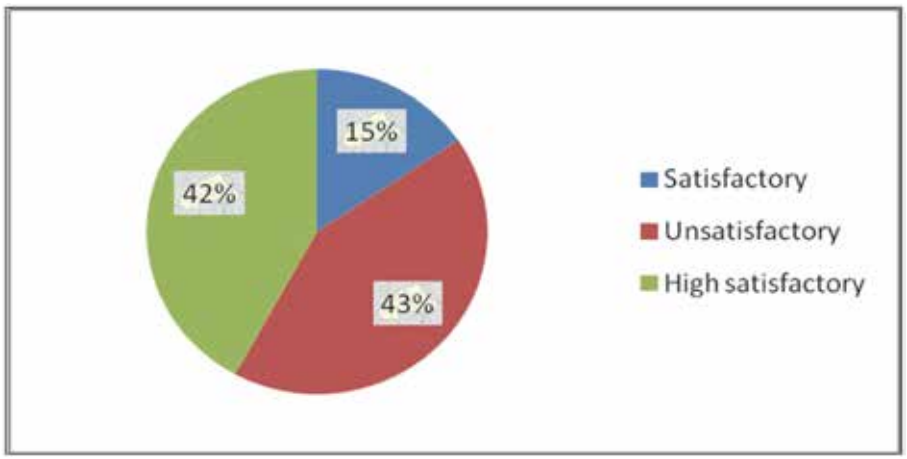
As they are not directly confronted to the constraints of applying pesticides in the fields, the distributors do not think a lot about the risks of pesticides on animals(named by only 45%of the distributors)and on environment, meaning water, the soil and the air.

5.1.2 Knowledge standard about the means to mitigate the effects of pesticides

The distributors' knowledge standard about the means to mitigate the effects of pesticides on environment is shown in graph 10.

We remark a low rate of distributors who have satisfactory knowledge about EPC management (only 15% of them).This finding confronts the one of those who say they have a professional consent (51,12%).The consent is not attributed only if the professional competences are judged satisfying. Besides, the knowledge of the effects mitigation measures would be one of the conditions to have an approval.

This finding will allow seeing that there is a need of vocational training and information regularly and adapted to the distributors. The conditions of granting the consent to those who want to sell pesticides should be reviewed.

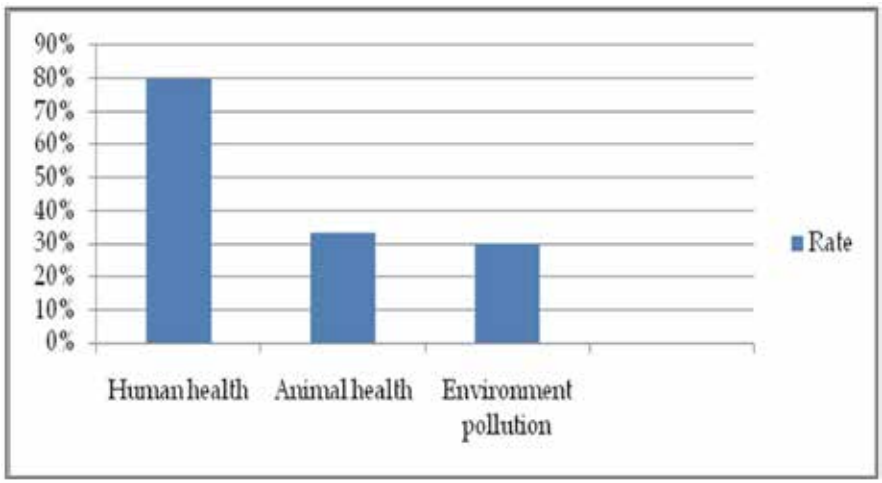


Graph 10. Distributors finding on mitigation

5.2 Producers knowledge about environmental problems

5.2.1 Knowledge of the risks sources

A great number of producers are aware of the risks on human health. Contrary to distributors (see graph8) producers perceive well the risks on animal health and about environmental pollution. This situation is shown in graph 11.

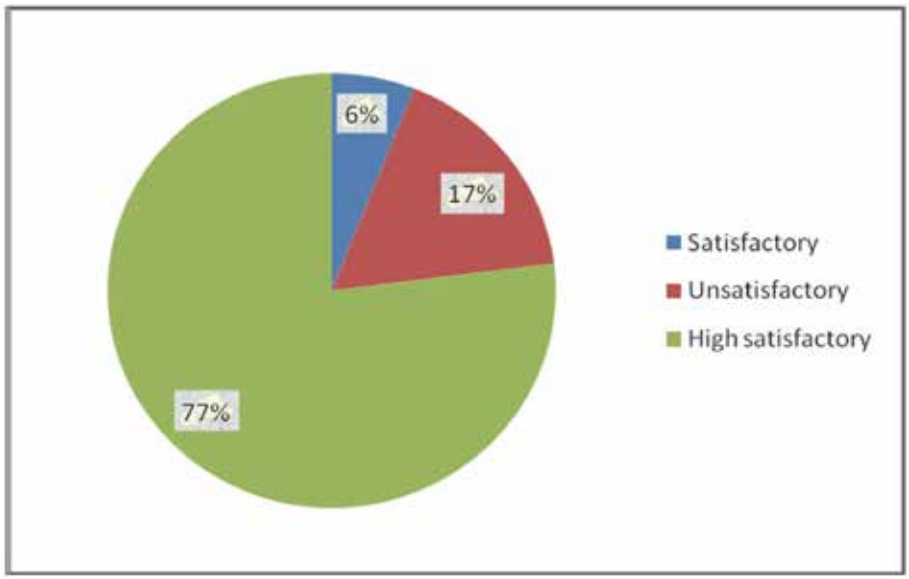


Graph 11. Consciousness of the risk generated by EPC

5.2.2 Knowledge about the measures to mitigate the effects

The measures to mitigate the effects used by the producers were evaluated. Graph 12 sums up the level of knowledge of these measures known by the agricultural producers.

The findings show that 77% of the producers have a very insufficient knowledge about the measures to lessen the effects. This percentage must attract the persons in charge of the APO attention so that they can invest themselves in the vocational training and in the sensibilisation of their members.



Graph 12. Mitigation knowledge by agricultural producers

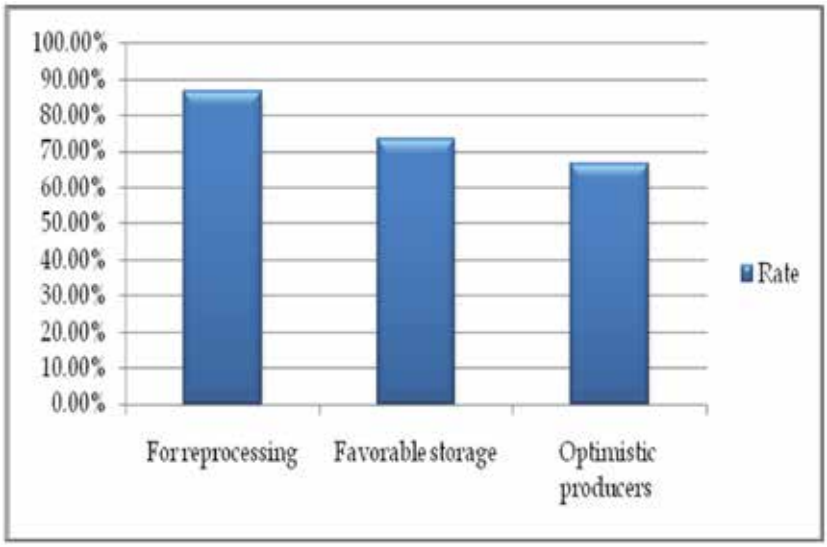
In this connection, the findings are the same as those obtained by the Benin Organization For The Promotion Of the Organic Agriculture (ODEPAB)(2006).Likewise, the salvation for reprocessing of the empty wrapping is and will remain the difficult work as long as it will be done with some skills and practices behavior. Indeed, in actual practice, our rural populations have a very limited view about the incurred risks through the use of the wrapping. No sensibilisation can convince them that there is a risk of using this or that poisonous wrapping. But it is when they are in front of a concrete case of poisoning that they are convincing now. A peulh point of view is that the plastic cans of 1-20l can be replaced with cucurbitaceous beaker and cancel some constraints due to transport, foods preservation such as milk and butter. Travelers and farmers value 4-20l plastic cans covered by basin fiber as the best means of cold water conservator than goatskin used in the past.

From the point of view of the real knowledge of our populations (rural population as well as urban one) about the used pesticides and their socio-cultural practices, the presence of these non-recovered wrapping constitutes effectively a threat for human health especially when a low rate of salvation of reprocessing comes to add to some factors such as the mode of management and the storing sites which are essentially located in cattle farm and agricultural areas (IFDC, 2004).

6. Prospects of the EPC management

6.1 Distributors' opinion

The risks due to the EPC and the measures to mitigate the effects mentioned by the distributors and the agricultural producers direct the reflexion to find the means and ways to manage the EPC. Some means were prospected for the distributors (graph 12).It is to institute a system of EPC reprocessing, of collect and of storage. This system aim is also to motivate all the members.



Graph 12. Distributors strategy for EPC managing

Moreover, the distributors propose some responsibilities to the different actors in other to insure a good management of the EPC. The propositions to attribute the responsibilities to all the actors are mentioned as follows:

The government: to install a unit to value the EPC in Ouagadougou; to install some compartments not far from each distributors to collect the EPC; to entrust to the concerned actors the management(production firms; suppliers; distributors; agricultural producers) with their respective different professional organizations(AGRODIA;FEPAB)

AGRODIA: to train and to sensitize the distributors about the risks caused by the EPC; to seek financers to insure well the EPC salvaging for reprocessing.

The distributors: to accept to store temporarily the EPC and to motivate the agricultural producers who are costumers to give back the EPC after using; to advise the agricultural producers about the risks caused by the EPC and about the measures to mitigate the effects.

The producers: to accept to store temporarily the EPC and to give back the EPC to the pesticides distributors units.

6.2 Producers' opinion

Convinced of the necessity to manage rationally the EPC, the agricultural producers have made the following propositions:

All of them are for a collection department of the EPC: 100% of the cases.

Some propose to review the pesticides packing manufacturing: 33% of the cases.

80% are optimistic about a good working of the EPC integrated management department.

All the producers think there is a lack of training about the EPC management. So, they think the responsibility returns to the responsibles of their Unions, Cooperatives and Organizations and especially to the FEPAB.

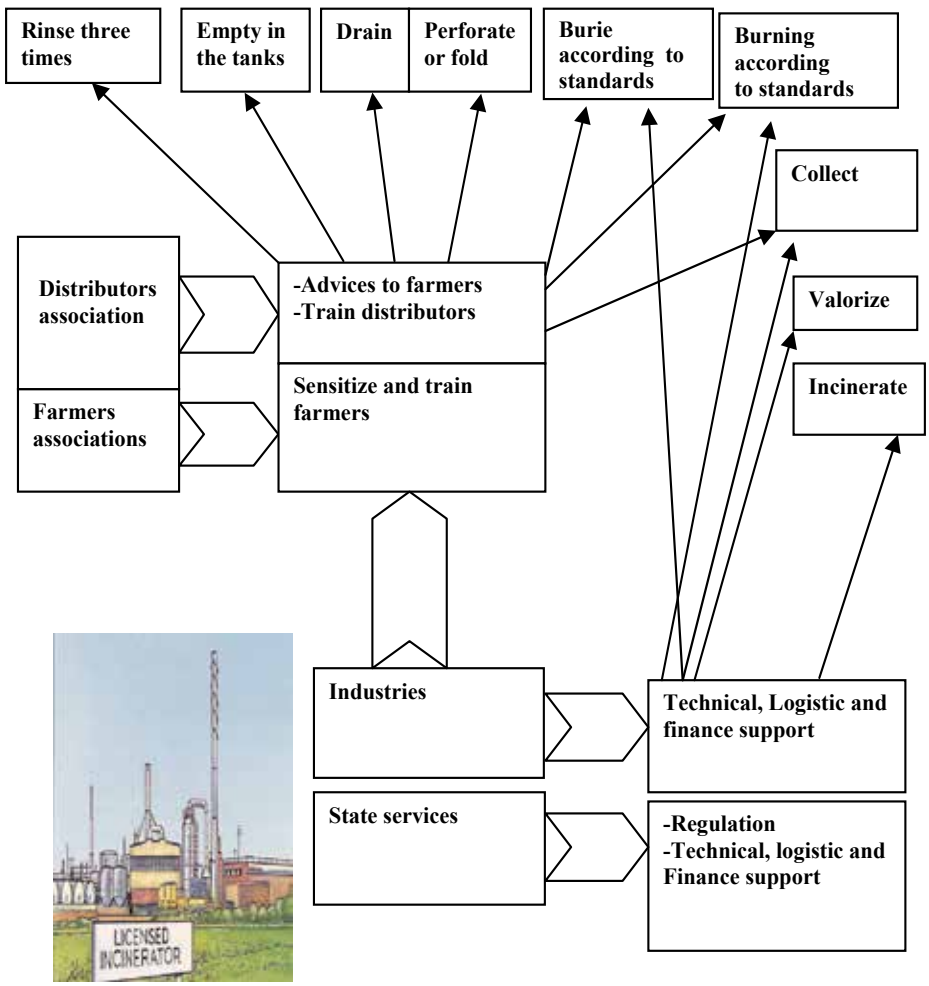
For the producers, AGRODIA should encourage its members by a motivation to collect the EPC.

For the majority of the interviewed producers, the government must demand the EPC to the distributors.

According to 100% of the interviewed producers, the pesticides salesmen must take back the EPC by giving a little discount on the sold products or by giving a free gift of product to some who give back the EPC.

6.3 The ways for a lastable management of the EPC

Given the issues raised concerning the management of the EPC, the diversity of actors involved in the pesticides domain, we urge all of those who are concerned to show a considerable interest about this topic. We propose the following scheme 1 that helps to position the various stakeholders, both technical and organizational domains. This scheme responding to a goal of our study also aims to bring together all the actors involved directly or indirectly in the management of the EPC; pushed for an exchange through workshops, seminars, conferences etc.; to install an official organization for the management of the EPC.



Scheme 1. Actors organization for EPC secure and sustainable management

7. Conclusion

This study has allowed emphasizing the actors, the risks and the prospects for the EPC management to give a sense of security. The actors are varied and come down to the firms, the distributors who are salesmen at the same time and the agricultural producers(market gardeners, paddy field farmers, flower gardeners).We will retain that in Ouagadougou area, the main sources of pesticides supplying are the distributors (SCAB, SAPHYTO), abroad firms, the shops in the markets. The dreaded risks are related to population poisoning, the ecological matrices contamination (water, soil). They are closely linked to the EPC management and elimination practices. These practices come down to the distributors and the agricultural producers as follows:

- The pesticides over packing stored with foods;
- The use of some new types of packing for the pesticides reprocessing;
- The storage of the empty packing on the shop counter after the reprocessing;
- The re-use of the cans by the salesmen for alimentary purposes (especially for drinking water);
- The anarchic abandoning of the empty packing behind the residences walls at the edge of the roads;
- The anarchic abandoning of the empty packing in the fields or in the houses by the producers;
- The burying of the empty packing in the water wells (wells and dams which are not lay out) by the producers and the distributors;
- The storage of empty packing at the children reaches under the fields' trees by the producers;
- The re-use for alimentary purposes, the throw in the living areas or in some lost wells;
- The open air burning.

Moreover, some factors such as the schooling level of the actors, a good knowledge about the risks and the disponibility of the actors constitute some ways to be prospected for the EPC elimination. All these mentioned factors are determinant for the elaboration and the carrying out of a rational EPC management project. Considering these relative concerns, it is urgent to plan an intensive campaign to inform-sensitize the administrative responsible, the distributors, the farmers and the whole populations about the dangers and the risks linked to the use of the pesticides and the EPC.

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5

Electrochemical Detoxification of Obsolete Pesticides Stocks

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1. Introduction

The disposal of pesticides can cause serious problems due to the chemical nature of the active ingredients in pesticide formulation and due to the large quantities of the unwanted products. These products undergo physical and chemical alterations either due to extended storage, beyond the recommended expiry date, or storage under improper conditions (high humidity and temperature). Commercial synthetic chemical pesticides are utilized in all countries and as a consequence reach even remote regions. Wherever pesticides are used, unusable or unwanted pesticides and empty pesticides containers have to be properly and safely managed. In many countries, large quantities of pesticides have been accumulated since they have lost their desirable characteristics. Although these products are not suitable for use, they still contain toxic compounds. Many surplus pesticides, still within their expiry limits, may become useless, when their future use is prohibited due to toxicological or environmental concerns. Food and Agricultural Organization of the United Nations (FAO) estimated that between 400.000 and 500.000 tones of obsolete pesticides are stocked in developing countries (FAO, 2001).

The biological degradation of pesticides is generally difficult due to their high content in toxic matter (Felsot, 1996; Zaleska & Hupka, 1999). An ideal treatment method for pesticide surplus should be non-selective, should achieve rapid and complete mineralization, and should be suitable for small-scale wastes (Krueger et al., 1984; Bourke et al., 1991). Today the main disposal method of obsolete pesticide stock is incineration, an impractical and expensive procedure. High-temperature incineration in dedicated hazardous waste incinerators is the currently recommended method for obsolete pesticide treatment. However, sophisticated incinerators do not exist in developing countries (FAO, 2000).

Various innovative technologies have been proposed for the disposal of obsolete pesticides. These technologies include photocatalytic oxidation (Pignatello & Sun, 1995; Chen et al., 1998), ultrasonic radiation (Kotronarou et al.,1997). The major disadvantage of these technologies is that they are designed for decontamination of aqueous solutions with very low active ingredient content, rather than highly concentrated obsolete pesticide stocks.

Electrochemical methods have been successfully applied in the purification of domestic sewage (Della Monica et al., 1980), olive oil wastewaters (Israilides et al., 1996), textile wastes (Vlyssides et al., 1999) etc.

The electrochemical reactions, which take place during the electrolysis of a chloride-containing solution (brine solution), are complicated and not entirely known. The electrochemical oxidation of aqueous solutions which contain organic matter, by the use of Ti/Pt anode, proceeds in two steps (Comninellis et al., 1992). The first step is the anodic discharge of the water, forming hydroxyl radicals which are absorbed on the active sites of the electrode surface M [ ].

$$H_2O + M[\,] \rightarrow M\left[OH^-\right] + H^+ + e \quad (1)$$

After this the absorbed hydroxyl radical oxidizes the organic matter.

$$R + M\left[OH^-\right] \rightarrow RO + M[\,] + H^+ + e^- \quad (2)$$

where RO represents the oxidized organic matter which can be produced continuously by the hydroxyl radicals which are also continuously formed, since the anodic discharge of the water goes on. The radicals $OH^{\cdot}$, O. and $HClO^{\cdot}$ have a very short life-time due to their high oxidation potential and are either decomposed to other oxidants (such as Cl_2, O_2, ClO_2, O_3, and H_2O_2) or oxidize organic compounds (i.e. direct oxidation). The primary (Cl_2, O_2) and secondary (ClO_2, O_3, and H_2O_2) oxidants that are produced from the destruction of radicals have quite a long life-time and are diffused into the area away from the electrode, thus continuing the oxidation process (indirect oxidation). Effective pollutant degradation is based on the direct electrochemical process (that takes place in a closed area around the electrode) because the secondary oxidants are not able to convert totally all the organic species into water and carbon dioxide.

In the last few years, interest in direct and indirect electrochemical oxidations of organic pollutants increased significantly. In the direct anodic oxidation, adsorbed $OH^{\bullet}$ radicals are produced from water oxidation at an anode made from Pt, PbO_2, doped SnO_2 (Comninellis & Pulgarin, 1993; Comninellis, 1994) or boron-doped diamond (BDD) (Weiss et al., 2007; Hachami et al., 2008):

$$H_2O \rightarrow OH^{\cdot}_{ads} + H^+ + e^- \quad (3)$$

This radical is the main mineralizing agent of organics, causing their conversion to CO_2, water and inorganic ions.

Recently, electrooxidation has received great attention due to the use of non-active boron-doped diamond (BDD) thin film electrodes, which possess high O_2-overvoltage that favors the production of great quantity of reactive BDD ($OH^{\cdot}$) with ability to completely mineralize organics, as reported for several aromatics (Marselli et al.,2003; Flox et al., 2009) and carboxylic acids (Weiss et al., 2007). The recent use of BDD thin-film as new anode material has shown that it possesses technologically important characteristics such as an inert surface with low adsorption properties, remarkable corrosion stability and extremely wide potential windows in aqueous medium (Panizza & Cerisola,2005). In comparison with conventional anodes such as Pt, PbO_2, doped PbO_2, doped SnO_2 and

IrO_2, the BDD anode has much greater O_2-overvoltage allowing the generation of greater amount of OH. from reaction (4) or (5) and hence, a quicker oxidation of aromatics and pesticides in acid and neutral media:

$$BDD(H_2O) \rightarrow BDD(HO^{\cdot}) + H^+ + e^- \quad (4)$$

in basic media (pH ≥ 10):

$$BDD(OH^-) \rightarrow BDD(HO^{\cdot}) + e^- \quad (5)$$

In this paper we report on a comparative study on the electrochemical oxidation and the degradation process in supporting electrolytes NaCl of a solution containing high concentration of Bupirimate and methidathion, respectively a systemic fungicide widely and insecticide used in agriculture fields. Two electrodes were selected for this investigation: BDD electrode and a metal oxide anode SnO_2.

2. Materials and methods

2.1 Chemicals

Bupirimate, a substituted pyrimidine (a sulphamate ester of ethirimol, 5-butyl-2-ethylamino-6-methyIpyrimidin-4-yl-dimethylsulphamate), is a local systemic fungicide that is effective in controlling mildew in roses and apples. Bupirimate formulation is commercially available in the NIMROD 25 EC (25% Bupirimate) (Figure 1). It was Purchased from AAKO (Morocco). All chemicals used in the experiments were of analytical pure grade and used without further purification. The concentration of bupirimate in wastewater for these experiments was 230 mg/L with a corresponding COD value of 1440 mgO_2/L.

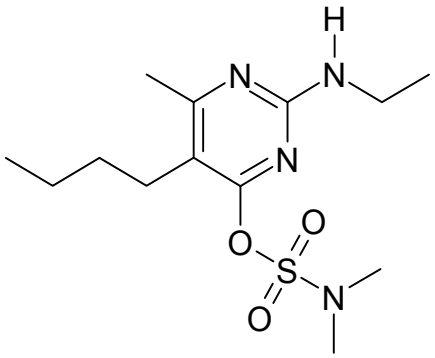

Fig. 1. Chemical structure of bupirimate.

Methidathion [O,O-dimethylS-(5-methoxy-1,3,4-Thiadiazoliny-l-3-methyl) dithiophosphate] (Figure 2) is a widely used organophosphorous insecticide, The commercial formulation Methidaxide (40 % methidation) was purchased from Bayer. The concentration of methidathion in wastewater for these experiments was 1.4 mM with a corresponding COD value of 2400 mgO_2/L. The sodium chloride used was of analytical-reagent grade and was obtained from Aldrich (Spain).

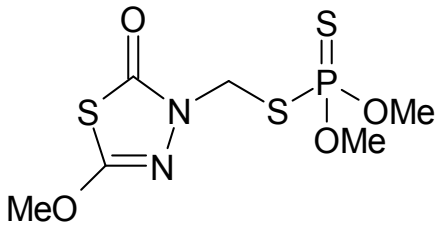

Fig. 2. Structural formula of methidathion

2.2 Electrolytic system

Electrochemical measurements were performed using a computer controlled by Potentiostat/Galvanostat model PGP 201 associated to "Volta-Master1" software. A conventional three electrodes cell (100 cm^3) thermoregulated glass cell was used (Tacussel Standard CEC/TH). The anode was a square plate of BDD electrode or SnO_2 with effective surface area of 1 cm^2, whereas the cathode was a platinum electrode, and the gap between electrodes was 5 mm. A saturated calomel electrode (SCE) was used as a reference.

Galvanostatic electrolysis was carried out with a volume of 75 mL aqueous solution of pesticide during 120 minutes. The range of applied current density was 20 to 60 $mAcm^{-2}$ and samples were taken, at predetermined intervals during the experiment, and submitted for analysis. In all cases, sodium chloride (NaCl) was added to the electrolytic cell at different concentrations.

Comparative degradation of bupirimate was studied by electrolyzing 75 mL of solutions containing 230 mg/L of initial pollutant and 2% NaCl of pH 6.2. In all trials, a constant current density of 60 $mAcm^{-2}$ was applied.

2.3 Analytical procedures

The UV–Vis spectra of bupirimate were recorded in 200–400 nm range using a UV–Vis spectrophotometer (UV-1700 Pharmaspec, Shimadzou) with a spectrometric quartz cell (1 cm path length). The Chemical Oxygen Demand (COD) values were determined by open reflux, a dichromate titration method.

The method used for the extraction of bupirimate was adapted from Charles and Raymond (Charles & Raymond, 1991). For each 2 ml of the sample, 100 mL of acetone was added and the mixture was stirred for 2 hours. The extraction was carried out respectively with 100 ml and 50 ml of acetone. After filtration, the residues in acetone were partitioned with saturated aqueous NaCl (30 mL) and dichloromethane (70 mL) in a separating funnel. The dichloromethane fraction was collected and the separation process with (70 mL) dichloromethane were combined and dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure at 40°C and the residues were dissolved in an acetone-hexane (1:9) mixture (10 mL). Samples were analysed by gas chromatography.

2.4 Gas chromatography analysis

Analysis of the bupirimate pesticide was carried out with a Hewlett–Packard 6890 gas chromatograph equipped with an ECD Detector, on-colum injection port, and HP-5 column (5 % diphenyl copolymer/95 % dimethylpolysiloxane) (25 m × 0.32 mm ID, 0.52 µm film thickness). The temperature programme applied in GC/ECD was as follows: 80 – 250 °C

at 15 °C/min, 80 °C (1.00 min). The injection volume was 1 µl. The temperature of the detector was 300 °C.

3. Results and discussion

3.1 Electrooxidation of bupirimate

3.1.1 Effect of the NaCl concentration

Figures 3-a and 3-b show the effect of the electrolyte concentration on the % COD for both BDD and SnO_2 respectively. As shown in this graph, the % COD increases with the increase of electrooxidation time but decreases with the amount of NaCl in the solution. This indicates that at low concentration of NaCl, the bupirimate removal ratios increased with time. The presence of a low concentration of chloride ions (2 % of NaCl) allows inhibiting the water discharge into oxygen, and promotes hydroxyl, chloride, and oxycloride radicals' formation. The increase of the NaCl concentration (> 3%) could cause a "potentiostatic buffering" by the chlorine redox system and, consequently, a decrease of the anode potential.

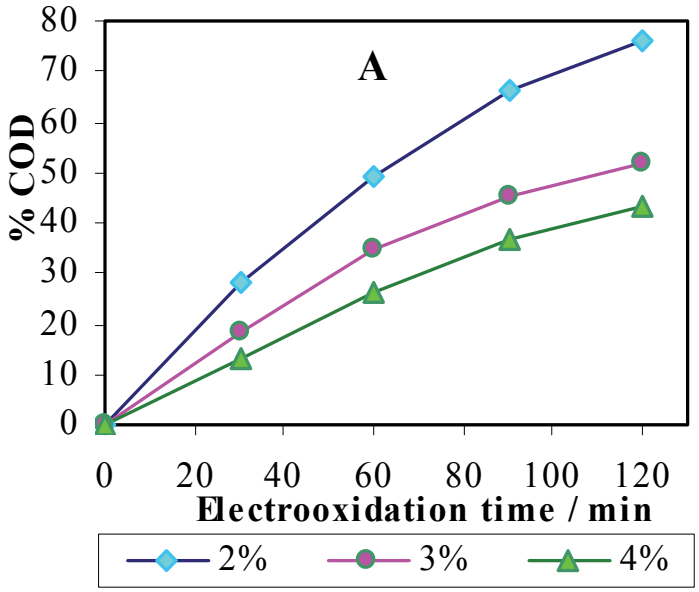

Fig. 3. a. Direct electrooxidation at BDD anode: effect of NaCl concentration on the %COD (230 mg.L^{-1} Bupirimate solution, 120 min, (60 mA.cm^{-2}, pH=6.2, and T=25 °C).

It is also possible that the presence of competitive reactions, in particular oxygen and chloride evolution due to recombination of radicals that becomes bigger with the increasing of NaCl concentration. The balance of all of these phenomena results in an optimum of NaCl concentration, which is 2 % mass of NaCl for the degradation of Bupirimate.

Kinetic studies were carried out to determine the COD reduction efficiency for both electrodes at different concentration of NaCl. For this purpose, the removal rate of COD was assumed to obey a first order kinetic as follows (Laviron ,1972).

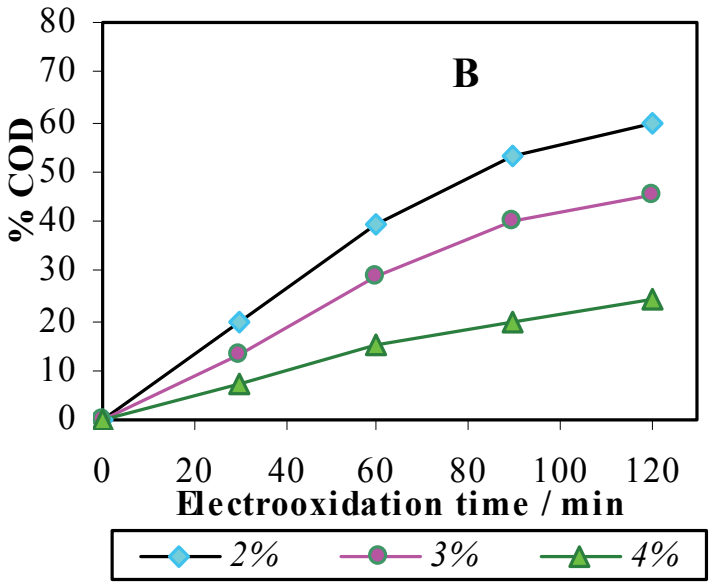

Fig. 3. b. Direct electrooxidation at SnO2 anode: effect of NaCl concentration on the %COD (230 mg.L^{-1} Bupirimate solution, 120 min, (60 mA.cm−2, pH=6.2, and T=25 °C).

$$d[COD]/dt = -K[COD] \quad (6)$$

The values of rate constant for different NaCl concentration are summarized in table 1. The rate constant, K was calculated for all mass of supporting electrolyte. At each mass of NaCl, COD reduction was estimated at different time intervals and using Eq. (4), rate constant K was calculated at each time interval and averaged. The effect of supporting electrolyte on rate constant increased with decreasing concentration of NaCl and the higher reaction rate constant (109x10-4 min^{-1}) was obtained at 2% of NaCl supporting electrolyte. This indicates that the bupirimate molecules were easily attacked by hydroxyl radicals at lower concentration of NaCl. These results are in agreement with previously reported results (Hachami et al., 2010; Hachami et al., 2008).

Electrode	NaCl concentration, %	Rate constant, K (min^{-1})	% COD
BDD	2	109x10^{-4}	74
	3	61x10^{-4}	53
	4	49x10^{-4}	44
SnO_2	2	85x10^{-4}	60
	3	57x10^{-4}	45
	4	35x10^{-4}	24

Table 1. Effect of NaCl concentration on the values of rate constant and the % COD.

3.1.2 Effect of the applied current density

The effect of applied current density on the electrochemical process was reported in several studies (Hachami et al., 2008; Radha et al., 2009). It is an important factor affecting the electrolysis kinetics. Degradation assays of 230 mg/L bupirimate solutions were performed using the BDD and SnO_2 electrodes at different current densities (Figures 4-a, 4-b and Table 2). Overall, COD removal efficiency increased with increasing applied current density. As illustration, when the current is increased from 20 $mAcm^{-2}$ to 60 $mAcm^{-2}$ %COD removal increased from 26 % to 74 % for BDD and from 24 % to 60 % for SnO_2.

Electrode	Current intensity ($mA.cm^{-2}$)	Rate constant, K (min^{-1})	%COD
BDD	20	$109x10^{-4}$	74
	40	$52x10^{-4}$	47
	60	$35x10^{-4}$	26
SnO_2	20	$80x10^{-4}$	60
	40	$75x10^{-4}$	58
	60	$28x10^{-4}$	24

Table 2. Effect of current intensity: values of rate constant and %COD.

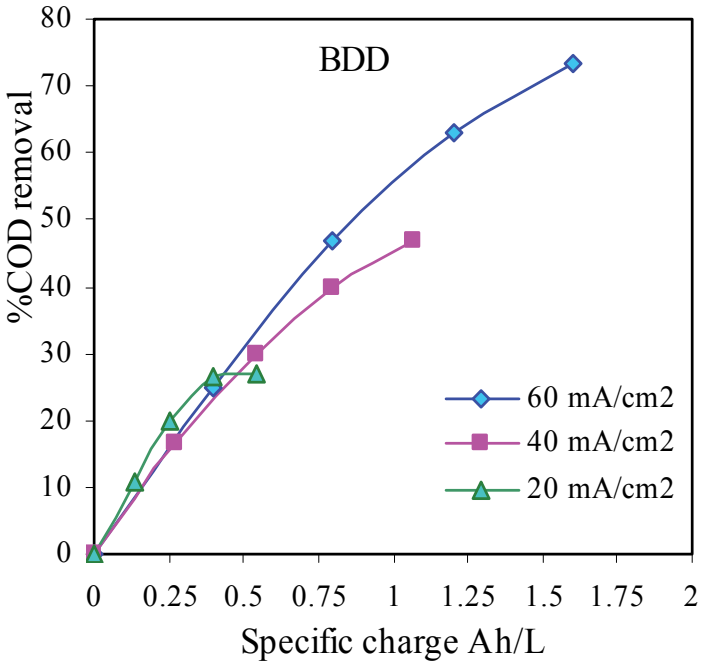

Fig. 4. a. Variation of %COD as a function of the specific charge passed during electrolysis of bupirimate performed with BDD and at several current densities (20, 40 and 60 $mAcm^{-2}$). Initial bupirimate concentration = 230 mg/L, pH = 6.2, T = 25 °C, electrolyte = 2 % NaCl.

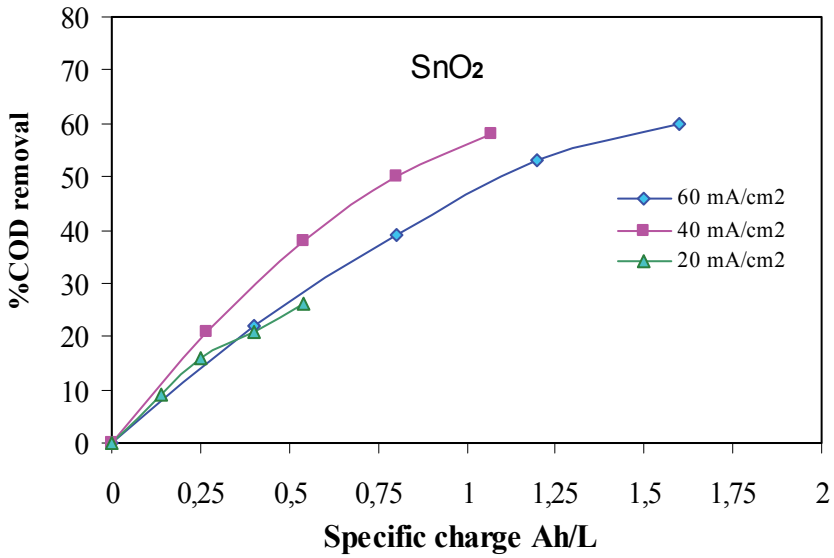

Fig. 4. b. Variation of COD removal as a function of the specific charge passed during electrolysis of bupirimate performed with SnO2 and at several current densities (20, 40 and 60 $mAcm^{-2}$). Initial bupirimate concentration = 230 mg/L, pH = 6.2, T = 25 °C, electrolyte = 2 % NaCl.

3.1.3 Effect of initial concentrations of bupirimate on the degradation efficiency

To investigate the electrooxidation efficiency on high concentration of bupirimate, the experiments of electrochemical degradation of 115, 230 and 345 mg/L bupirimate solutions were carried out with a selecting current density and NaCl concentration. As shown in Figures 5-a and 5-b, complete bupirimate removal can be achieved on 115 mg/L concentration of bupirimate for BDD anode as the electrolysis time was extended. The trends of normalized COD are moderately overlapped and same electrolysis-times are required to achieve the best values of COD abatement for two anodes BDD and SnO2. This indicates that the oxidation rate and process efficiency are directly proportional to organic matter concentration. This outcome is in agreement with the data reported by Panizza and Cerisola (Panizza & Cerisola, 2007, 2008).

The electrolysis time for complete removal of bupirimate was proportional to the concentration of bupirimate. In conclusion, the BDD anode performs well for electrochemical degradation of high concentration of bupirimate solution with appropriate current density and NaCl concentration as supporting electrolyte.

3.1.4 Effect of pH at the BDD anode

The effect of initial pH on the degradation of bupirimate solutions was investigated. The pH of the effluent was adjusted using H_2SO_4 and NaOH (initial pH was 6.2). Experiments were carried out in four different pH (11, 8, 5 and 2.5) at a constant current density of 60 $mAcm^{-2}$ using the boron doped diamond electrode as the anode. The initial concentration of bupirimate was 230 mg/L (Figure 6). The decrease of the pH 11 to 2.5 leads to a decrease of

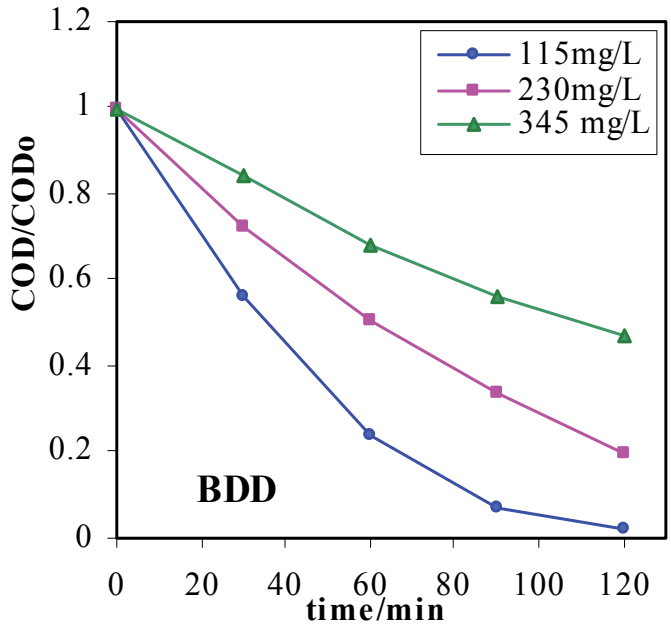

Fig. 5. a. Influence of bupirimate initial concentration on the normalized COD during BDD-anodic oxidation. Operating conditions: electrolyte = 2 % NaCl, current density = 60 $mAcm^{-2}$, T = 25 °C.

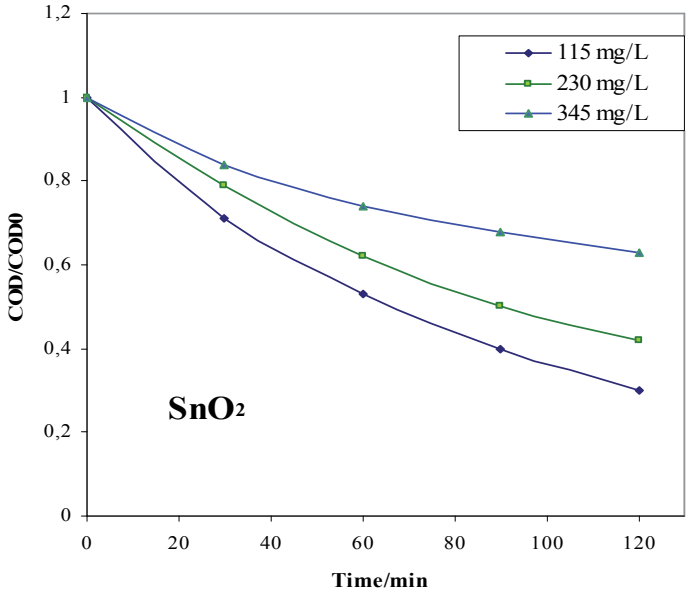

Fig. 5. b. Influence of bupirimate initial concentration on the normalized COD during SnO_2 anodic oxidation of bupirimate. Operating conditions: electrolyte = 2 % NaCl, current density = 60 $mAcm^{-2}$, T=25°C.

the absorbance of the peaks located at 240 nm and 310 nm. At pH=5, the peak located at 310 nm disappeared. This result indicates that the reaction rate is less in basic condition, which indicates that the OH- is unstable in basic condition. Decrease in pH increases the hydroxyl radicals, which favours the rate of oxidation (Yavuz & Savas, 2008).

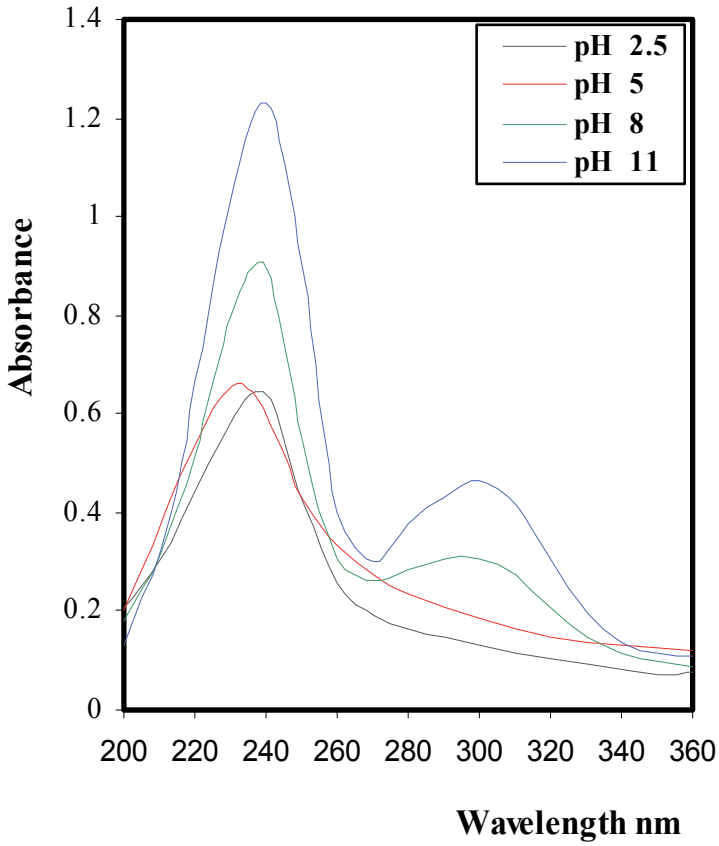

Fig. 6. Effect of initial pH on the variations of UV spectra for 2 h of the electrooxidation assays performed at a BDD anode. Bupirimate initial concentration = 230 mg/L, current density = 60mAcm^{-2}, electrolyte = 2 % NaCl.

3.1.5 Comparative study of electrochemical degradation efficiency on BDD and SnO_2 electrodes

The comparative study of electrochemical degradation of bupirimate was also performed on BDD and SnO_2 electrodes. The absorption spectral changes during galvanostatic electrolysis in aqueous solution are shown in figures 7-a and 7-b. It can be observed that the absorption spectrum of bupirimate is characterized by a band in the UV region with its maxima located at 240 nm and by a band in region located at 310 nm. The absorbance peak at 240 nm can be attributed to the sulfamate, while the absorbance at 310 nm is probably due to the presence of amine group in the pyrimidine pesticide. For BDD electrode, the absorption of the band located at 310 nm decreased sharply with the time and disappeared after 120 min (Figure 7-a). The same observation could be made for SnO_2 electrode but with slower arte.

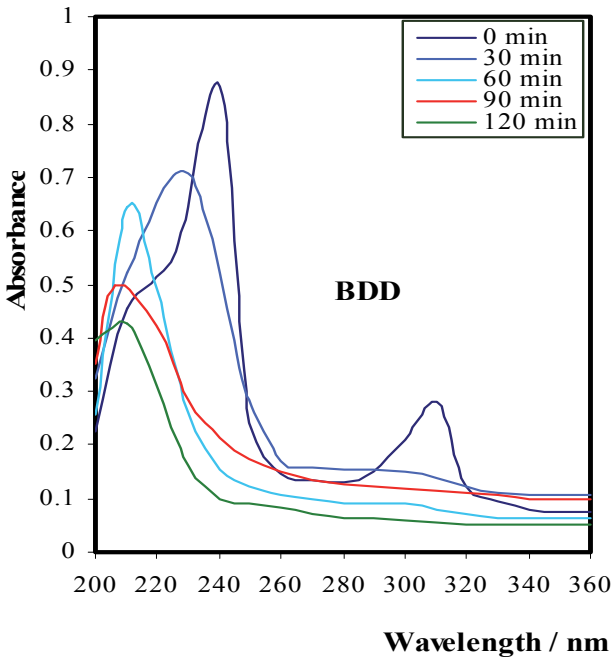

Fig. 7. a. UV spectra for the electrooxidation assays performed at the BDD anode. Bupirimate initial concentration = 230 mg/L, current density = 60 mAcm^{-2}, pH = 6.2, electrolyte = 2 % NaCl.

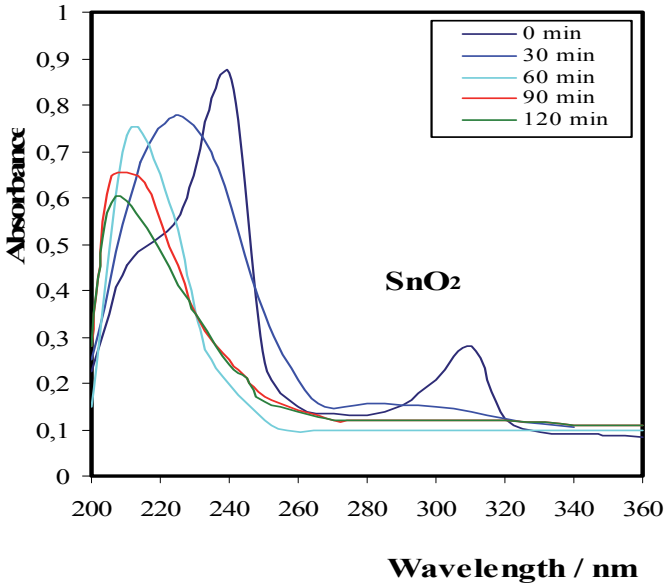

Fig. 7. b. UV spectra for the electrodegradation assays performed at the SnO_2 anode. Bupirimate initial concentration = 230 mg/L, current density = 60 mAcm^{-2}, pH = 6.2, electrolyte = 2 % NaCl.

The concentration of bupirimate was measured using GC and the variations of bupirimate concentration with electrolysis time for the two anodes are shown in figure 8. At the same electrolysis time, the rate of electrodegradation of bupirimate is different for both anodes. The reaction rate is fast on the BDD anode, while the reaction rate is relatively slow on the SnO_2 anode. Table 3 indicates that different electrodes exhibit different performance in the rate of electrochemical degradation of pesticide. These results show that the % of abatement bupirimate found by GC is the same as analyzed by COD.

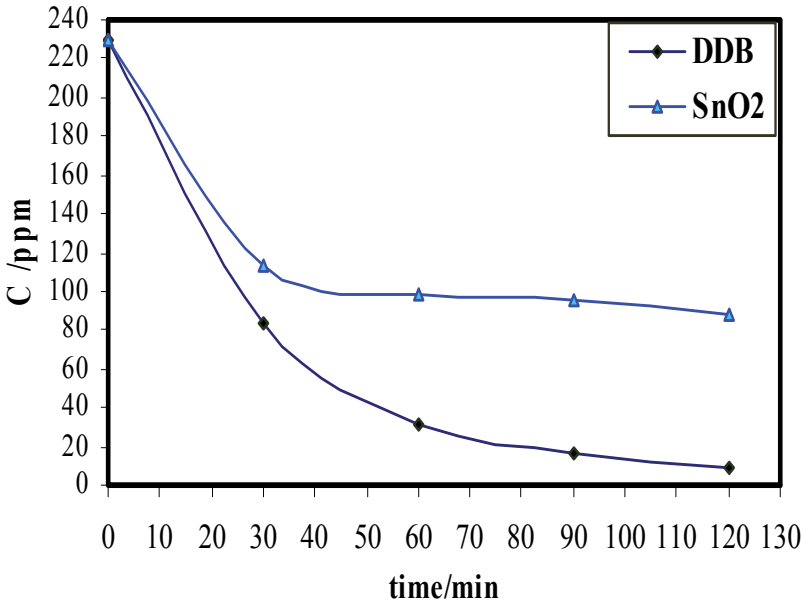

Fig. 8. Electrolysis time dependence of bupirimate concentration for two anodes (BDD, SnO_2). Bupirimate initial concentration = 230 mg/L, current density = 60 $mAcm^{-2}$, pH = 6.2, electrolyte = 2 % NaCl.

Anodes	Rate constant, K (min^{-1})	%COD
BDD	288.10^{-3}	74
SnO_2	80.10^{-3}	59

Table 3. Apparent rate constants of bupirimate removal fitted by a first order model and %COD for BDD and SnO_2 anodes.

3.1.6 Conclusion

The electrochemical degradation of high concentration bupirimate in sodium chloride-mediated wastewater at a BDD electrode was investigated in comparison with SnO_2 electrode. In 2 % of NaCl, the electrochemical degradation efficiency of bupirimate on BDD electrode was much greater than that on SnO_2 with a COD removal of 74 % on BDD anode and 59 % on SnO_2 anode. The different experimental conditions tested using the BDD anode allow us to conclude that the increases of the pH of the solutions, from acidic solution (pH = 2.5) to basic solution (pH = 11), slightly decreases the rate of absorbance of the peaks

located at 240 nm and 310 nm. When comparing the performances of both anode materials, at 60 $mAcm^{-2}$, the degradation efficiency is much higher for the BDD anode than that of SnO_2 anode. This means that the rate of mineralization is higher for the BDD anode. However, at 20 and 40 $mAcm^{-2}$, identical values are obtained for both anodes, with a COD removal of 26 and 58 %, respectively. Furthermore, the increase in initial bupirimate concentration from 230 mg/L to 345 mg/L increases the absolute removal of COD. These results lead to the conclusion that the BDD electrode is the most efficient compared to SnO_2.

3.2 Electrooxidation of methidathion

3.2.1 Electrooxidation of methidathion by anodic boron doped diamond electrode

3.2.1.1 Effect of chloride concentration

We observed that the application of electrolysis in this pesticide have the ability to reduce considerably the COD. For example, for 2 % mass NaCl and 3 % NaCl the achieved reduction was 85 % and 72 % respectively, while for 4 % NaCl was 56 %.

The mechanism of electrochemical mineralization can be direct, in this case there is oxidation of methidathion on the electrode or indirect via some mediators like chlorinated species or other radicals (Tatapudi & Fenton, 1994; Lin et al.,1998).

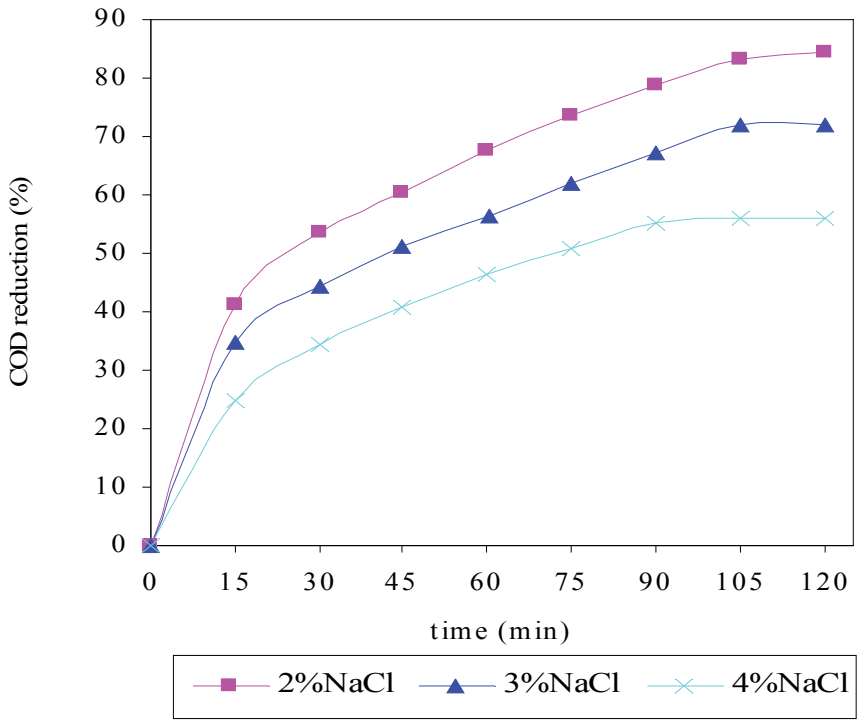

Fig. 9. The chemical oxygen demand (COD) reduction (in %) for methidathion 1.4 mM solution for 120 min of electrolysis at 60 mA and 25 °C.

Since some oxidant compounds that are produced during oxidation of water (like O2, O3 or hydroxyl radical) or oxidation of chlorine ions following Eq (7) to (9):

$$Cl^- \Leftrightarrow Cl^{\cdot}_{ads} + e^- \quad (7)$$

$$Cl^- + Cl^{\cdot}_{ads} \Leftrightarrow Cl_2 + e^- \quad (8)$$

$$Cl_2 + H_2O \Leftrightarrow HClO + H^+ + Cl^- \quad (9)$$

As cited in reference (Bonfatti et al., 2000), at pH higher than 4.5 the complete dismutation of Cl_2 into HClO and Cl^- is occurred. An explanation of the mediating role of chloride ions has been proposed by Bonfatti et al (Bonfatti et al., 2000). The presence of a weak concentration of chloride ions allows to inhibit the water discharge into oxygen, and to favorise hydroxyl or chloride and oxycloride radicals, which are very powerful oxidants. It can be explain why until 2 % of NaCl concentration the COD removal increases with NaCl concentration. Increasing the chloride concentration more than 3 % cause a "potentiostatic buffering" by the chlorine redox system and consequently a decrease of the anode potential. Another possibility is the presence of competitive reactions, in particular oxygen and chloride evolution due to recombination of radicals that becomes bigger with the increasing NaCl concentration. The balance of all these phenomena results that there is an optimum of NaCl concentration which is 2 % mass of NaCl for the degradation of methidathion. Figure 10 illustrates that the pH during the electrolysis is significantly reduced.

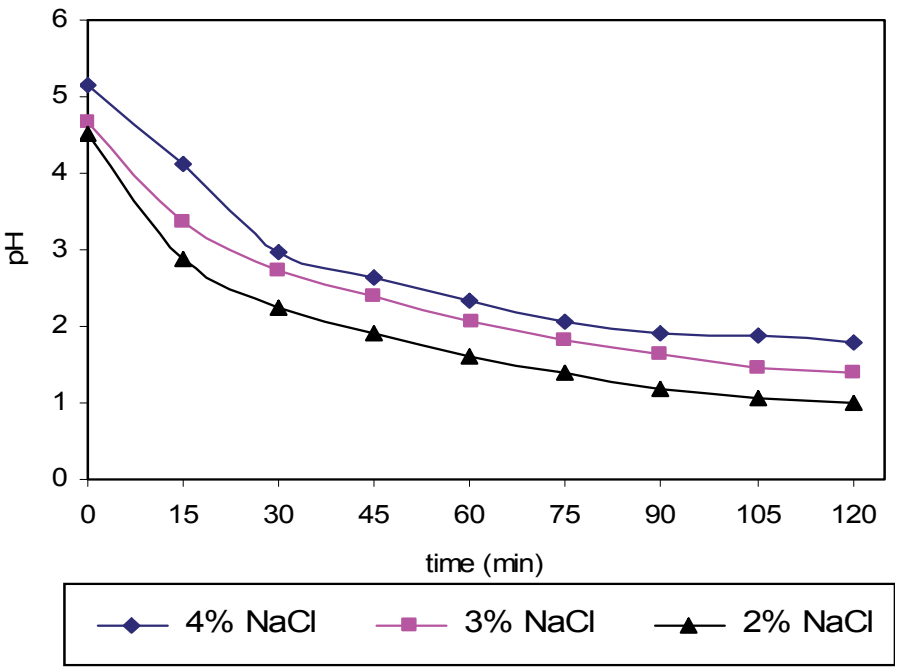

Fig. 10. pH reduction for methidation 1.4 mM solution for 120 min of electrolysis at 60mA and 25 °C.

Finally the pH in all cases became strong acidic. It is obvious that the continuous addition of high levels of organic matter in the electrolytic cell, resulted in the drop of pH. The electrolysis was more effective in terms of %COD reduction when the pH was in the acid

range. This drop of the pH, during pesticides degradation, was also reported by Bonfatti et al that while the mineralization goes to completion and the solution pH get more and more acidic.

3.2.1.2 Effect of applied current

Applied current is an important factor affecting the electrolysis kinetics and process economics. The effect of applied current on the electrochemical process was demonstrated in several studies. In Figure 11 the % COD reduction for the methidathion is presented under different current inputs (chlorides = 2 %).

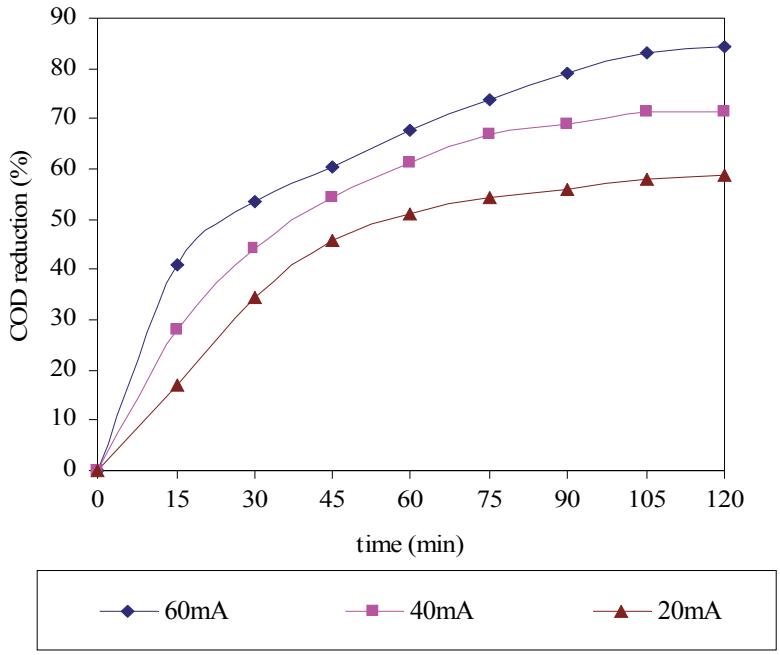

Fig. 11. % COD reduction for methidathion 1.4 mM under different current inputs (chlorides= 2%) and 25°C

These studies concluded that applied current increases the rate of electrochemical oxidation process. The COD of methidathion was observed to fall with pseudo first-order kinetics, on all the surface studied. This is related to the dependence of the rate of oxidation on the rate of formation of the oxidising species at the electrode surface. The pseudo first-order constant of methidathion (k) varies from 0.0073 s^{-1} (20 mA) to 0.0146 s^{-1} (60 mA). This is exemplified in Figure 12 where the pseudo first-order plot is presented. From these results it was calculated that the best applied current is 60 mA.

3.2.1.3 Effect of temperature

In Figure 13 the % COD reduction for methidathion at different temperatures under current input 60 mA is presented. It is observed that for 25 °C and 65 °C the achieved reduction was 85 % and 66 % respectively. The COD of methidathion was observed to fall with pseudo first-order kinetics Figure 14. The pseudo firstorder constant of methidathion (k) varies from 0.0131 s^{-1} (25 °C) to 0.0077 s^{-1} (65 °C).

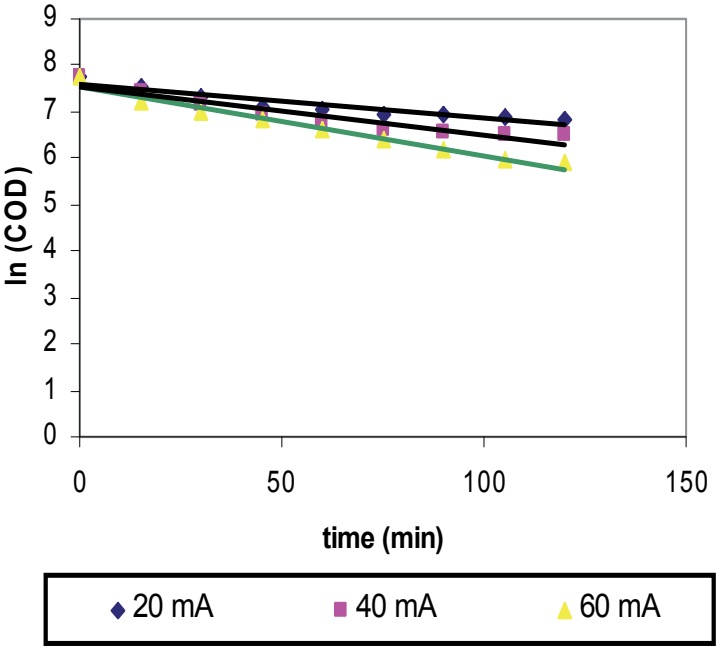

Fig. 12. Pseudo first-order plot oxidation of methidation 1.4 mM in 2% NaCl at 25°C under different current inputs (COD at a given time, t,during electrolysis).

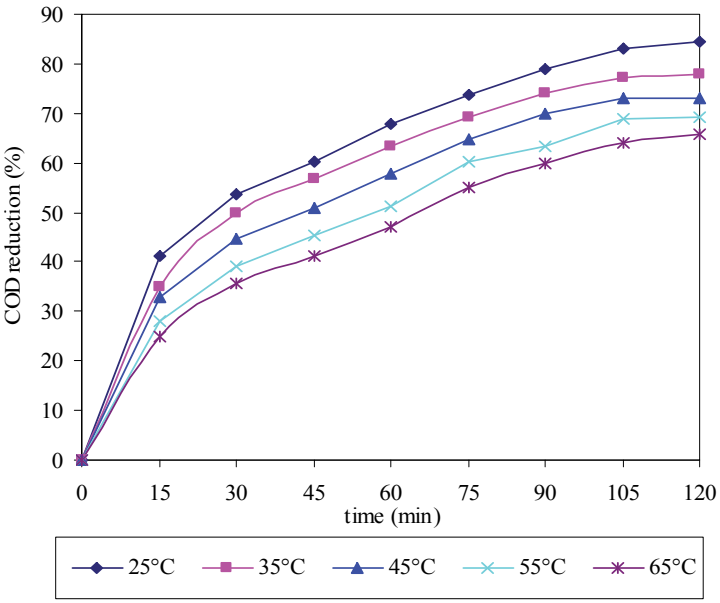

Fig. 13. COD reduction (in %) for methidathion 1.4 mM solution in 2 % NaCl at 25 °C at different temperatures.

The effect of temperature on the rates of constant was modelled using the Arrhenius plots, are shown in Figure 15. The apparent activation energies were determined by

$$K = A.\exp(-E_a / RT) \quad (10)$$

where K is rate constant, A is constant, Ea is the activation energy, T is the temperature (K) and R is the gas law constant.

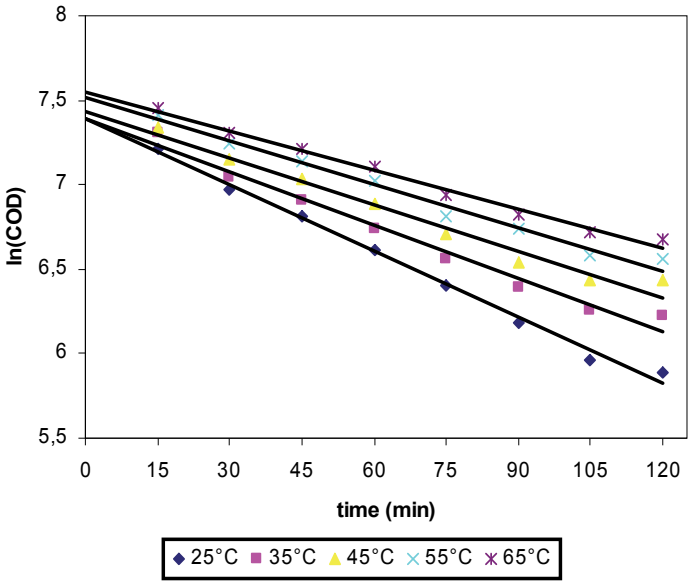

Fig. 14. Pseudo first-order plot oxidation of methidation 1.4 mM in 2% NaCl at 60 mA under different temperatures (COD at a given time, t,during electrolysis).

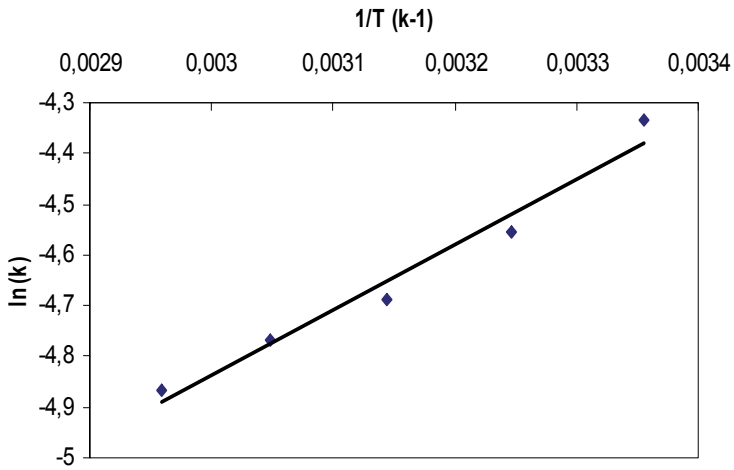

Fig. 15. Arrhenius plote of methidation 1.4 mM in 2% NaCl at 60 mA at various temperatures.

The obtained activation energy (-10.75 kj) indicate that the electrochemical degradation is complex.

3.2.2 Electrooxidation of methidathion by anodic SnO_2 electrode

3.2.2.1 Effect of applied current

In Figure 16 the COD for the methidathion is presented under different current inputs (chlorides = 2%). These studies concluded that the rise of applied current increases the rate of electrochemical oxidation process. In fact the COD abatement is faster as the applied current is greater, but the results are a logical consequence of the major quantity of charge passing in solution. On the other hand, the application of the highest applied current can be suggested in order to obtain the complete abatement of the organic content in the smallest time, obviously, the efficiency of the oxygen evolution reaction is larger at higher applied current.

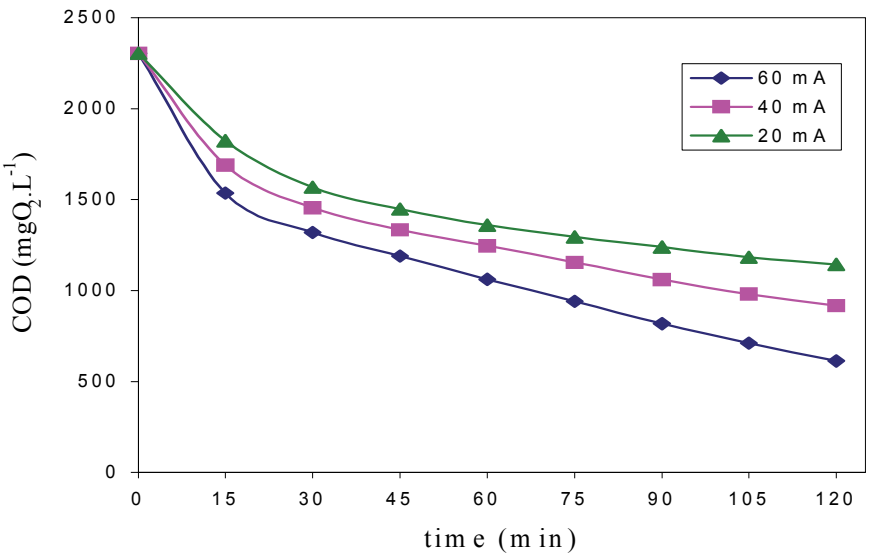

Fig. 16. % COD reduction for methidathion 1.4 mM under different current inputs (chlorides= 2%). 25°C and volume of treated solution: 75 cm^3

An important factor in the electrochemical treatment of organic containing effluents is the energy necessary to achieve the desired results. For an electrochemical reactor under galvanostatic conditions, the electrolysis energy is given by the equation (11):

$$E = \int_t U(t)\, I\, dt \tag{11}$$

The cell voltage U(t) is given by the following sum of terms:

$$U = U^0 + \eta_A + \eta_C + \sum RI \tag{12}$$

where, U_0 is the potential at nil current, η_A and η_B are respectively the anodic and cathodic overvoltages and Ω(RI) represent the ohmic drop through the solution of resistance R. The cell voltage was directly measured during electrolyses.
The energy consumption after 50 % reduction of COD of aqueous methidation solution is summarized in Table 4.

	COD input (mg)	Time of electrolysis (min)	Energy consumption (kWh/g COD).
20 mA	2304	115	$6.12\ 10^{-4}$
40 mA	2304	75	$9.61\ 10^{-4}$
60 mA	2304	48	$9.72\ 10^{-4}$

Table 4. Energy consumption after 50 % reduction of COD.

As can be seen, an increase in applied current caused the decrease of the time of electrolysis under 50% percent COD reduction while the energy consumption value increased. At lower current, the voltage is insufficient to mediate side reactions. Therefore, the energy is utilized for the degradation reaction only. However, under high current, the side reactions of oxygen evolution are more dominant.
The COD of methidathion was observed to fall with pseudo first-order kinetics, on the entire surface studied. This is related to the dependence of the rate of oxidation on the rate of formation of the oxidising species at the electrode surface. The pseudo first-order constant of methidathion (k) varies from 0.0043 s^{-1} (20 mA) to 0.0086 s^{-1} (60 mA). This is exemplified in Fig. 17 where the pseudo first-order plot is presented. These results show that the rate constant depends on the applied current.

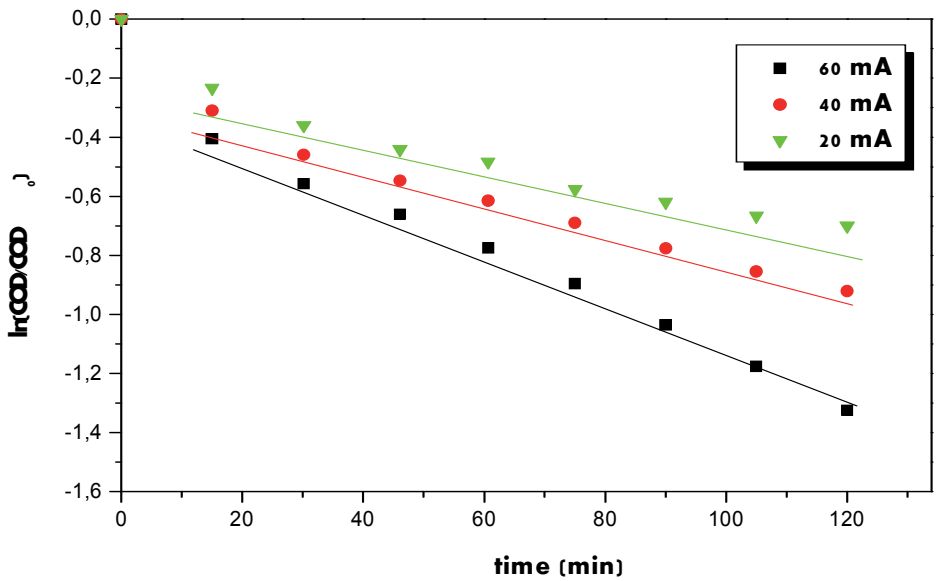

Fig. 17. Pseudo first-order plot oxidation of methidation 1.4 mM in 2% NaCl at 25°C and volume of treated solution: 75 cm3 under different current inputs (COD_t and COD_0 at t=0 and at a given time, t, during electrolysis).

3.2.2.2 Effect of temperature

To determine the effect of temperature on the % COD reduction for methidathion, experiments were carried under current input 60 mA for which reduction for methidathion is more important. Figure 18 shows the % COD reduction for methidathion at different temperatures. It was observed that, the % COD reduction decrease with temperature, for 25°C and 65°C the achieved reduction was 75 % and 50 % respectively.
The COD of methidathion was observed to fall with pseudo first-order kinetics. The pseudo first-order constant of methidathion (kI) varies from 0.0085 s^{-1} at 25 °C to 0.0038 s^{-1} at 65 °C (Fig. 19).

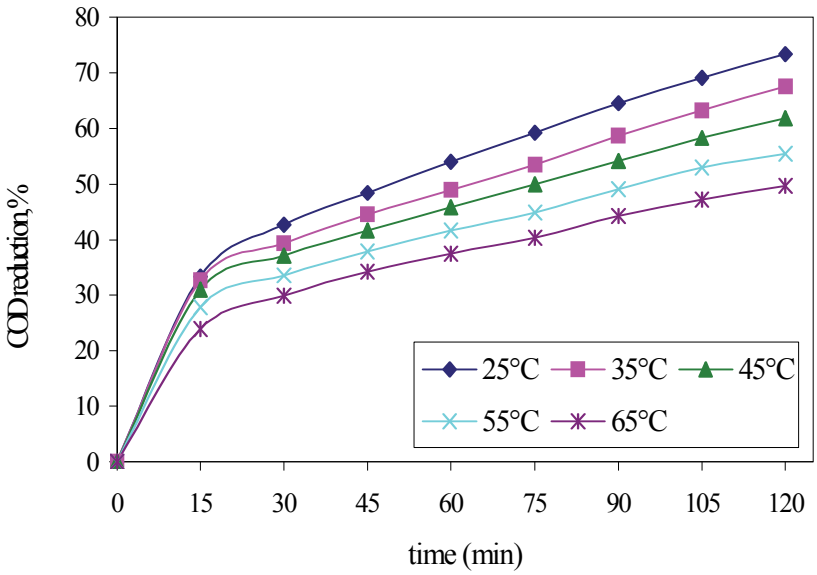

Fig. 18. % COD reduction for methidathion 1.4 mM in 2% NaCl at 25°C and volume of treated solution: 75 cm^3 at different temperatures.

The values of the rate constant (k) at different temperatures are listed in Table 5.

T (K)	k_I (s^{-1})
298	0.0085
308	0.0068
318	0.0056
328	0.0046
338	0.0038

Table 5. k_I as a function of temperature.

The linear least square fit of all the data points provided by the experiments at different temperatures leads to the Arrhenius parameters (activation energy and preexponentiel factor):

$$k_I(T) = (9.80 \pm 0.30)x10^{-6} \exp(2016 \pm 10 / T)\ s^{-1}$$

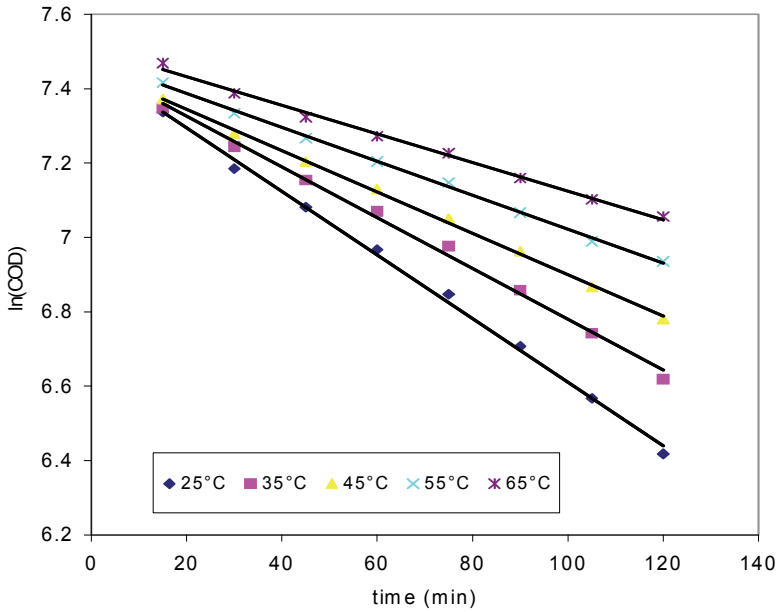

Fig. 19. Pseudo first-order plot oxidation of methidation 1.4 mM in 2% NaCl at 60 mA and volume of treated solution: 75 cm^3 under different temperatures (COD at a given time, t, during electrolysis).

As can be seen the rate constant k_I exhibits a negative temperature coefficient which confirms that the electrochemical degradation proceed via a complex mechanism.

3.2.3 Conclusion

The application of electrolysis in pesticide has the ability reduce the COD. For 2 % NaCl the achieved COD removal was 85 % and 75 % for BDD and SnO_2 respectively. In all cases, the pH of electrolysis was significantly reduced after 45 min for both electrodes. The COD of methidathion was observed to fall with pseudo first-order kinetics, on the entire surface studied. The applied current increases the rate of electrochemical oxidation process.
The effect of temperature shows that for 25 °C and 65 °C by BDD achieved was 85 % and 66 % respectively. The rate constant k_I obtained exhibits a negative temperature coefficient which confirms that the electrochemical degradation proceeds via a complex mechanism.
The COD of methidathion was observed to fall with pseudo first-order kinetics, on the totally surface studied.
The applied current increases the rate of electrochemical oxidation process.
The activation energy indicates that the process of electrochemical degradation is a complex one.

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6

Advances in Analytical Methods for Organophosphorus Pesticide Detection

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1. Introduction

Environmental pollution by organic chemicals continues to be one of the world's leading challenges to sustainable development. Modern developed and developing countries utilize millions of synthetic organic compounds in their civilian, commercial, and defense sectors for an ever-expanding diversity of uses (Ariese et al., 2001). Common applications include plastics, lubricants, refrigerants, fuels, solvents, preservatives, surfactants, dispersants and pesticides. As a result of widespread global usage coupled with improper handling practices, many of these organic compounds enter the environment and cause air, water, and soil pollution. For example, pesticides and herbicides are applied directly to plants and soils, while accidental releases originate from spills, leaking pipes, underground storage tanks, waste dumps, and waste repositories. Many pesticides are sprayed in large amounts with only 1% reaching the intended target. Some of these contaminants have long half-lives and thus persist to varying degrees in the environment. They migrate through large regions of soil until they reach water resources, where they may present an ecological or human-health threat (Karr & Dudley, 1981). Organisms, vegetation, animals and humans are affected by various chemicals through absorption, inhalation or ingestion. These contaminants pose serious to fatal health hazards, such as asthma, birth defects and deaths. Therefore, environmental monitoring is required to protect the public and the environment from possible organic toxins released into the air, soil, and water.

The United States Environmental Protection Agency (U.S. EPA) has imposed strict regulations on the concentrations of many environmental contaminants in air and water (U.S. EPA, 2010). However, current monitoring methods for most organic contaminants are costly and time-intensive, and limitations in sampling and analytical techniques exist (United States Geological Survey, U.S.G. S., 2010). Thus, there is a great demand for development of quick, simple and reliable methods for the detection of organic-based agricultural pesticides. In this chapter, advancements in methods to detect organophosphorus (OP) pesticides are discussed.

1.1 Structure of OP Compounds

OP pesticides are synthetic esters, amides, or thiol derivatives of phosphoric, phosphonic, phosphorothioic, or phosphonothioic acids. Table 1 lists the names of the most commonly used OP pesticides (International Programme on Chemical Safety, INCHEM, 2010; Pesticide Action Network, PAN, 2010; Ullmann's Agrochemicals, 2007). The chemical structure of all

OP pesticides consists of a central phosphorus atom, with either a double bonded oxygen (P = O), which are termed oxon pesticides, or a double bonded sulfur atom (P = S), which are termed thion pesticides, as shown in Figure 1. Structurally, both oxons and thions vary in the single-bonded R_1, R_2 and X groups attached to the central pentavalent phosphorus atom. However, R_1 and R_2 generally tend to be alkoxy, aryloxy and thioalkoxy groups, while X is a labile leaving group.

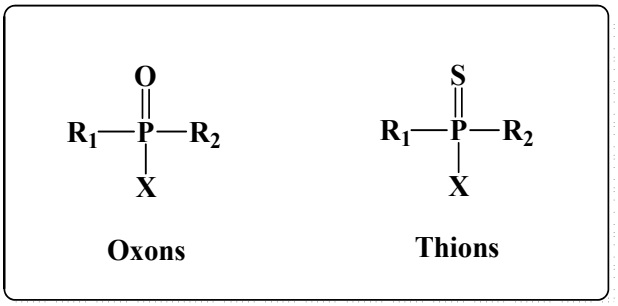


Fig. 1. General chemical structure of oxon and thion OP compounds.

1.2 OP Compounds and their toxicity

Pesticides are described as chemicals that kill or slow down the growth of undesirable organisms. Pesticides include herbicides, insecticides, fungicides, and nematocides (Celik et al., 1995). OP pesticides constitute the most widely used insecticides available today. This class of compounds has achieved enormous commercial success as a key component in the arsenal of agrichemicals, and is currently an integral element of modern agriculture across the globe. According to the U.S. EPA, about 70% of the insecticides in current use in the US are OP pesticides (U.S. EPA, 2010). Although OP compounds are considered safer than organohalides, they are still highly neurotoxic to humans and in some cases their degradation products have the potential to be more toxic with chronic exposure. OP pesticides are efficiently absorbed by inhalation, ingestion, and skin penetration. They are strong inhibitors of cholinesterase enzymes that function as neurotransmitters, including acetylcholinesterase, butylcholinesterase, and pseudocholinesterase. These enzymes are inhibited by binding to the OP compound. Upon binding, the OP compound undergoes hydrolysis leading to a stable phosphorylated and a largely unreacted enzyme. This inhibition results in the accumulation of acetylcholine at the neuron/neuron and neuron/muscle junctions or synapses.

Each year OPs poison thousands of humans across the world. In fact, in 1994, an estimated 74,000 children were involved in common household pesticide related poisoning or exposures in the United States (U.S. EPA, 2010). In a more recent study, it was found that children exposed to OP pesticides were more likely to be diagnosed with attention deficit hyperactivity disorder (ADHD) (Bouchard et al., 2010). Exposure has been attributed to frequent use of OPs in agricultural lands and their presence as residues in fruits, vegetables, livestock, poultry products and municipal aquifers (G. L. Liu & Lin, 1995). For example, typical pesticide concentrations that flow into aqueous waste range from 10,000 to 1 ppm (Gilliom et al., 1999; U.S.G.S., 2010). Pesticides are influenced by a number of biological, chemical and physical processes once they enter the environment. Figure 2 shows the

possible routes of environmental exposure of OP pesticides to humans and wildlife (Vermeire et al., 2003). While many OP pesticides can degrade via microbial or environmental processes, some of the pesticides are consumed by organisms, or they could leach into ground water. Once a pesticide enters ground water it can remain there for considerable periods of time. In ground water, there is little sunlight exposure, which slows down the degradation of OP pesticides and increases their potential risks to the environment and human health.

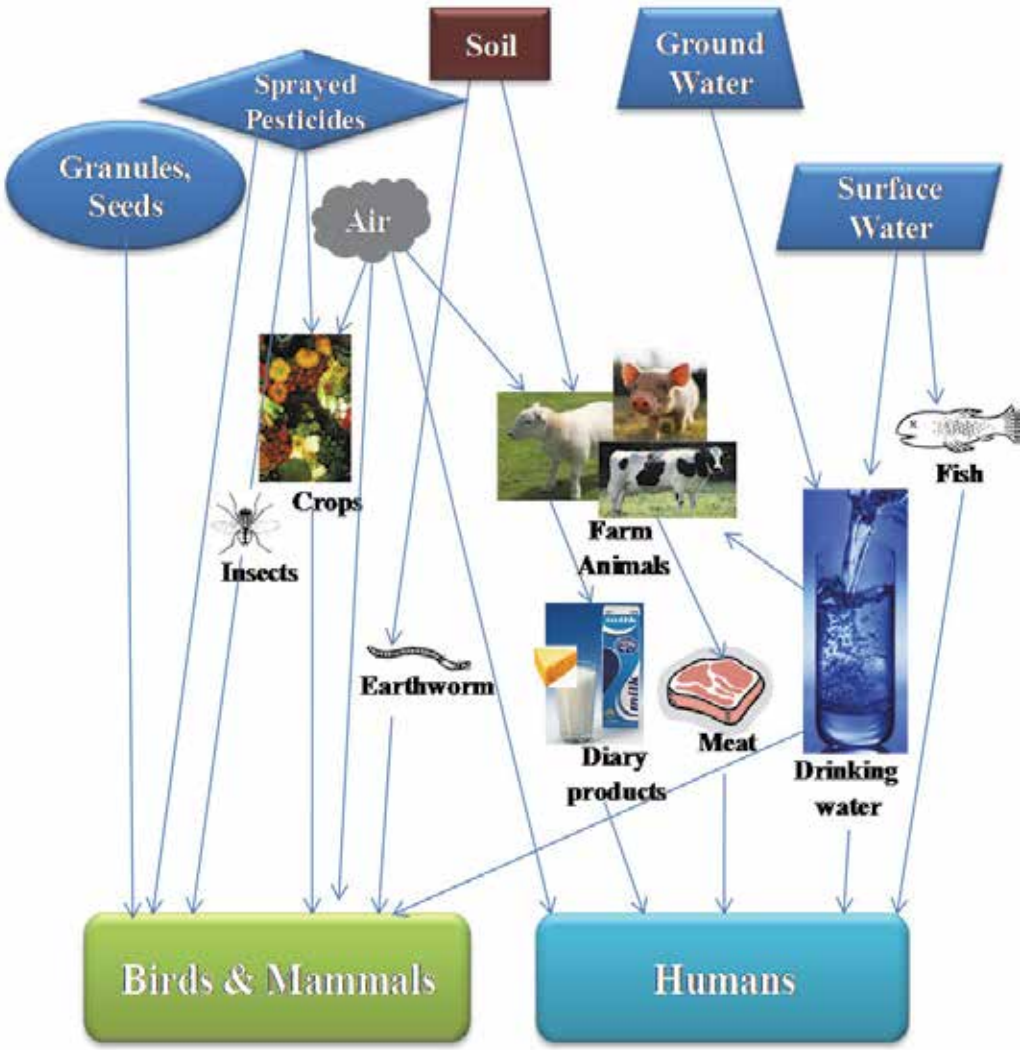


Fig. 2. Schematic representation of the possible routes of environmental exposure of OP pesticides to humans and wildlife. Adopted from Reference Vermeire et al., 2003.

No	OP Name	Structure (Thions: 1–17; Oxons: 18 – 29)	LD_{50}, mg/kg *		WHO Acute Hazard§	IARC Carcinogens‡	U.S. EPA Carcinogens†
			Oral	Dermal			
1	Parathion		1	21	Ia	3, Unclassifiable	C, Possible
2	Fonofos		8–17	147	Ia	N/A	E, Unlikely
3	Azinphos-methyl		11–13	220	Ib	N/A	Not Likely
4	Coumaphos		16–41	1,000	Ib	N/A	Not Likely
5	Methidathion		25–48	1,546	Ib	N/A	C, Possible

Table 1.

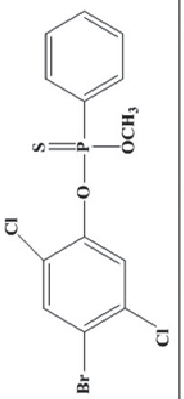
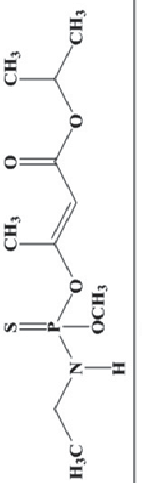
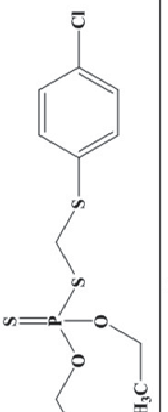
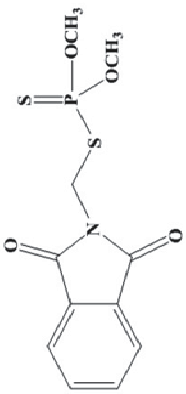
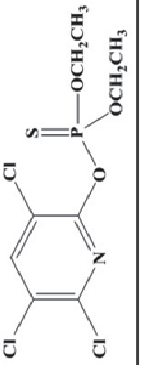
6	Leptophos		45–53	>800	N/A	N/A	N/A
7	Propetamphos		75–82	2,300	Ib	N/A	Not Likely
8	Carbophenothion		98–120	190–215	N/A	N/A	N/A
9	Phosmet		113–160	>1,500	II	N/A	Suggestive
10	Chlorpyrifos		135–165	2,000	II	N/A	E, Unlikely

Table 1. *Cont.*

11	Fenthion		214–245	330	II	N/A	E, unlikely
12	Fenitrothion		250	>3,000	II	N/A	E, unlikely
13	Dichlofenthion		270	6,000	N/A	N/A	N/A
14	Dicapthon		330–400	790–1,250	N/A	N/A	N/A
15	Diazinon		300–850	2,150	II	N/A	Not Likely

Table 1. *Cont.*

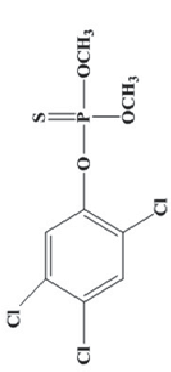
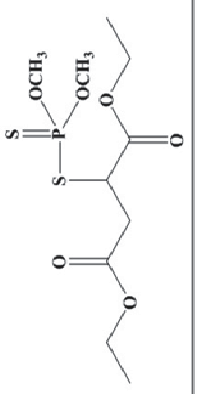
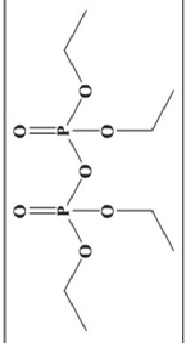
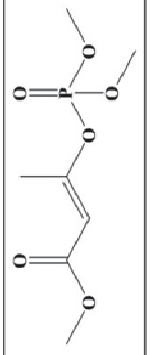
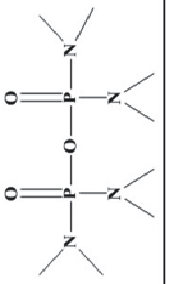
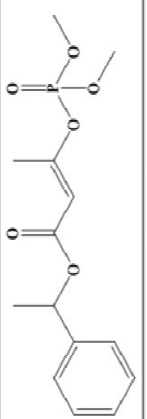
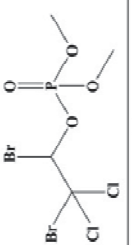
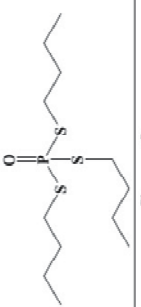
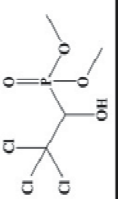
16	Ronnel		1,250–2,630	2,000	N/A	N/A	N/A
17	Malathion		5,400–5,700	>2,000	III	3, Unclassifiable	Suggestive
18	Tetraethyl pytophosphate (TEPP)		0.5	2.4	N/A	N/A	N/A
19	Mevinphos		3.7–6.1	4.2–2.7	Ia	N/A	N/A
20	Schradan		10	15	N/A	N/A	N/A

Table 1. *Cont.*

21	Monocrotophos		18–20	112–126	Ib	N/A	N/A
22	Phosphamidon		24	107–143	Ia	N/A	C, Possible
23	Oxydemeton methyl		47–52	158–173	Ib	N/A	Not Likely
24	Ethoprophos		61	26	Ia	N/A	Likely
25	Dichlorvos		56–80	75–107	Ib	2b, Possible	Suggestive

Table 1. *Cont.*

26	Crotoxyphos		74–110	202–375	N/A	N/A	N/A
27	Naled		250	800	II	N/A	E, Unlikely
28	Tribufos		560–630	>2,000	N/A	N/A	Likely (high doses), Not likely (low doses)
29	Trichlorfon		400–800	>2,000	II	3, Unclassifiable	Likely (high doses), Not likely (low doses)

- Toxic interactions of organophosphorus compounds with any given biological system are dose-related. Their toxicity is expressed in terms of the lethal dose (LD) which will kill 50% of the animal species (LD_{50}). LD_{50} values are generally expressed as amount per unit weight (e.g., $mg \cdot kg^{-1}$).
- Toxic interactions of organophosphorus compounds with any given biological system are dose-related. Their toxicity is expressed in terms of the lethal dose (LD) which will kill 50% of the animal species (LD_{50}). §WHO = World Health Organization, acute hazard classify: Ia = extremely hazardous to human health; Ib = highly hazardous; II = moderately hazardous; III = slightly hazardous. ǂ IARC = International Agency for Research on Cancer. † EPA = Environmental Protection Agency.

Table 1. Common OP pesticides classified by oxon/thion structure and oral LD_{50} toxicities (Gilliom et al., 1999; Vermeire et al., 2003; Walker, 1972) .

2. Advances in the detection of OP pesticides

Significant advances toward the development of detection methods for OP compounds have been reported in the literature (Jenkins et al., 1997a, 1999b; Jenkins et al., 2001; Rudzinski et al., 2002; Russell et al., 2003; Sohn et al., 2000; Steiner et al., 2005). Analysis of OPs in environmental and biological samples is routinely conducted using various analytical techniques, including nuclear magnetic resonance (NMR) spectroscopy (Ross & Biros, 1970), gas, liquid or thin layer chromatography, and mass spectrometry (Steiner et al., 2005). A variety of approaches have been investigated for new sensors, including enzymatic assays (Russell et al., 2003**)**, molecular imprinting coupled with luminescence (using lanthanides) (Jenkins et al., 1997a, 1999b; Jenkins et al., 2001; Rudzinski et al., 2002), colorimetric methods (Michel at al., 1973; Novak et al., 1979; Wallace et al., 2005), surface acoustic waves (Ngehngwainbi et al., 1986; Nieuwenhuizen & Harteveld, 1997), fluorescent organic molecules (Van Houten et al., 1998; Yamaguchi et al., 2005; S. W. Zhang & Swager, 2003), and interferometry (Sohn et al., 2000). The most common ways for detecting OP pesticides are chromatographic methods coupled with different detectors and different types of spectroscopy, immunoassays, and enzyme biosensors based on inhibition of cholinesterase activity (Evtugyn et al., 1996; Sherma, 1995; Trojanowicz, 2002). Recently, advances have risen toward designing and developing optical sensors, *i.e.*, colorimetric or fluorimetric chemosensors or reagents. One of the most convenient and simple means of chemical detection is the generation of an optical signal, for example, changes in absorption or emission bands of the chemosensor in the presence of the target analyte. Optical outputs have been used extensively in recent years for the development of chemosensors for ion or neutral molecule recognition and sensing based on supramolecular concepts (Martínez-Máñez & Sancenón, 2003). Unfortunately, although the utility of optical detection is becoming increasingly appreciated in terms of both qualitative and quantitative analysis, the number of optical sensors currently available for OP compound detection is quite limited. We review recent advances in (1) mass spectrometric techniques, and (2) enzymatic assays and chemosensors for detection of OP pesticides.

3. Determining OP pesticides using mass spectrometric techniques

Mass spectrometry (MS) techniques have been utilized for the detection and quantification of various OP pesticides due to their low detection limits in the part per billion (ppb) and part per trillion (ppt) range, as well as the selective detection of analytes in multi-residue samples. The mass spectrometry techniques are often coupled with separation methods like gas chromatography (GC) or liquid chromatography (LC), and other analyte introduction methods such as inductively coupled plasma (ICP), or fiber introduction (FI). The following sections describe extraction methods, sources of ionization, analyzers, and data acquisition modes generally chosen for GC-MS, LC-MS, and other MS configurations specifically for the detection of OP pesticides.

3.1 Gas chromatography

GC-MS has been a widely practiced OP detection method of choice over the last decade for various sample types; biological (Barr et al., 2002; Tsatsakis et al., 2008), environmental (Castro et al., 2001; Goncalves & Alpendurada, 2005; Hildebrandt et al., 2007; Kristenson et al., 2004; Miranda et al., 2008; Riederer et al., 2010; Sabik et al., 2003; Tahboub et al., 2005;

Toledano et al., 2010; Villaverde et al., 2008;), agricultural (Berrada et al., 2010; Kirchner et al., 2008; Kristenson et al., 2001; Mastovska et al., 2004), and food products (Fontana et al., 2010; Sasamoto et al., 2007; Schellin et al., 2004; Zheng et al., 2007). GC-MS offers sensitive and selective determination of OP analytes. Optimization of instrument limits of detection (LODs) and quantification (LOQs), and analysis time has been investigated by adapting various combinations of extraction methods, ionization sources, and analyzers for the determination of OP pesticides.

3.1.1 Extraction methods

Sample types vary for OP pesticide analysis from fruits and vegetable sources to biological specimen and environmental samples; thus extraction and clean-up methods used for sample pretreatment play an important role, and influence the results provided by the instrument technique. Solid phase extraction (SPE) is a frequently used technique for various sample matrix types, including aqueous samples (Aguilar et al., 1997; Baugros et al., 2008; El-Kabbany et al., 2000; Gupta et al., 2008), biological specimen (Adachi et al., 2008; Park et al., 2009; Raposo et al., 2010), and clean-up of solid samples (Diaz-Cruz & Barcelo, 2006; Shuling et al., 2007a). SPE is an efficient method for isolating and concentrating solutes from relatively large volumes of liquid. The technique can be very effective, even when the solutes are present at extremely dilute concentrations (e.g. ppb). Limits of the technique include extensive time and labor expense, and requirement of large quantities of high-quality solvents. As there is a trend to simplify and miniaturize sample preparation, and to minimize organic solvents used, solid phase microextraction (SPME) and matrix solid-phase dispersion (MSPD) reduce the effect of the aforementioned disadvantages. MSPD extraction is a microscale preparation technique used with solid sample matrices, such as soil (Shen et al., 2006) and fruit (Ramos et al., 2008) matrices that reduce the required amount of sample and solvent to efficiently extract analytes of interest. SPME is a sorptive extraction method in which analyte enrichment occurs through portioning between the polymer and the aqueous phase according to the distribution constant. A silica fiber coated with a stationary phase is immersed in an aqueous sample, followed by injection and desorption by temperature in the GC injector. The quantity of analyte extracted by the fiber is proportional to its concentration in the sample as long as equilibrium is reached. The attraction of SPME for OP pesticide detection is that the extraction is fast and simple and can be done without solvents, and detection limits can reach parts per trillion (ppt) levels for certain compounds. SPME has been applied to aqueous samples (Bagheri et al., 2010; Berceiro-Gonzales et al., 2007; Garcia-Rodriguez et al., 2008; Menezes Filho et al., 2010; Natangelo et al., 1999), biological specimen (Beltran et al., 2001; Hernandez et al., 2002), food samples (Lambropoulou & Albanis, 2002; Zambonin et al.; 2004), textiles (Zhu et al., 2009), soil (Zambonin et al., 2002), and sewage samples (Basheer et al., 2006). Polydimethylsiloxane (PDMS) fibers are shown to have good extraction efficiency and are thus often used (Berceiro-Gonzales et al., 2007; Garcia-Rodriguez et al., 2008; Lambropoulou & Albanis, 2002). However, Natangelo *et al*. were able to use a small 10 mL sample aliquot for extraction by a divinylbenzene-based carbowax, followed by thermal desorption at 250°C for 15 min for a total extraction time of 70 min (Natangelo et al., 1999). Shorter extraction times have been reported as low as 30 min (Menezes Filho et al., 2010; Beltran et al., 2001; Zhu et al., 2009). Restricted application of SPME may be due to the coated material on the fiber, as well as expense and fragility of the fibers during the extraction process. Alternatively, Basheer *et al*. packed multi-walled carbon nanotubes (MWCNT) inside a sheet

of porous polypropylene membrane for supported micro-solid-phase extraction (μ-SPE) (Basheer et al., 2006). Adsorption of the analytes occurred through π-π electrostatic interactions between the analytes and the large surface area of the MWCNT, yielding good repeatability of the extractions (RSD 2-8%) and low LODs (1-7 pg/g).

Stir bar sorption extraction (SBSE) is another popular extraction technique that is based on equilibrium sorption opposed to adsorption. Extraction by this method is performed by adding a suitable sample amount to an extraction vessel, then adding a polydimethylsiloxane (PDMS) coated magnetic stir bar, and stirring for a period of time, followed by relocation of the stir bar to a glass desorption tube in a thermal desorption unit (Benanou et al., 2011). Application of this extraction technique has been mainly applied to aqueous samples (Leon et al., 2003; Leon et al., 2006; Ochiai et al., 2006; Ochiai et al., 2008; Perez-Carrera et al., 2007; Serodio & Nogueira, 2004), a few specific liquid food types (Lavagnini et al., 2011; Zuin et al., 2006), and even a solid food sample that required extraction of 85 pesticides in vegetable, fruit, followed by green tea by an alcohol and dilution with water (Ochiai et al., 2005). For the sorptive enrichment techniques, SPME and SBSE, extraction is significantly influenced by aqueous volume, extraction and desorption time, desorption solvent and ionic strength. For instance, addition of a NaCl solution aids to target solutes with low partition coefficients and improves sensitivity (Leon et al., 2003; Ochiai et al., 2008). Alternatively, addition of methanol favors the extraction of apolar compounds, thus making the analytes more available for partitioning (Lavagnini et al., 2011; Ochiai et al., 2005).

As an alternative to SPE methods, liquid-phase microextraction (LPME) is cheap, rapid, and easily automated, in which only microliters of solvents are required to concentrate analytes from aqueous samples. LPME involves the distribution of a solute between two immiscible liquid phases. Therefore, the extraction solvent must be stable during extraction time, its polarity should be compatible with that of the fiber, and it should be water immiscible. Longer exposure time results in higher extraction efficiency. Furthermore, other parameters such as rate of agitation can enhance extraction efficiency, and the addition of salt can increase extraction yields for compounds with low octane-water partition coefficients. Examples utilizing LPME include the determination of ethoprop, diazinon, disulfoton and fenthion from lake water samples (P. Chen & Huang, 2006), and other OP pesticides from wastewater (Basheer et al., 2007). The optimal LPME parameters adopted by Chen et al. were as follows: 20 mL aqueous sample, 15% w/v NaCl, cyclohexane extraction solvent, 50 min extraction time, a stirring rate of 700 rpm, and a polypropylene hollow fiber was incorporated (P. Chen & Huang, 2006). Polypropylene is highly compatible with a broad range of organic solvents, and it strongly immobilizes the solvents, thus preventing leakage of the organic phase during extraction. Another miniature liquid-phase extraction technique recently investigated for OP pesticide detection in natural water samples is single-drop microextraction (SDME) (H. Chen et al., 2009). 1.5 μL of toluene was exposed via the tip of a microsyringe directly immersed in a 5 mL aqueous sample, followed by stirring at 800 rpm at a pH of 5, for extraction of OP analytes before transferring the single drop to a GC-MS detection system. Inter- and intra-day RSD values were below 5.4 and 6.1%, respectively.

Supercritical fluid extraction (SFE) is an alternative to the solvent-intensive isolation procedures, like SPE or LLE, especially for environmental samples (Goncalves at al., 2006; S. R. Rissato, M. S. Galhiane, B. M. Apon, et al., 2005) and complex food matrices (Norman & Panton, 2001; Rissato et al., 2004; S. R. Rissato, M. S. Galhiane, A.G. de Souza, et al., 2005). Advantages of SFE that have been discussed in the literature include: rapidity, simplicity,

great analyte selectivity, good extraction efficiency, no need of a clean-up step, suitability for thermally labile compounds, automation, solventless or near solvent free character and reduced environmental hazard (Goncalves at al., 2006; Norman & Panton, 2001; Rissato et al., 2004;S. R. Rissato, M. S. Galhiane, B. M. Apon, et al., 2005; S. R. Rissato, M. S. Galhiane, A. G. de Souza, et al., 2005). The greatest advantage of supercritical fluids, however, is the fact that they have densities and solvating powers comparable to the density of liquids, which can be continuously varied by one order of magnitude by varying the temperature and pressure of the extraction vessel. CO_2 is frequently used as a supercritical fluid due to its suitable critical temperature (31.2 °C) and pressure (72.8 atm), since it can be easily removed by reducing its pressure (S. R. Rissato, M. S. Galhiane, B. M. Apon, et al., 2005; Norman & Panton, 2001; Rissato et al., 2004; S. R. Rissato, M. S. Galhiane, A. G. de Souza, et al., 2005). Analysis of a honey and soil sample prepared by SFE required 400 bar CO_2 pressure and a CO_2 modifier, acetonitrile and methanol, respectively (S. R. Rissato, M. S. Galhiane, B. M. Apon, et al., 2005; Rissato et al., 2004).

Recently, Morgan's group at the University of South Carolina adapted disposable pipette extraction (DPX), a dispersive SPE method that uses loosely contained sorbent that is mixed with sample solutions in a pipette tip, for the extraction of OP pesticides from high fat content food (H. Guan et al., 2009), as well as fruit and vegetable samples (H. Guan et al., 2010). The method involved dynamic mixing of DPX sorbent with solutions that provide rapid equilibration, partitioning, and enhanced contact between analytes and solid-phase sorbent for more rapid performance and sensitivity (H. Guan et al., 2009). Guan et al. studied the effectiveness of weak anion exchange mechanisms to remove fatty acid matrix interferences from cocoa beans (50% fat) prior to multiresidue pesticide analysis (H. Guan et al., 2009). Average recoveries reached 100% for most targeted pesticides studied, with relative standard deviations below 10%.

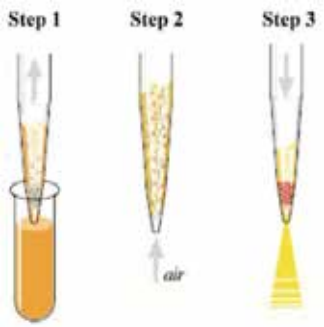


Fig. 3. Schematic representation of DPX cleanup extraction method. After blending and filtering, the sample solution is aspirated into the DPX tip (step 1), and then air bubbles are created by aspirating air into the tip causing mixing (step 2); the solution is then dispensed to a GC vial after a short exposure time (step 3). (Taken with permission from Reference H. Guan et al., 2009)

Microwave-assisted extraction (MAE), a rapid heating process that yields an accelerated kinetic dissolution of the matrix when a microwave field is applied to the sample, has been explored through, hydrological samples (Gfrerer et al., 2003), working air quality samples (Esteve-Turrillas et al., 2008), and olive oil (Fuentes, E.; Báez, M. E. & Quiñones, A., 2008). Scientists at the University of Chile incorporated atmospheric pressure microwave-assisted

liquid-liquid extraction (APMAE) for the determination of OP pesticides in olive and avocado oil and where LOQs ranged from 0.004 to 0.015 µg/g (Fuentes, E.; Báez, M. E. & Diaz, J., 2009). The APMAE glass system employed for this extraction consisted of an Erlenmeyer flask, with 5 g of oil in 5 mL diluted *n*-hexane, attached to an air-cooled condenser where 15 mL of acetonitrile was added. The system was put into a microwave oven and heated for 13 min at 150 W. After cooling, the upper layer of the solution was transferred to a test tube before GC-MS/MS analysis. Power, time of extraction, volume of extracting solvent, and the step of dilution of oil with *n*-hexanes were assessed to optimize the extraction efficiency of the aforementioned extraction set-up.

3.1.2 Ionization sources

There are various pathways to provide the ionization energy required to generate the fragment and /or molecular ion for an OP analyte of interest during GC-MS detection, namely electron impact (EI), chemical ionization (CI), and atmospheric pressure chemical ionization (APCI). Electron impact is a widely used hard impact ionization source implemented in MS detection of OP compounds. It is robust and generates standard mass spectra that can be compared with those in established MS libraries. EI-MS detection is also rapid, sensitive, and accurate, minimizing the possibility of releasing false positive and false negative results (Araoud et al., 2007; Albero et al., 2003; Aybar-Munoz et al., 2003; Aybar-Munoz et al., 2005; Elfein et al., 2003; Frenich et al., 2007; Haib et al., 2003; Ling et al., 2006; Tarbah et al., 2001;). To accurately determine toxic OP pesticide exposure, for malathion in pregnant women in the Philippines, researchers employed electron impact (EI) ionization (+70 eV), which led to OP pesticide detection limits of low ppm in umbilical cord blood (Corrion et al., 2005) and hair samples (Posecion et al., 2006).

Mass spectrometry techniques have successfully been applied to determine OP pesticides in food samples. To greatly optimize the sensitivity and selectivity of the EI-MS system, selective ion monitoring (SIM) mode was utilized to monitor 3 particular ion analytes of interest in various OP pesticides containing samples, including textiles (Zhou et al., 2007), biological samples (Lacassie et al., 2001), environmental samples (Berger-Preiss & Elflein, 2006; Patsias & Papadopoulou-Mourkidou, 1996; Stiles et al., 2008; Wang et al., 2007), agricultural (Aguera et al., 2002a; Nguyen et al., 2008; Stajnbaher & Zupancic-Kralj, 2003; Wong et al., 2007), and different food matrices (Albero, B.; Sanchez-Brunete, C. & Tadeo, J. L., 2004; Wong et al., 2004). For example, Rissato *et al*. showed that GC-EI-MS detection in the SIM mode was used to detect high concentrations of malathion residues in original honey samples inundated by mosquito control methods during a three year time period prior to the year of study (Rissato et al., 2007).

Low probability of obtaining molecular ion ($M^{+\cdot}$) in EI spectra, due to extensive fragmentation, is a drawback of EI screening reliability (Portoles et al., 2010). Soft ionization techniques such as chemical ionization (CI) would overcome this issue. CI results in a significant increase in selectivity, while allowing the simultaneous confirmation and quantification of trace levels, e.g. ppb, of pesticides in complex matrices (Hernando et al., 2001; R. Húšková, E. Matisová, L. Švorc, et al., 2009; Jover & Bayona, 2002; Kolberg et al., 2010; Li et al., 2010; Russo et al., 2002;). Due to OP pesticides having electronegative elements and being capable of producing a negative ion, chemical ionization is mostly utilized under negative ion (NI) mode. Negative chemical ionization (NCI) offers several advantages of EI including limited fragmentation, simple mass spectra with reduced interferences from ions derived from the sample matrix, better signal-to-noise ratio, and

higher sensitivity and selectivity at ultratrace concentrations (ppt) (R. Húšková, E. Matisová, S. Hrouzková, et al. 2009). Húšková *et al*., confirmed the advantages of GC-NCI-MS through the determination of dimethoate, chlorpyrifos-methyl, malathion, and diazinon in non-fatty food matrices, fruits and vegetables (R. Húšková, E. Matisová, S. Hrouzková, et al. 2009). The instrument's LODs and LOQs were up to 3 orders of magnitude lower for NCI compared to EI ionization, with analyte levels in the ppt range.
Portoles *et al*. applied atmospheric pressure chemical ionization (APCI) to GC quadrupole time-of-flight (QTOF) MS, operating in NI mode, and found that using water as a modifier, and investigating the highly abundant MH^+ ions yielded optimum parameters to perform wide-scope screening of OP pesticides in agricultural products (Portoles et al., 2010). They suggested that the corona discharge needle, using N_2 as an auxiliary gas, generates $N_2^{+\cdot}$ and $N_4^{+\cdot}$ ions that react with water or any proton source to indirectly transfer protons to the analyte of interest as shown in Figure 4.

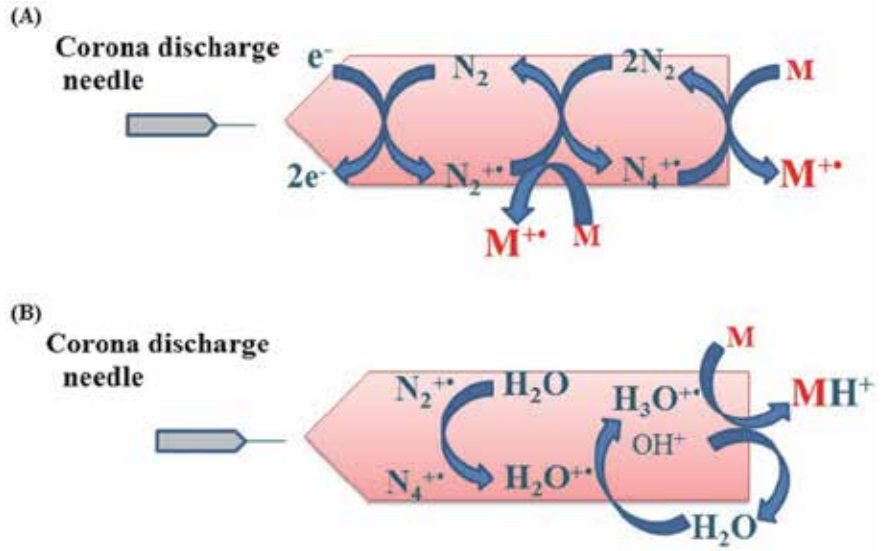


Fig. 4. Molecule reactions when using APCI source: (A) N2 transfer conditions; (B) proton transfer conditions. (Modified and Taken with permission from reference Portoles et al., 2010).

Amendola *et al*. aimed to optimize the ionization conditions for detecting 19 OP pesticides by investigating effects of source temperature, influence of electron energy, emission intensity, and gas pressure of the ionizing gases isobutane, methane, ammonia in methane, and pure ammonia (Amendola et al., 2002). They found that the different ionizing gases generate significant differences in the mass spectra of several of pesticides investigated. Additionally, it was determined that the use of pure ammonia reduces the background noise thereby improving the overall sensitivity of the method to reach low LODs (< 1 µg/L).
In determining OP pesticide content of various matrices, both hard (e.g. EI) and soft (e.g. CI) ionization methods have been primarily used to generate analyte ions of interest. EI is a robust source, which produces standardized mass spectra that can be compared with those from commercially available MS libraries. However extensive fragmentation is a drawback for some applications when multiresidue screening is necessary. Alternatively, CI overcomes these limitations and provides better sensitivity and selectivity. Furthermore a

combination of EI and CI in GC tandem mass spectrometry has also proven useful in agricultural samples (Aguera el al., 2002b; Sanchez et al., 2006).

3.1.3 Mass analyzers

The impact of analyte ionization is realized through the choice of analyzer available. Ion trap (IT) and triple quadrupoled (QqQ) detectors have been coupled with gas chromatography for the determination of OP pesticides in assorted sample matrices. Ion trap (IT) mass spectrometry is a low cost and easy to operate analyzer, which is based on a selected parent ion and whole mass spectrum of the daughter ions that result in high sensitivity and selectivity of target analytes (Boy-Bolan et al., 1996; Ma et al., 2001; Na et al., 2006; Shuling et al., 2007b; Tao et al., 2009). While there is the possibility of obtaining higher order MS spectra from the large amounts of fragments generated in IT-MS, there is also a limit to low scan speeds that result in longer analysis time (Frenich et al., 2008). Frenich et al. demonstrated the robustness of IT-MS in comparison to QqQ-MS through intra-day and inter-day precision; it was found that the RSD values obtained by IT-MS were two times lower than the RSD values obtained by QqQ as shown in Figure 5. In addition to the low scan speeds, IT analysis suffers from sample matrix limitations, where for example, high fat content matrices result in low signal to noise ratios and require additional sample clean-up stages (Frenich et al., 2008).

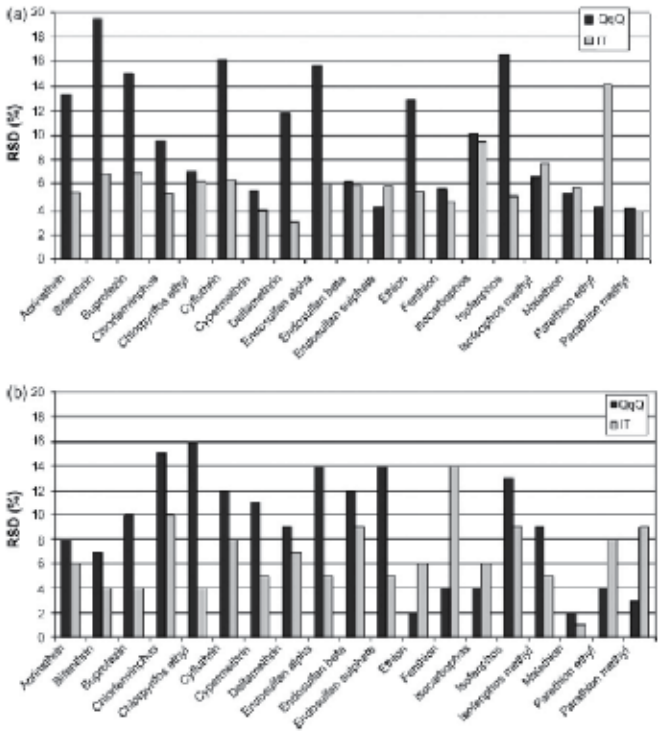


Fig. 5. Inter-day (a) and intra-day (b) precision values, expressed as relative standard deviation (RSD), obtained with the QqQ and the IT analyzer for solvent standards at 50 µg/L. (Taken with permission from Reference Frenich et al., 2008).

Tandem mass spectrometry (MS/MS) incorporates two or more analyzer stages to better resolve and select analytes of interest. The first stage selects the precursor ion after ionization, and in the latter stage fragment ions obtained from collision-induced dissociation (CID) of the precursor ion with an inert gas (e.g. helium or argon) are analyzed. MS/MS methods can be carried out in either tandem-in-time, performance of a sequence of events recorded in an ion storage device; or tandem-in-space, where two different instruments are coupled together for the analysis. Low ppb LODs in aqueous (Esteve-Turrillas et al., 2007; Rubio et al., 2006), fruit and vegetable samples (Gonzalez-Cubelo et al., 2011; Martinez Salvador et al., 2006; Moreno et al., 2006; Rawn et al., 2006; Schachterle & Feigel, 1996; Wong et al., 2010), and food products (Guardia-Rubio et al., 2006; Mezcua et al., 2007; S. N. Sinha, V. K. Bhatnagar, et al., 2011; Yague et al., 2005) were established by GC-MS/MS; still LODs in ppt have also been established utilizing this method (Garcia-Rodriguez et al., 2010; Goncalves & Alpendurada, 2004). Triple quadrupole (QqQ) analyzers represent 3-stage tandem-in-space detectors that are commonly used in pesticide residue analysis. The system offers the advantages of high scan speed that allows for simultaneous determination of roughly 25-30 target compounds at a time, thereby reducing analysis time and enhancing selectivity such that false positive results are minimized. (A. Garrido Frenich, J.L. Martínez Vidal et al., 2006; A. Garrido-Frenich, R. Romero-González, et al., 2006; Pitarch et al., 2007; P. Plaza Bolaños, J. L. Fernández Moreno, et al., 2007; P. Plaza Bolaños, A. Garrido Frenich, et al., 2007; Qu et al., 2010). Qu *et al.* incorporated QqQ-MS to examine leeks, representing complex matrices of pigments and sulfur, for OP pesticide contamination (Qu et al., 2010).
Time-of-flight (TOF) mass spectrometry detects OP pesticides by way of recording the ion *m/z* ratio as a result of the time it takes the particles to reach a microchannel plate detector. TOF-MS can have higher spectral acquisition rates with accurate mass information, yet suffer limited dynamic ranges as compared to quadrupole instruments (Hayward & Wong, 2009). Nevertheless, more recently TOF-MS instruments have been employed for the determination of OP pesticides in agriculture (Banerjee et al., 2008; Cochran, 2008; Hayward & Wong, 2009), food (Patil et al., 2009), and surface waters (Matamoros et al., 2010).

3.1.4 Data acquisition modes

Data acquisition modes are another important process for the GC-MS detection system and can enhance detection sensitivity and selectivity. For an extensive range of ion spectra, MS systems typically run in full scan mode. However, that could be time consuming, especially for routine analysis of various commodities and environmental samples. Data acquisition modes that limit particular analyte *m/z* ratios that are transmitted and/or detected by the instrument include selective ion monitoring (SIM), selective ion storage (SIS), and selective reaction monitoring (SRM). SIM significantly increases sensitivity of OP pesticides detection examined in a range of agricultural (Albero, B.; Sanchez-Brunete, C.; Donoso, A. et al., 2004; Gelsomino et al., 1997; Lambropoulou & Albanis, 2003; W. Zhang et al., 2006), food products (Jeong et al., 2008; Jiang et al., 2009; Mastovska at al., 2001; Wong et al., 2006), biological (S. Liu & Pleil, 2002; Simonelli et al., 2007), and environmental (S. Liu & Pleil, 2002) matrices (Berhanu et al., 2008; Lambropoulou et al., 2004). EI generates large quantities of ion fragments, thus SIM is often operated during the analysis of specific analytes in attempts to increase the sensitivity and selectivity of OP pesticide detection (Albero et al., 2004; Berger-Preiss & Elflein, 2006; Nguyen et al., 2008; Rissato et al., 2007; Stajnbaher & Zupancic-Kralj, 2003; Wong et al., 2004; Wong et al., 2007; Zhou et al., 2007). SIM has also led to ppb LODs in other GC-MS instrument configurations (Hayward & Wong, 2009; Russo et al., 2002).

Through special control of an ion trap during its ionization phase, only preselected ions of analytical interest may be stored in selective ion storage mode (SIS) for detection even during the co-elution of multi components, or complex matrices (Ma et al., 2001; Shuling et al., 2007b). Shuling *et al*. were able to determine 100 pesticides (ppm) in vegetables within 35 min upon employing GC-IT-MS in the SIS mode (Shuling et al., 2007b). Selected reaction monitoring is a highly specific analysis method that when performed with tandem MS, permits simultaneous measurement of a high number of MS/MS transitions, thus providing chromatographic peaks with an adequate number of scans (Frenich et al., 2008; Pitarch et al., 2007; P. Plaza Bolaños, A. Garrido Frenich, et al., 2007; Qu et al., 2010). In this type of experiment a specific fragment ion of a particular parent ion is detected, thus reducing the SRM chromatogram to represent only ions of interest. Frenich *et al*. noted that sensitivity in SRM depends on the efficiency of the isolation of the precursor ions, the CID fragmentation yield and the specificity of the selected transitions when choosing the ionization source and analyzer combination (Frenich et al., 2008).

3.2 Liquid chromatography

Often times, pesticide analysis via GC-MS experimental conditions may not be most effective. An alternative method is the application of liquid chromatography mass spectrometry (LC-MS), a rapid and efficient alternative (Alder et al., 2006; Sharma et al., 2010). LC-MS is gaining popularity with the advancing of soft ionization techniques that generate molecular ions that can be more easily monitored (John et al., 2008). For more detailed information, we refer the readers to the references (John et al., 2008) and on the specific application of LC-MS for OP determination for food safety (Pico et al., 2006) and biological exposure monitoring (Hernandez et al., 2005).

3.2.1 Extraction methods

The range of OP pesticide polarities precludes the establishment of a universal procedure for efficient sample preparation. Compounding that issue is the complexity of sample matrices, for example, biological specimen contain lipids, proteins, and salts; or food samples, which include sugars, amino acids, and pigments. Several extraction techniques have been studied for spectral optimization. Precipitation extraction is a simple and frequently used method for clinical specimen with rapid performance with a low cost (Inoue et al., 2007). Inoue *et al*. deproteinated serum samples by acetonitrile precipitation to analyze 10 OP pesticides (Inoue et al., 2007). The supernatant was directly injected for LC-APCI-MS measurements, yielding recoveries over 60%. Ultrasonic solvent extraction (USE) is another low cost and effective preparation method for multi-residue OP pesticides at ppb levels (Garcia-Valcarcel & Tadeo, 2009; Pan et al., 2008). USE provides efficient contact between sample matrix and solvent by increasing analyte-extractant interface contact, with acoustic cavitation, mechanical function and thermal function having direct effects on the extraction efficiency. The procedure proposed by Pan *et al*. allowed extraction of 6 pesticides in a single step with a small volume of ethyl acetate for a short 35 min sonication time (Pan et al., 2008). This optimized extraction method provided analyte recovery rates over 83% and low ppb LOQs. Determining trace levels of pesticides in fruits by pressurized liquid extraction (PLE) offers low solvent use and short extraction times, with recoveries ranging from 58% to 97% (Blasco et al., 2005). Prior to analysis by a combined IT/QqQ LC-MS, samples were extracted at 75 °C and 1500 psi, using ethyl acetate as the extraction solvent and acidic alumina as they drying agent (Blasco et al., 2005). Another liquid extraction method used

ethyl acetate to extract 57 multi-residues in one single determination step at 0.01 mg/kg (Jansson et al., 2004).

SPE is a widely used technique for sample preparation, mainly due to optimization of extraction efficiency by appropriate choice of SPE sorbent material (John et al., 2008). On-line SPE-LC mass spectrometers fitted with turbulent-flow columns allowed complete OP extraction from surface and drinking water with good recoveries in less than 14 min (Asperger et al., 2002; Koal et al., 2003). OP pesticides extracted from fruits and vegetable samples with acetonitrile and dispersive SPE, with primary or secondary amines as the sorbent, rendered simultaneous determination, with recoveries in the range of 70-100% and RSD values less than 8% (M. Liu et al., 2005). Blasco *et al*. applied SPME and SBSE extraction methods to determine OP contamination in honey samples (Blasco et al., 2004; Blasco et al., 2008.). They optimized parameters affecting the sorption process, such as sample volume (25 mL), sorption and desorption times (120 min total extraction time), ionic strength (30% w/w NaCl), elution solvent (methanol), and dilution, and found that SBSE exhibited higher concentration capability, and accuracy (5-20 times) and sensitivity (10-50 times) greater than that of SPME (Blasco et al., 2004). It has been demonstrated that both self-contained working separation steps, and on-line multistep separation techniques prove viable in OP pesticide determination in various matrices. If further recommendation is needed, Niessen *et al*. provide a review on matrix effects in quantitative pesticide analysis using LC-MS (Niessen et al., 2006). Here, we have highlighted selective and efficient extraction techniques.

3.2.2 Ionization sources

Efficient ionization of OP analytes is necessary for reliable and sensitive measurement. Hard ionization impact, e.g. EI, generate a full range of fragment ions that offer detailed information. On the other hand, soft ionization impact, e.g. APCI and ESI, produce molecular ions that can be more easily monitored for specific analytes. Here we discuss the ionization sources used to detect trace levels of OP toxicants.

Thermospray ionization is generally carried out by filament activation, whereby emission of the electron beam from the heated filament supports ionization of the vaporized mobile phase and analytes. Analysis takes place in either positive (PI) or in negative (NI) ionization mode (Barcelo et al., 1993; Lacorte & Barcelo, 1995). Upon monitoring two main ions, for example, $[M+H]^+$ and $[M + NH_4]^+$ for PI detection mode, and $[M-H]^-$ and $[M+HCOO]^-$ for NI detection mode, it is possible to obtain LODs in the ppb range (Barcelo et al., 1993; Lacorte & Barcelo, 1995). LC-MS instruments with APCI have been widely utilized for trace detection of OP levels in a range of samples from the environment (Ingelse et al., 2001; Schreiber et al., 2000; Slobodnik et al., 1996) to agriculture (Mol et al., 2003; Titato et al., 2007) and biological specimen (Inoue et al., 2009), due to APCI's lack of signal suppression from matrix components. Ingelse *et al*. demonstrated APCI usefulness for detecting very polar OP pesticides in water (Ingelse et al., 2001). APCI-MS was performed in PI mode, with the nebulizer heated to 400°C and 50 psi held for curtain, nebulizer, and auxiliary gases. They found that solvent mixtures of methanol/water (50/50 v/v) improved analyte response by a factor of 2, and the addition of 0.1% acetic acid also improved the signal-to-noise ratio for the polar compounds. Fernández and collaborators used APCI LC-MS to detect trace levels of OP pesticides in honeybees as a means to assess pesticide exposure in agricultural fields (Blasco et al., 2003; Fernández et al., 2001a, 2002b, 2003c; Ghini et al., 2004). Optimized to achieve better sensitivity and specificity for a range of OP pesticides,

APCI parameters were established as having a 350°C vaporizing temperature with nitrogen as the nebulization gas and drying gas, and a corona current of 4 µA and 25 µA for PI and NI mode, respectively (Blasco et al., 2003; Fernandez et al., 2001; Fernandez et al., 2002; Fernandez et al., 2003; Ghini et al., 2004).

Electrospray ionization (ESI) is another commonly incorporated ionization technique in LC-MS that is used for rapid and sensitive OP detection. For optimal OP detection in clinical samples, for example, urine and serum, ESI has been integrated in LC tandem mass spectrometry (Araoud et al., 2010; John et al., 2010; Olsson et al., 2003). The LC-ESI-MS/MS detection system exploited by John *et al*. proved robust and highly selective for simultaneous quantification of pesticides in porcine urine and plasma, with established intra- and inter-day RSD precision values between 1-14% and accuracy values ranging between 90-115% (John et al., 2010). Kmellar *et al*. used an acetonitrile-based QuEChERS preparation method and ESI in PI mode for high water, high sugar, and high acidic content produce commodities (Kmellar et al., 2008). Mixed soft, medium, and strong matrix effects were observed for the compounds studied, yet more than 90% of the investigated compounds had LODs in the ppb range, thus supporting ESI robustness. ESI has also demonstrated efficacy for polar OP pesticide detection in aqueous samples (Kuster et al., 2008; Molina et al., 1994). While not utilized as often as the aforementioned soft impact ionization techniques, direct-EI provided sensitive detection of OP pesticides commonly distributed in local sugar beet cultivation water. The direct-EI interface mechanism is based on the formation of aerosols in high vacuum conditions followed by a quick droplet desolvation and final vaporization of the solute prior to ionization (Cappiello et al., 1996). Azinphos-methyl, parathion-methyl, azinphos-ethyl, and parathion-ethyl were detected at a concentration level of ~3 ng/L in real sugar beet water cultivation samples.

	positive mode			negative mode		
compound	SIM ion (*m/z*)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	SIM ion (*m/z*)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
azinphos-ethyl	346	10	32	185	8	25
bromophos		nd	nd	349	5	15
chlorpyriphos-methyl	322	10	32	302	7	21
coumaphos	363	6	18	361	4	12
diazinon	305	2	6	275	17	51
dimethoate	230	4	12	214	5	15
fenofos	247	2	6	153	15	45
heptenophos	251	4	12	235	30	90
malathion	285	6	18	157	13	42
methidathion	145	90	200	287	8	24
paraoxon	248	25	75	232	8	25
parathion-ethyl		nd	nd	262	2	7
parathion-methyl		nd	nd	138	1	4
phenthoate	321	60	180	319	8	24
phosalone	368	12	36	338	4	13
phosmet	160	15	45	157	20	60
pirimiphos-ethyl	334	1	4	304	5	15
pirimiphos-methyl	306	2	7	290	7	21
pyrazophos	374	3	9	372	1	3
quinalphos	299	4	12	169	10	30
triazophos	314	5	15		nd	nd
vamidothion	288	4	12	272	5	17

Table 2. Limits of detection (LOD) and quantification (LOQ) in both ionization modes of APCI and the ion used for quantification in LC-MS to assess OP levels in honeybees. (Taken with permission from Reference Fernandez et al., 2001)

3.2.3 Mass analyzers

Optimized accuracy for measurement of analyte mass results in more reliable and precise data. Tandem mass detectors enhance the selectivity and sensitivity of analytes studied in

complex matrices, especially when coupled to various column chromatography separation techniques; liquid-liquid (Sancho et al., 2000), hydrophilic interaction liquid chromatography (Hayama et al., 2008), high pressure liquid chromatography (Salm et al., 2009), and ultra performance liquid chromatography (G. Chen et al., 2011). G. Chen *et al*. developed optimum tandem analyzer parameters, including ion transition, collision energy, and cone voltage for analysis of OP pesticides in multi-residues of tea matrices, which also contain pigments, organic acids, and caffeine interferents (G. Chen et al., 2011). LOQs were measured at 0.01 mg/kg across all pesticides, and reproducibility for OP pesticides analyzed was less than 29% RSD. More recently, triple quadrupole tandem mass spectrometry (QqQ), operating with ESI, has been exploited as a highly accurate and precise method to analyze OP constituents of environmental (Barco-Bonilla et al., 2010; Rodrigues et al., 2007) and food samples (Chung & Chan, 2010; S. Guan et al., 2011). S. Guan *et al*. optimized performance and sensitivity of the QqQ-MS/MS by using high-purity nitrogen as the collision gas, and operating quadrupoles Q1 and Q3 at unit resolution to produce the highest intensity for the main fragment (S. Guan et al., 2011). A good linear relationship between response and concentration over a selected range was established, with LODs as low as 0.06-0.15 µg/kg and LOQs of 0.2-0.5 µg/kg for 9 OP pesticides.

Other tandem MS/MS hybrid systems, namely between ion trap and quadrupole analyzers, are also utilized for trace level pesticide detection in food (C. Ferrer et al., 2005), human blood (Salm et al., 2009), and drinking water (S. N. Sinha, K. Vasudev, et al., 2011). Salm *et al*. established compound dependent parameters of the HPLC-IT/Q-MS/MS that led to reduced sample preparation and low LODs (Salm et al., 2009). Parameters were set for dwell times between 50-100 ms, declustering potentials between 20-50 V, and collision energies between 30-35 eV, depending on the type of OP pesticide. Excellent chromatographic separation using time-of-flight LC-MS was provided in a 30 min interval by using narrow accurate mass windows (0.05 Da) in the determination of food and water OP pesticide contaminants (I. Ferrer & Thurman, 2007).

3.2.4 Data acquisition modes

Applying an appropriate MS scan mode helps to optimize selectivity and limits of detection. When matrices are less complex, full-scan or simple SIM mode are sufficient. However, there are other data acquisition modes that may be more appropriate to use, as is the case with tandem MS. Multiple reaction monitoring (MRM) is a highly specific monitoring mode that improves signal-to-noise ratios and maximizes sensitivity as a result of only data on the analyte of interest is collected. This in turn allows for faster flow rates into the ion source and quicker analysis. Typically two MRM transitions (parent ion → daughter ion) and the corresponding MS conditions are chosen for each pesticide under analysis, as demonstrated for 9 OP pesticides determined in fruit and vegetable samples by LC-ESI-QqQ-MS/MS as shown in Table 3 (S. Guan et al., 2011). MRM is frequently exploited for APCI (Ingelse et al., 2001; Mol et al., 2003) and ESI (Araoud et al., 2010; Chung & Chan, 2010; S. Guan et al., 2011; John et al., 2010) ionization techniques as more abundant molecular parent ions are generated. Additionally, other selective data acquisition modes have been used alongside APCI and ESI, including selected ion-recording (SIR) (Titato et al., 2007) and selected reaction monitoring (SRS) (Hayama et al., 2008; Raina & Sun, 2008).

3.3 Other types of MS

While coupled GC and LC mass spectrometry systems are the primary choice for OP determination, other mass spectrometry methods have also been investigated. For instance,

Retention time (min)	Analyte	Parent ion	Daughter ion (p1/p2)	Fragmentor potential (V)	Collision energy (V)
1.24	Methamidophos	142.0	94.0 125.1	80 80	15 10
2.98	Monocrotophos	224.0	127.1 192.6	100 100	20 5
3.35	Mevinphos	225.2	193.3 127.1	80 80	10 15
4.97	Methidathion	303.2	85.0 145.1	80 80	10 5
5.44	Parathion-methyl	264.2	232.1 125.2	120 120	15 20
5.95	Malathion	331.3	127.1 285.2	80 80	10 5
6.95	Parathion-ethyl	292.2	236.0 264.0	120 120	10 5
7.51	Diazinon	305.2	169.0 153.2	160 160	20 20
8.50	Ethion	385.0	171.2 199.0	80 80	15 5

Table 3. Parent/daughter ions and conditions used for MRM mode of LC-ESI-QqQ-MS/MS analysis. (Taken with permission from Reference S. Guan et al., 2011).

fiber introduction mass spectrometry (FIMS) is a relatively new technique which directly couples SPME and MS without chromatographic separation. In this technique, the SPME fiber containing the sorbed analytes is directly introduced into the ionization chamber of a mass spectrometer and placed between two EI filaments, which cause desorption of the extracted species directly in the region of maximum ionization power (Cesar da Silva et al., 2007). Cesar da Silva *et al*. have shown FIMS to be an effective alternative to determine OP pesticides, offering simplicity, speed, selectivity, and robustness with low LOD and LOQ values. Fiber coating was important as the PDMS/PVA composite coated fiber has been shown to provide fast detection and better signal resolution than PDMS/DVB coating.

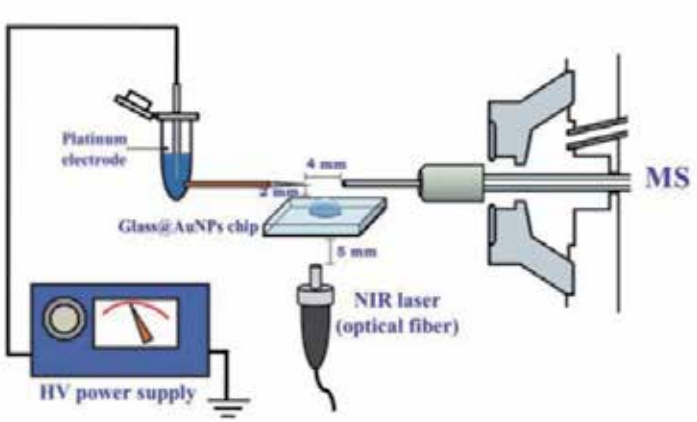


Fig. 6. Representation of the TDAMS system used to detect small OP analytes. (Taken with permission from Reference (Lin et al., 2010))

Lin *et al*. studied multilayered gold nanoparticles (Au NPs) as an NIR energy absorber and sample holder for sample deposition at ambient conditions during a thermal desorption-based ambient mass spectrometry (TDAMS) analysis of small OP compounds, e.g. methamidophos (Lin et al., 2010). A NIR laser diode (808 nm) was employed as the thermal desorption source to liberate molecules from the self-assembled Au NP layers prior to ESI and IT analysis. Because the analysis was carried out under ambient conditions, direct analysis at atmospheric pressure with minimal sample preparation offered advantages of speed and easy use. Figure 6 represents the setup of the TDAMS system (Lin et al., 2010). While this MS detection system offers speed and simplicity, it is only suitable for small organics, and one must be mindful to the configuration of the laser focusing beam which is critical.

Inductively coupled plasma mass spectrometry (ICP-MS), coupled to capillary electrophoresis (CE) (Wuilloud et al., 2005; Yang et al., 2009) or GC (Fidalgo-Used et al., 2005a, 2006b; Profrock et al., 2004; Vonderheide et al., 2003), is another approach used for OP analysis. For OP detection, ICP-MS monitors ^{13}P levels as a means to identify OP analytes in a given matrix. Argon sustained plasma ICP-MS methods suffer from low ionization efficiency and interference from polyatomic ions $^{14}N^{16}O^{1}H^{+}$ and $^{15}N^{16}O^{+}$, which overlap phosphorus's only isotope peak at m/z = 31; thus different experimental conditions have been exploited as a means to overcome these limitations. The addition of small percentages of nitrogen in the carrier flow has proven to enhance the sensitivity for GC coupled ICP-MS (Fidalgo-Used et al., 2005a; Vonderheide et al., 2003); for example, a N_2 flow rate between 8-8.5% permitted ruelene detection in the ppt level (Fidalgo-Used et al., 2006b). Using He as a collision gas in a collision cell was found to further reduce background noise without affecting analyte signal (Profrock et al., 2004; Vonderheide et al., 2003). Wuilloud *et al*. also noted that selection of a proper make-up electrolyte solution helps to eliminate polyatomic interferences, as is the case when using a sodium borate solution (Wuilloud et al., 2005). Yang *et al*. employed a collective sample introduction technique that allowed for the simultaneous determination and quantification of OP pesticides in vegetable samples (Figure 7) (Yang et al., 2009). The collective sample-introduction technique reduced the dilution of analyte and makeup volume, and narrowed the peak width, which resulted in higher sensitivity, lower LOD, and better electrophoretic resolution as compared to continuous sample-introduction.

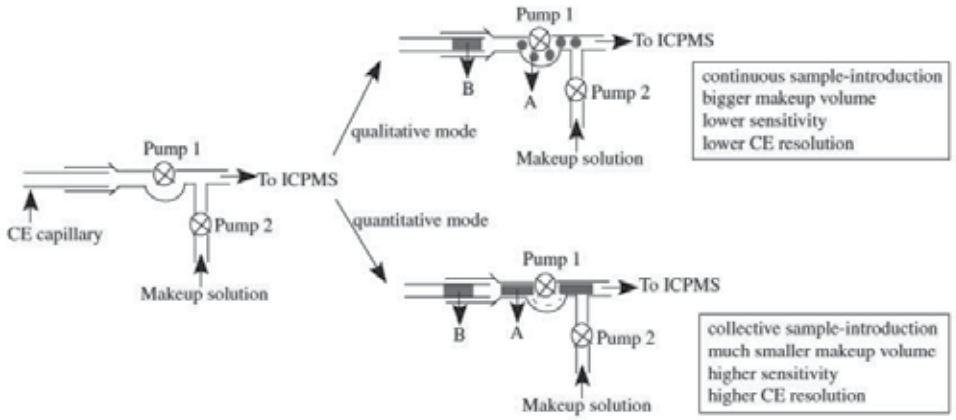


Fig. 7. Schematic of continuous sample-introduction (qualitative mode) and collective sample-introduction (quantitative mode). (Taken with permission from Reference Yang et al., 2009).

Herein, mass spectrometry and experimental design based on extraction methods, ionization, detectors, and data acquisition modes have been discussed. Gas and liquid chromatography remain the most prevalently used detection systems for regular analysis of trace levels of OP pesticides in environmental, clinical, and food product samples. MS methods involving ICP or direct fiber introduction, although highly useful, have not been employed as frequently. Given these consideration and the recent advances it is clear that MS analysis offers high sensitivity and selectivity for accurate determination of OP pesticides.

4. Optical sensors

The past decade has shown several advancements in the development of selective biosensors and chemosensors for the detection of OP pesticides via optical and fluorescence spectroscopy. Below we highlight some of the most notable work reported recently in the literature. Additional information is also available in a review article published by Obare and coworkers (Obare et al., 2010).

4.1 Fluorescence-based biosensors for OP compounds

Fluorescence-based sensors, both biosensors and chemosensors, offer significant advantages over other conventional methods for detection of OP compounds. The principal advantages of fluorescence are its high single-molecule sensitivity and in most cases almost an instantaneous response. Fluorescence methods are capable of measuring concentrations of analytes 10^6 times smaller than absorbance techniques (Brufani et al., 1986). Thus, fluorescence techniques have been widely used in molecular biology and analytical chemistry but not extensively in the detection of OP pesticides. To date, a number of sensitive biosensors based on acetylcholinesterase (AChE) or butyryl cholinesterase (BChE) inhibition have been developed and used for OP compound detection (Cao et al., 2004; Brufani et al., 1986; Burnworth et al., 2007; Evtugyn et al., 1996; Lei et al., 2005; Mionetto et al., 1994; Palleschi et al., 1992; Rogers et al., 1991; Russell et al., 2003). In general, enzyme-based sensors for the detection of OP compounds can be broadly categorized into two major classes based on the enzyme employed-(1) AChE or (2) hydrolase (OPH).

Hydrolysis of acetylcholine by AChE produces one proton per substrate molecule resulting in an increase in the acidity of the solution. This forms the basis for AChE-based sensors. Rogers *et al*. used a pH-sensitive fluorescent dye, consisting of AChE linked to the pH-sensitive compound fluorescein isothiocyanate (FITC) (Rogers et al., 1991). The enzyme-dye adduct was immobilized on a quartz fiber which was attached to a fluorescence spectrophotometer. In the absence of an OP compound, the labeled AChE was able to hydrolyze acetylcholine leading to a decrease in pH which resulted in the reduction of the FITC fluorescence intensity due to interruption of the fluorophore's conjugation upon protonation. However, in the presence of diisopropylfluorophosphate (DFP) and subsequently acetylcholine, it was observed that 90% of the enzyme activity was lost, which was quantified by a less pronounced reduction of the fluorescence intensity. This biosensor was found to be very sensitive (capable of detecting nanomolar (nM) concentrations of paraoxon when exposed to the solution containing the analyte for ten minutes), and it demonstrated some selectivity toward different OP pesticides.

The second family of biosensors utilizes OPH as the enzymatic sensor for the detection of OP compounds. The mode of action of OPH is different from AChE; it catalytically

hydrolyses the OP compound, as illustrated in Figure 8, instead of covalently binding to it. Thus, instead of measuring the enzyme inhibition, detection methods involving OPH allow for a more direct measurement of OP compounds. Nowadays, OPH is widely used as a biosensor because of its ability to hydrolyze a wide range of compounds containing P-O, P-F, P-S, or P-CN bonds (Burnworth et al., 2007; H. Cao et al., 2007). Hydrolysis of the OP compounds led to the stoichiometric production of two protons which can be monitored and directly correlated to the amount of OP substrate (Dave et al., 1993). For instance, X. Cao *et al.* labeled OPH with FITC and deposited the resulting material onto silanized quartz slides in the form of Langmuir-Blodgett films thus creating organized monolayers of the enzyme-based sensors (X. Cao et al., 2004). It was demonstrated that this OPH based enzyme sensor showed enhanced sensitivity and could detect the analyte at nM concentrations.

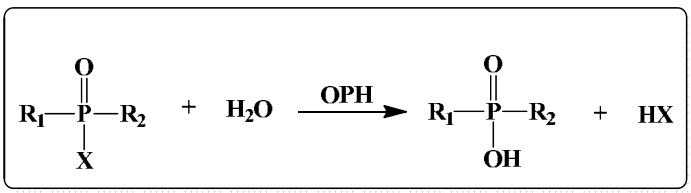


Fig. 8. Mechanism for the hydrolysis of OP compounds by OPH.

A number of biosensors have been developed based on fluorescence polarization immunoassays (FPIA) (Lee et al., 2005; Kim et al., 2003; Kolosova et al., 2003; Kolosova et al., 2004; Tang et al., 2008). One example reported by Kolosova *et al*. showed the use of a monoclonal antibody for the detection of parathion-methyl using FPIA (Kolosova et al., 2003). The sensing unit comprised a parathion-methyl derivative linked to fluorescein. Binding to parathion methyl or other closely related compounds was confirmed by measuring the intensity of emitted polarized light which indicated antibody binding. Despite the susceptibility of interference with different components existing in some matrices and the wide determinative range, the FPIA method is highly specific and reproducible and without complicated cleanup the method meets the performance criteria for detecting parathion-methyl.

In summary, enzyme based sensors are both very sensitive and selective in their approach to detect OP compounds. Furthermore, OPH based enzyme sensors offer distinct advantages over AChE-based systems. While these approaches towards OP detection have been significant, the inhibition-based biosensors suffer from three drawbacks: (1) the enzymes easily lose activity in the event of environmental or handling factors, therefore these enzymes may provide false positive signals, (2) the sensors require baseline testing prior to sample application and lengthy incubation times to allow enzyme-analyte interaction, and (3) due to the irreversible nature of cholinesterase enzyme inhibition, inhibition-based sensors cannot be reused without regeneration of enzyme activity. In addition, the lifetime of these sensors is limited by enzyme degradation.

4.2 Fluorescence-based chemosensor detection methods

Recently, a number of innovative methods for the detection of OP compounds based on optical chemosensors have been reported in the literature. Delattre and co-workers reported a cyclodextrin (CD) based fluorescent sensor for the detection of pesticides in water (Delattre

et al., 2009). D-Glucopyranose units in CDs form truncated cone-shaped molecules with a hydrophobic cavity, which can induce the inclusion phenomena of a guest, as shown in Figure 9. The dipole of the macromolecular system varies with the entry of a guest molecule. A modified β-cyclodextrin, pyridinoindolizin-β-cyclodextrin, was used to detect pesticides and herbicides, linadane, parathion, malathion, imidacloprid, atrazine, and simazine, through an inclusion complex between the pesticide or herbicide and the hydrophobic cavity of the macrocycle. This interaction leads to fluorescence quenching of the fluorophore. An advantage of this fluorescence sensor is the ability to quantify concentration data via fluorescence intensity concentration-dependence.

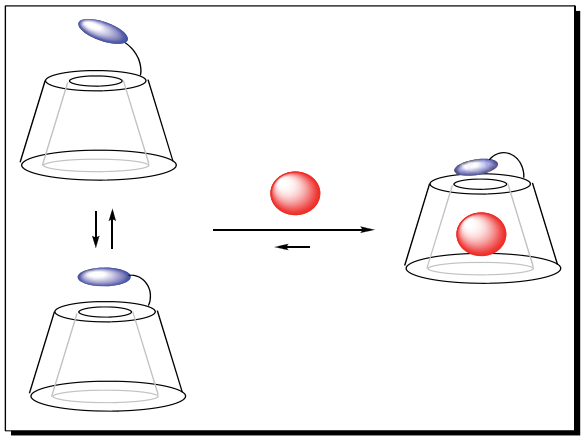


Fig. 9. Inclusion phenomena of a guest in CDs molecules. Reproduced with permission from Reference (Delattre et al., 2009), published by Bentham Science, 2009.

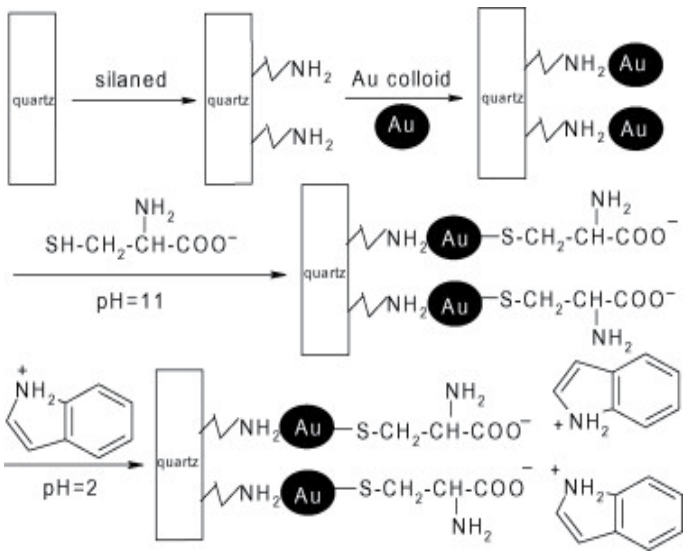


Fig. 10. Representation of the formation of the indole-based SAM sensor. (Reproduced with permission from Reference Sun et al., 2008)

A self-assembled multilayer (SAM) consisting of amino-silanized quartz functionalized with gold nanoparticles and coated with indole via a L-cysteine linker was fabricated as shown in Figure 10 (Sun et al., 2008). When the SAM sensor was exposed to the pesticide, the indole group of the sensor on the modified film was oxidized to a fluorescent indoxyl group. The oxidation process depended on the pesticide concentration and was reflected by changes in intensity. The sensor was capable of detecting methylparathion and monocrotophos in the ppm and ppb range, respectively. An advantage of the indole-based SAM sensor is that it could detect OP pesticides in ionic and other environmental species, but it was subject to interference at 20 equivalents of Fe^{3+} ions.

4.3 Sensors with multiple modes of signal transduction

There is a growing awareness and trend toward the development of multimodal systems reminiscent of living organisms that utilize multiple senses to intelligently respond to multiple stimuli in real-world environments. A major advantage of mutimodal sensors is the minimization of false positives. With this in mind, we have recently developed and reported new chemosensors with multimodal sensing capabilities for analytes such as saccharides (Beaudoin & Obare, 2008) and toxic OP compounds (De et al., 2010). Our design strategy, shown in Figure 11, utilizes and couples the electrophilic reactivity of the pentavalent phosphorus atom of the phosphoryl and thiophosphoryl groups of toxic OPs to a nucleophilic fluorophore capable of recognizing and reporting sensor—analyte interactions. We have shown that the azastilbene, dimethyl-[4-(2-quinolin-2-yl-vinyl)-phenyl]-amine (DQA), Figure 12, recognizes, reacts with and responds to the pesticides: ethion, malathion, parathion, and fenthion. DQA binding to either of the above mentioned pesticides resulted in changes of the UV-visible and cyclic voltammogram of DQA (De et al., 2010) indicating the selective binding.

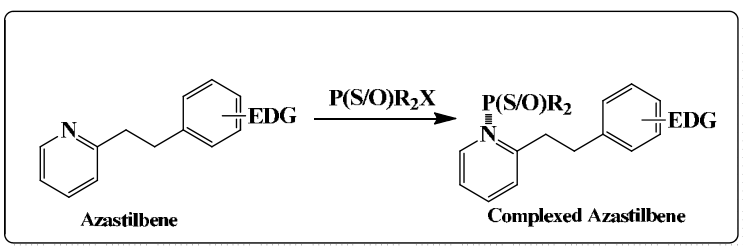
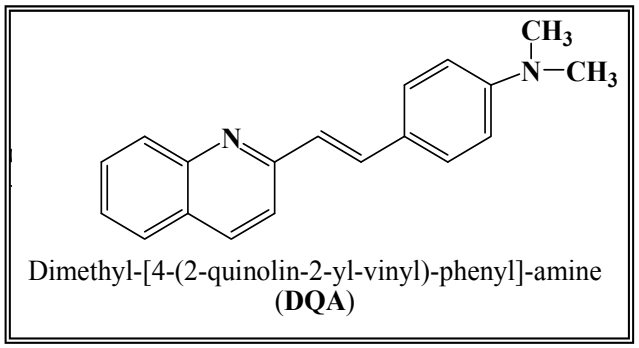


Fig. 11. Schematic representation of uncomplexed (*left*) and complexed (*right*) azastilbene (De et al., 2010). (EDG = electron donating group).

DQA was titrated with the pesticides, with ethion, malathion, parathion and fenthion and changes in the UV-visible absorbance spectrum of DQA were measured. As shown in Figure 13a, increase in ethion concentration to a solution of DQA in acetonitrile resulted in the decrease in the UV-visible absorbance intensity at 385 nm, and was accompanied by formation of two new peaks at 325 nm and at 500 nm. Similar behavior was observed in the case of malathion, except two new peaks arise at 330 nm and 505 nm, as shown in Figure 13b. In both cases two isosbestic points were observed at 340 nm and 425 nm for ethion, and at 335 nm and 430 nm for malathion. Furthermore, we observed that titration of parathion to a solution of DQA in acetonitrile did not result in the quenching of the 325 nm peak,

however, a new peak at 505 nm formed and increased in intensity with an increase in parathion concentration as shown in Figure 13c. On the other hand, addition of fenthion to the DQA solution did not show any notable changes in the original absorbance of DQA as shown in Figure 13d. Changes in the UV-visible absorbance spectrum show that DQA is efficient in distinguishing between the four OP pesticides and results in different colored solutions with different λ_{max} values. The method of continuous variation was used to determine the stoichiometry of DQA with ethion, malathion and parathion. In each case, it was found that a 1:1 DQA-OP complex formed. Based on the 1:1 stoichiometry, binding constants were calculated to be 6.5×10^4 M^{-1}, 1.1×10^4 M^{-1}, and 0.2×10^4 M^{-1} for ethion, malathion, and parathion, respectively. At the end of the DQA titrations with ethion, malathion and parathion, the solution color had changed from yellow to red-orange, orange and peach-orange, respectively. The same color changes in DQA were also observed when saturation concentrations of OP pesticides were added. No color change was observed when fenthion was added to DQA.



Dimethyl-[4-(2-quinolin-2-yl-vinyl)-phenyl]-amine
(**DQA**)

Fig. 12. Chemical Structure of DQA.

Molecules that provide optical and electrochemical signals are ideal for developing sensors that offer dual signal transductions (Ko & Park, 2006). Cyclic voltammograms were acquired using a BAS CV50 electrochemical workstation using glassy carbon as the working electrode, a platinum wire as the counter electrode, and Ag/AgCl as the reference electrode. The electrolyte was a 0.1 M solution of tetrabutyl ammonium hexafluorophosphate ($TBAPF_6$). DQA was found to have a formal potential (E^0) at 860 mV *vs.* Ag/AgCl. Changes in the electrochemical waves of DQA with 1 equivalent of the pesticides ethion, malathion, parathion and fenthion were measured. In the case of ethion, malathion and parathion, the DQA-OP complex formed had significantly different redox characteristics relative to DQA, Figure 14. The DQA/ethion complex showed three redox waves at $E_{1/2}$ = −875 mV *vs.* Ag/AgCl, $E_{1/2}$ = −500 mV *vs.* Ag/AgCl and $E_{1/2}$ = +500 mV *vs.* Ag/AgCl. The cyclic voltammogram of the DQA/malathion complex was also different relative to that of DQA; in this case two waves at $E_{1/2}$ = −1,498 mV *vs.* Ag/AgCl and $E_{1/2}$ = −870 mV *vs.* Ag/AgCl) corresponding to DQA-malathion complex were observed.

The formation of a DQA/parathion complex also demonstrated significant changes in the redox behavior ($E_{1/2}$ = −1,072 mV *vs.* Ag/AgCl, $E_{1/2}$ = −773 mV *vs.* Ag/AgCl) in comparison to DQA. As expected, there were no changes in the redox behavior of DQA with the addition of fenthion. The observed DQA-OP reactions can be explained by Lewis acid-base or nucleophile-electrophile interactions between the quinolinyl nitrogen and the OP

phosphorus atoms. Reactions of electrophiles (for example, proton, metal cations, and carbon-based) with 4-dimethylamino styrylazaaromatics occurs exclusively at the 'ring' (pyridyl, quinolinyl) nitrogen (Allen & Dunaway-Mariano, 2004). This generally results in the formation of the corresponding quaternary pyridinium and quinolinum salts. It is thus reasonable to assume that electrophilic phosphorus reactants will also react preferentially at the azaaromatic 'ring' nitrogen. Furthermore, our computational calculations done by GAUSSIAN 03 program suite (Frisch et al., 2004) reveals, as expected, that the electrostatic potential at the quinoline nitrogen is higher relative to the dimethylamino nitrogen. One common mechanistic pathway for phosphoryl transfer reactions is *via* concerted $S_N2(P)$ processes in which a nucleophilic attack on phosphorus leads to expulsion of the leaving group. In these S_N2 scenarios, the reaction rate for the thiophosphoryl transfer is expected to be highly dependent on the leaving group. This in turn will affect the binding constant of the incoming nucleophile. This interpretation is consistent with our results since, for example, it is known that the *p*-nitrophenolate anion of parathion is a much better, more stable leaving group than the phenolate anion of fenthion. Thus, parathion has a stronger binding constant than fenthion to DQA. The interaction of DQA with each OP pesticide relies on the stability of the leaving group - the more stable the OP leaving group, the more likely it will dissociate upon interaction with the nucleophilic DQA quinolinyl nitrogen.

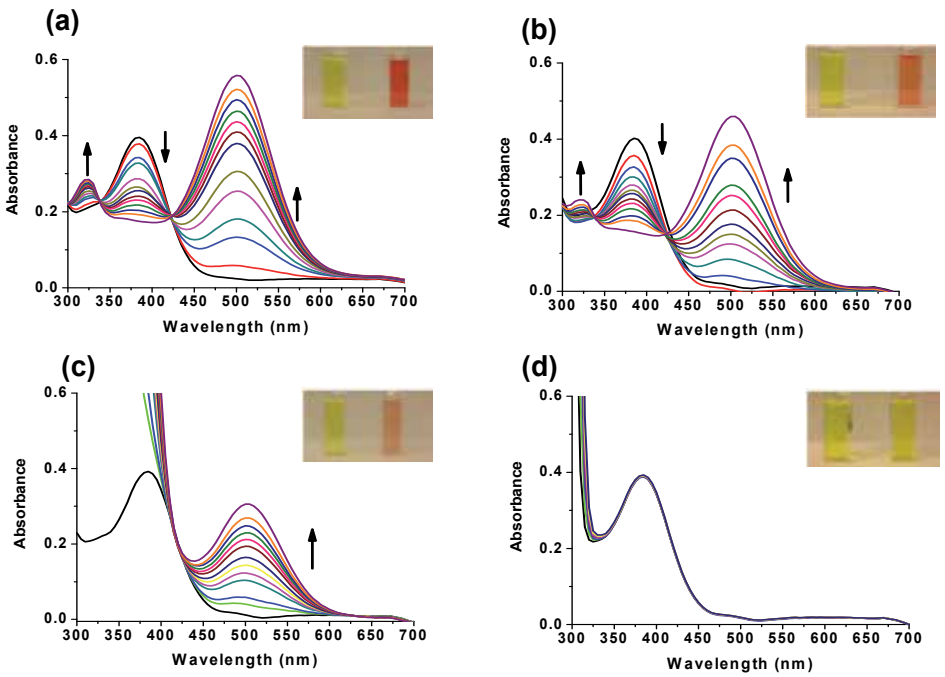


Fig. 13. Changes in UV-visible absorbance of DQA upon binding to OP pesticides: (a) titration with ethion; (b) titration with malathion; (c) titration with parathion; and (d) titration with fenthion. In each case the direction of the arrow indicates concentration of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 μM.

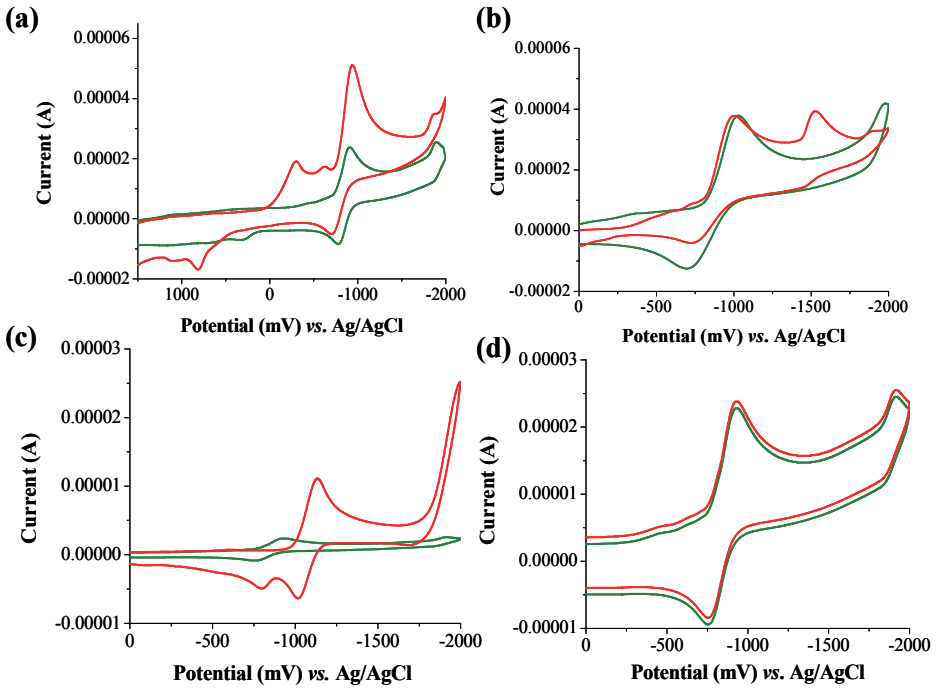


Fig. 14. Cyclic voltammograms of DQA before and after addition of (a) ethion, (b) malathion, (c) parathion, and (d) fenthion.

The optical and electrochemical changes of the azastilbene DQA when exposed to ethion, malathion, parathion and fenthion shows the potential of azastilbenes as viable structural motifs for development of multimodal chemosensors. Azastilbenes have demonstrated the capability of distinguishing between various pesticides, which is important for both environmental as well as homeland security applications. Future work on this project to further develop our azastilbene-based multimodal chemosensors for toxic organophosphates and other important toxic analytes is continuing.

5. Future perspectives

Significant progress has been achieved toward the development of detection methods for toxic OP pesticides. The most common and effective developments have been in mass spectrometric techniques as well as in the development of optical biosensors and chemosensors. While the mass spectrometric methods offer high sensitivity and specificity, they require well trained technicians and experts to run the analysis. In recent years there have been advancements in the development of miniaturized devices that are expected to be portable and operable *in situ*. Such advancements will enable the rapid detection of OP pesticides and in turn lead to improved quality of life. Biosensors and chemosensors are easier to develop for *in situ* analysis. Biosensors offer improved selectivity relative to chemosensors, however, biosensors require careful control of environmental conditions, for example, temperature and pH, otherwise the biosensor could degrade. Chemosensors are expected to have much more robustness and there continue to be increasing research developments in this area. It is clear that future improvements in this area will require the

design of new chemosensors with additional modes for signal transduction. Such sensors will play an important role in minimization or elimination of false-positives. Due to the structural similarity of OP compounds, it is also paramount that the designed sensors are fabricated such that they are highly selective toward specific OP compounds.

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7

Organophosphorus Pesticides Analysis

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1. Introduction

The organophosphorus (OP) pesticides are synthetic esters, amides, or thiol derivatives of the phosphoric, phosphonic, phosphorothioic, or phosphonothioic acids (Corbett et al., 1984; Eto, 1974; Gupta, 2006; Hassall, 1982; Quin, 2000). The structural diversity of this family of compounds is reflected in their physicochemical and biological properties: vapour pressure, solubility in water, chemical stability and toxicity (Corbett et al., 1984; Hassall, 1982; WHO, 1986), which determine their specific application (Hassal, 1982). Compared to the mostly banned in U.S. and Europe organochlorine pesticides, the OP ones are less persistent in the environment, are not subject of bioaccumulation and biomagnification, and do not release toxic break down products (Krieger, 2001). These features justify their application in the agricultural and veterinary practices of the modern world. In 1999 the OP insecticides represented ≈37% of the pesticides in use at a global scale (Table 1) and 72% of the insecticides used in U.S. (Kiely et al., 2004). The top ten OP insecticides active ingredients include malathion, chlorpyrifos, terbufos, diazinon, methyl-parathion, phorate, acephate, phosmet, azinphos-methyl, and dimethoate (Kiely et al., 2004).

Consumption, tons	Year								
	1990	**1991**	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**
OP insecticides	49446	48919	52472	53961	50321	63998	66620	71338	65578
Pesticides total	318528	311415	304063	276699	307901	327488	320934	324942	193491
% OP	15.52	15.70	17.25	19.50	16.34	19.54	20.76	21.95	33.89

Consumption, tons	Year								
	1999	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
OP insecticides	62653	48685	32507	13829	15495	20944	26724	18629	12377
Pesticides total	167912	252176	245064	179724	180897	202817	232515	234688	105940
% OP	37.31	19.31	13.26	7.69	8.56	10.33	11.49	7.94	11.68

Table 1. Summary of the annual OP insecticides consumption (metric tons of active ingredients) and of the annual total pesticides consumption (metric tons of active ingredients), according to the Statistics Division of the Food and Agriculture Organization of the United Nations (FAOSTAT, 1990-2007).

Nevertheless, because of the high acute toxicity of the OP pesticides (Eddleston et al., 2008; Gupta, 2006; Roberts & Aaron, 2007), as well as because of the registered chronic effects (Gupta, 2006), the OP pesticides residues limits in food, drinking water and environmental

samples are subject of regulation and control. The European Council Directive 98/83/EC on the quality of water intended for human consumption (Council Directive 98/83/EC, 1998) sets the limit value of the individual pesticides in drinking water at 0.1 μg L^{-1} and that of the total pesticides at 0.5 μg L^{-1}. According to the U.S. Environmental Protection Agency Office of Ground Water and Drinking Water (OGWDW), the health advisory levels for some OP pesticides in drinking water are: diazinon 3 μg L^{-1}, parathion-methyl 2 μg L^{-1}, disulfoton 1 μg L-1, fenamiphos 2 μg L-1, etc., the following 22 OP pesticides being on the U. S. National Pesticide Survey List: diazinon, dichlorfos, dicrotophos, dimethoate, diphenamiphos, sulfone, disulfoton, disulfoton sulfone, disulfoton sulfoxide, fenamiphos sulfone, fenamiphos sulfoxide, fenitrothion, methyl paraoxon, mevinphos, monocrotophos, omethoate, parathion ethyl, phosphamidon, stirophos, terbufos, tetrachlorvinphos, and merphos. At this time, four European Council Directives (Council Directive 76/895/EEC, 1976; Council Directive 86/362/EEC, 1986; Council Directive 86/363/EEC, 1896; Council Directive 90/642/EEC, 1990) set the maximum residue limits (MRLs) for pesticides in food commodities. Other organisations involved in establishing the pesticide residues levels are the World Health Organization (WHO), the Food and Agricultural Organization of the United Nations (FAO), the Codex Alimentarius Commission, and the U.S. Environmental Protection Agency (EPA). Currently, EPA is reassessing pesticide residue limits in food to ensure that they met the safety standard established by the Food Quality Protection Act of 1996 (FQPA, 1996). Some relevant data on MRLs are presented in Table 2.

Pesticide	MRLs (varie according to the product, mg kg^{-1})		Pesticide	MRLs (varie according to the product, mg kg^{-1})	
	EC	Codex		EC	Codex
acephate	0.05-0.2	0.01-50	malathion	0.02-8	0.01-20
azinphos-methy	0.01-0.5	0.05-10	Parathion-methyl	0.02-5	0.05-1
chlorpyrifos	0.05-5	0.01-5	phorate	0.02-1	0.05-0.1
diazinon	0.01-5	0.01-5	phosmet	0.05-10	0.05-0.2
dimethoate	0.02-2	0.05-5	terbufos	0.01	0.05-0.3

Table 2. Maximum residue limits (MRLs) of pesticides in or on food and feed of plant and animal origin, set by the European Council (EC) regulation No 396/2005 (Reg. EC No 396/2005), and MRLs in food set by the Codex Alimentarius Commission (Codex pesticides residues in food online database, 1996)

The MRL of a number of OP pesticides is set up at or about the limit of their determination by the currently available analytical methods. Thus, in this work are reviewed the developed during the last years (2005-2011) procedures for OP pesticides analysis, including sample pretreatment and determination in the context of the implementation of modern reliable, high sensitive, selective, rapid, cost-effective and environmental friendly analytical techniques for OP pesticides residues quantification. This overview comments on their advantages and limitations.

2. Organophosphorus pesticides analysis

The revision of 115 original publications covering the period 2005-2011 and of 40 reviews demonstrated that the techniques applied for OP pesticides analyses are mostly

chromatographic (gas chromatography and liquid chromatography), electrochemical, immunochemical, and biosensors based ones (Fig. 1).

Fig. 1. Techniques applied for OP pesticides analysis (2005-2011)

2.1 Chromatographic methods for OP pesticides analysis

Chromatography is considered as a powerful analytical technique regarding the analysis of complex matrices. However, the method requires sample pretreatment, which reaches 60% of the total analysis time. Hence, the development of time-saving, jointly with effective and economic procedures is of crucial importance. Some recent publications provide an overview of the promising techniques: solid-phase extraction, solid-phase microextraction, stir-bar sorptive extraction, matrix solid-phase dispersion, solvent extraction, liquid-phase microextraction, super critical fluid extraction, ultrasonication extraction, microwave-accelerated extraction, and membrane-assisted methods, applied to various matrices (Beyer & Biziuk, 2008; Gilbert-López et al., 2009; Hyötyläinen & Riekkola, 2008; Picó et al., 2007; Pinto et al., 2010; Rial-Otero et al., 2007).

The tendencies in the application of gas chromatography and liquid chromatography to pesticides residues determination in environmental samples and food, together with sample preparation procedures are revised by several authors (Le Doux, 2011; Pareja et al., 2010; Sharma et al., 2010; Yusà et al., 2009). The trends in liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry development are highlighted in the published in 2007, 2009, and 2010 reviews (Kuster et al., 2009; Malik et al., 2010; Petrovic et al., 2010; Soler & Picó, 2007).

The original research works devoted to OP pesticides analysis applying various chromatographic techniques: gas chromatography-mass spectrometry (Baugros et al., 2008; Chen & Huang, 2006; Cortés-Aguado et al., 2008; Cunha et al., 2009; Filho et al., 2010a, 2010b; Hassan et al., 2010; Kolberg et al., 2011; Lavagnini et al., 2011; Lesueur et al., 2008a, 2008b; Li et al., 2011b; Libin et al., 2006; López-Feria et al., 2009; Nguyen et al., 2008; Nguyen et al., 2010; Pang et al., 2006; Rodrigues et al., 2010; Silva et al., 2008; Sinha et al., 2006; Toledano et al., 2010; Wang et al., 2007; Wang et al., 2011; Wu et al., 2011; Yang et al., 2011), gas chromatography-tandem mass spectrometry (Barco-Bonilla et al., 2010; Camino-Sánchez et al., 2008; Frenich et al., 2006; Fuentes et al., 2008, 2009; García-Rodríguez et al., 2010; Lee et al., 2008; Qu et al., 2010; Walorczyk, 2008; Walorczyk & Gnusowski, 2009), gas chromatography-ion trap mass spectrometry (González-Rodríguez et al., 2008; Przybylski & Hommet, 2008), gas chromatography time-of-flight mass spectrometry (Hernández et al., 2010; Portolés et al., 2011), high performance liquid chromatography (Akkad et al., 2010; Al-Degs et al., 2009; He et al., 2009; Pérez-Ruiz et al., 2005; Wu et al., 2010; Zhu et al., 2007), high performance liquid chromatography-electrospray ionization-mass spectrometry (Sinha et al., 2010), liquid

chromatography-ion trap-triple stage mass spectrometry (Blasco et al., 2005; Lesueur et al., 2008b), liquid chromatography-mass spectrometry (Inoue et al., 2007; Liu et al., 2005, 2006), liquid chromatography tandem mass spectrometry (Baugros et al., 2008, 2009; Chung & Chan, 2010; Dagnac et al., 2009; Díaz et al., 2008; Dujaković et al., 2010; García-Valcárcel & Tadeo, 2009; Hernández et al., 2006; Kujawski & Namieśnik, 2010; Pang et al., 2006; Pinxteren et al., 2009; Radišić et al., 2009; Salma et al., 2009), and nano-liquid chromatography (Buonasera et al., 2009) comment on the sample preparation procedure, the detection system, and the analytical performances of the method with emphasis on its optimization.

Sample preparation procedures include a large variety of techniques: solid-phase extraction (Al-Degs et al., 2009; Dujaković et al., 2010; Portolés et al., 2011; Wang et al., 2010; Yang et al., 2011; Zhu et al., 2007), solid-phase extraction using carbon nanotubes (López-Feria et al., 2009; Wang et al., 2007), solid-phase microextraction (Chai et al., 2009; Cortés-Aguado et al., 2008; Filho et al., 2010a, 2010b; Tsoutsi et al., 2006), solid-phase microextraction using a new sol-gel hybrid coating (Ibrahim et al., 2010), solid-phase dispersion (Libin et al., 2006; Radišić et al., 2009; Ramos et al., 2009; Silva et al., 2008), headspace-solid-phase microextraction (Rodrigues et al., 2010), microwave-assisted extraction coupled to solid-phase extraction or different clean-up methods (Fuentes et al., 2008, 2009), single-drop microextraction (Ahmadi et al., 2006; Xiao et al., 2006; Pinheiro et al., 2009), cloud point extraction coupled with ultrasonic-assisted back-extraction (Zhao et al., 2011), solvent extraction (Dugo et al., 2005; Fenoll et al., 2007; Georgakopoulos et al., 2009; González-Rodríguez et al., 2008; He et al., 2009; Hernández et al., 2006; Liu et al., 2005; Pang et al., 2006; Sinha et al., 2006, 2010; Walorczyk & Gnusowski, 2009), accelerated solvent extraction and gel permeation clean-up (Wu et al., 2011), membrane-assisted solvent extraction (Pinxteren et al., 2009), ultrasonic solvents extraction (García-Valcárcel et al., 2009; Lesueur et al., 2008b; Wu et al., 2010), liquid-liquid extraction (Kujawski & Namieśnik, 2010; Nguyen et al., 2010), low density miniaturized homogeneous liquid-liquid extraction (Hassan et al., 2010), liquid-liquid extraction and low temperature purification (Pinho et al., 2010), liquid extraction and programmed temperature vaporization (García-Rodríguez et al., 2010), dispersive liquid-liquid microextraction (Cunha et al., 2009), pressurized liquid extraction (Barco-Bonilla et al., 2010; Baugros et al., 2009; Blasco et al., 2005), hollow fiber sorptive extraction (Li et al., 2011b), hollow fiber-protected liquid-phase microextraction (Chen & Huang, 2006), stir-bar sorptive extraction-thermal desorption (Lavagnini et al., 2011), as well as the developed in 2003 (Anastassiades et al., 2003) quick, easy, cheap, effective, rugged and safe (QuEChERS) method (González-Curbelo et al., 2011; Kolberg et al., 2011; Lee et al., 2008; Lesueur et al., 2008a; Nguyen et al., 2008; Walorczyk, 2008). Some of these techniques like solid phase extraction and solid-phase microextraction in particular are commonly accepted. Advantage of the solid phase microextraction is the possibility of automation. Liquid-phase microextraction, as a relatively new procedure, does not find a large application at this time. The developed approaches are intended to reduce the sample preparation time, the solvent consumption and the amount of the sample, and to achieve high selectivity, applying simple, rapid, effective, and inexpensive methods, compatible with modern analytical techniques.

Other methods aimed to ensure a high sensitivity of the determination, in concert with the reliable sample preparation technique, apart of the mentioned above, are: gas chromatography with nitrogen-phosphorus detection (Fenoll et al., 2007; Georgakopoulos et al., 2009; Pagliuca et al., 2005; Ravelo-Pérez et al., 2008), gas chromatography with flame thermionic detection (Tsoutsi et al., 2006), gas chromatography with electron capture detection (Chai & Tan, 2009; Guardia-Rubio et al., 2007; Ibrahim et al., 2010; Pinho et al.,

2010; Ramos et al., 2009), gas chromatography with flame photometric detection (Ahmadi et al., 2006; Wang & Du, 2010; Xiao et al., 2006; Zhao et al., 2011), gas chromatography with flame ionization detection (Pinheiro, & Andrade, 2009), liquid chromatography with electrochemical flow detection (Trojanowicz, 2010), liquid chromatography with UV detection (Buonasera et al., 2009), high performance liquid chromatography with UV detection (Zhu et al., 2007), high performance liquid chromatography with fluorimetric detection (Pérez-Ruiz et al., 2005), and high performance liquid chromatography with diode array detection (Wu et al., 2010).

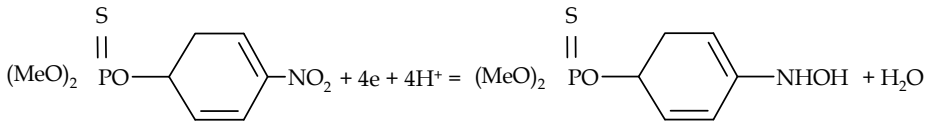
Relevant data collected for the period 2010-2011, revealing the analytical performances of some selected chromatographic methods are summarized in Table 3.

Pesticide	Detection Method	Sample preparation	LOD	References
Atrazine	LC-MS/MS	SE	0.06 μg L^{-1}	Sinha et al., 2010
	GS-MS	TOTAD	0.05 μg L^{-1}	Toledano et al., 2010
Chlorpyrifos	GC-MS	LLSE	0.13 μg kg^{-1}	Hassan et al., 2010
	GC-ECD	LLE	14.0 μg kg^{-1}	Pinho et al., 2010
Diazinon	GC–QqQ MS/MS	DSPE	0.37μg kg^{-1}	Qu et al., 2010
Dichlorvos	GC-MS	ASE	6.00 μg kg^{-1}	Wu et al., 2011
	GC-MS	HS-SPME	3.80 μg L^{-1}	Rodrigues et al., 2010
	GC–QqQ MS/MS	DSPE	1.50 μg kg^{-1}	Qu et al., 2010
Paraoxon	GC-MS	ASE	1.10 μg kg^{-1}	Wu et al., 2011
Malathion	GC-MS	ASE	0.20 μg kg^{-1}	Wu et al., 2011
	GC-MS	SPME	0.03 μg L^{-1}	Filho et al., 2010b
	GS-MS	TOTAD	0.07 μg L^{-1}	Toledano et al., 2010
	GC/PFPD	SPE	0.03 μg L^{-1}	Wang et al., 2010
	GC-MS	SPME	2.00 μg kg^{-1}	Filho et al., 2010a
	GC–QqQ MS/MS	DSPE	0.22 μg kg^{-1}	Qu et al., 2010
Parathion	GC-MS	ASE	0.20 μg kg^{-1}	Wu et al., 2011
	GC-MS	HS-SPME	4.70 μg L^{-1}	Rodrigues et al., 2010
	HPLC-DAD	UASEME	0.10 μg L^{-1}	Wu et al., 2010
	GS-MS	TOTAD	0.12 μg L^{-1}	Toledano et al., 2010
	GC/PFPD	SPE	0.02 μg L^{-1}	Wang et al., 2010
	GC–QqQ MS/MS	DSPE	1.50 μg kg^{-1}	Qu et al., 2010
Parathion-methyl	GC-MS	ASE	0.80 μg kg^{-1}	Wu et al., 2011
	GC-MS	HS-SPME	10.9 μg L^{-1}	Rodrigues et al., 2010
	HPLC-DAD	UASEME	0.10 μg L^{-1}	Wu et al., 2010
	GC-MS	SPME	0.02 μg L^{-1}	Filho et al., 2010b
	GC/PFPD	SPE	0.03 μg L^{-1}	Wang et al., 2010
	GC-MS	SPME	5.00 μg kg^{-1}	Filho et al., 2010a
	GC–QqQ MS/MS	DSPE	0.75 μg kg^{-1}	Qu et al., 2010
Phosmet	GC-MS	ASE	0.50 μg kg^{-1}	Wu et al., 2011
	HPLC-DAD	UASEME	0.10 μg L^{-1}	Wu et al., 2010
Phorate	GC-MS	ASE	0.70 μg kg^{-1}	Wu et al., 2011
	GC–QqQ MS/MS	DSPE	0.62 μg kg^{-1}	Qu et al., 2010
Trichlorfon	GC-MS	SPME	0.07 μg L^{-1}	Filho et al., 2010b
	GC-MS	ASE	5.1 μg kg^{-1}	Wu et al., 2011

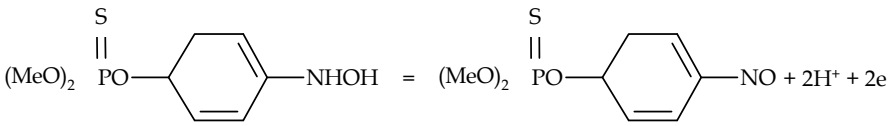
Table 3. Analytical performances of some chromatographic methods applied to OP pesticides analysis, namely LOD.

2.2 Electrochemical methods for OP pesticides analysis

The electrochemical activity of the OP pesticides containing nitro-phenyl groups: parathion-methyl, parathion, paraoxon, fenitrothion, etc. makes possible their direct electrochemical determination. The mechanism of the redox process, selecting parathion-methyl as a model, is the following:



Parathion-methyl



The electrochemical method of choice applied to OP pesticides analysis, considering the period 2005-2011, is square-wave voltammetry (Li and al., 2011; Parham and Rahbar, 2010; Sbaï et al., 2007; Tan et al., 2010; Wang and Li, 2008). It is recognized as a high sensitive regarding the detection of organic molecules. Current efforts are directed toward sensitivity and selectivity improvement by chemical modification of the electrode surface. The reported techniques include: nano-ZrO_2 modification of a carbon paste electrode (Parham and Rahbar, 2010), fabrication of ZrO_2/Au nano-composite films on alumina substrates (Wang and Li, 2008), electrodeposition of molecularly imprinted porous silicate (Tan et al., 2010) or of gold-sodium dodecylbenzene sulfonate nanoparticles onto a glassy carbon electrode (Li and al., 2011a), tetrasulfonated phtalocyanine electrodeposition combined with Nafion coating of carbon fibres (Sbaï et al., 2007), etc. ZrO_2 nanoparticles in particular, used as a selective sorbents for solid-phase extraction, demonstrate excellent performance in OP pesticides detection, because of their affinity toward the phosphate group on OP pesticide molecule (Parham and Rahbar, 2010; Wang and Li, 2008).

The developed electrochemical methods are applied for OP pesticides determination in pears (Li and al., 2011; Tan et al., 2010), and in water samples (Parham and Rahbar, 2010). Some relevant data revealing the sensitivity of the determinations are presented in Table 4.

Pesticide	Electrode	Electrode modification	LOD	References
Parathion-methyl	CFME	poly-NiTSPc/Nafion	0.1 mg L^{-1}	Sbaï et al., 2007
Parathion-methyl	GC	imprinted silicate	2.5 μg L^{-1}	Tan et al., 2010
Parathion-methyl	GC	nano-ZrO_2	2.0 μg L^{-1}	Parham and Rahbar, 2010
Parathion-methyl	GC	nano-Au/SDBS	25 μg L^{-1}	Li and al., 2011
Parathion	GC	ZrO_2/Au	3.0 μg L^{-1}	Wang and Li, 2008

Table 4. Analytical performances of square wave voltammetry, applied to OP pesticides analysis, namely LOD.

The analysis of the reported studies confirms that the electrochemical methods have the advantage to be rapid, sensitive, selective, and accurate. In addition, they use affordable, portable, and miniaturized instrumentation. These characteristics make them appropriate for the "*in field*" determination of the low persistent in the environment OP pesticides.

2.3 Immunochemical methods for OP pesticides analysis

Only few immunochemical methods for OP pesticides analysis were reported during the surveyed period 2005-2011. Gui and al. (2006) synthesize two haptens of the OP insecticide triazophos and develop an enzyme-linked immunosorbent assay based on monoclonal antibody, demonstrating high affinity and specificity to triazophos. Guo and al. (2009) investigate two formats of gold-labeled antibody lateral-flow strips for the simultaneous detection of triazophos and of the carbamate pesticide carbofuran. The application of the immunogold labeling technique in immunoassays is reviewed by Lai et al. (2010). Garcés-García and al. (2006) point out the development of plate immunoassays for routine determination of residues: diazinon, fenthion, malathion, and chlorpyrifos in extra virgin olive oil. The achieved by the mentioned methods LOD is shown in Table 5.

Pesticide	Method	LOD	References
Diazinon	ELISA	46 μg L^{-1}	García and al., 2006
Fenthion	ELISA	10 μg L^{-1}	García and al., 2006
Malathion	ELISA	16 μg L^{-1}	García and al., 2006
Chlorpyrifos	ELISA	17 μg L^{-1}	García and al., 2006
triazophos	ELISA	0.1 μg L^{-1}	Gui and al., 2006

Table 5. Analytical performances of some immunochemical methods, applied to OP pesticides analysis, namely LOD.

2.4 Biosensors based methods for OP pesticides analysis

The overview of the publications covering the period 2005-2011 shows that almost all of the described biosensors for OP pesticides analysis are electrochemical ones. The OP pesticides determination is based on the quantification of the acetylcholinesterase inhibition, they provoke. The enzyme activity is determined by electrochemically monitoring the thiocholine formed upon enzymatic hydrolysis of acetylthiocholine. Three alternative routes are explored as response-generating electrochemical reactions:

i. Direct electrochemical oxidation of thiocholine at 0.80 V/Ag, AgCl (Ivanov et al., 2010; Marinov et al., 2010; Ovalle et al., 2009):
$2(CH_3)_3N^+(CH_2)_2SH \rightarrow (CH_3)_3N^+(CH_2)_2S\text{-}S(CH_2)_2N^+(CH_3)_3 + 2H^+ + 2e^-$

It is important to note that potential lowering and hence interferences elimination could be achieved by using nanostructured materials for electrode modification. Nanoparticles reduce the working potential by catalysing the electrochemical thiocholine oxidation (Du, 2007).

ii. Mediated thiocholine oxidation at lower electrode potential (0.1÷0.45 V/Ag, AgCl), using cobalt phtalocyanine (Alonso et al., 2010; Law & Higson, 2005), tetracyanoquinodimetane (Hildebrandt et al., 2008) or hexacyanoferrate (III) (Ovalle et al., 2009) as electron mediators in a heterogeneous or in a homogeneous phase:
$2(CH_3)_3N^+(CH_2)_2SH + 2M_{ox} \rightarrow (CH_3)_3N^+(CH_2)_2S\text{-}S(CH_2)_2N^+(CH_3)_3 + 2M_{red}$
$M_{red} \rightarrow M_{ox} + e^-$

iii. Chemisorption of thiocholine at -0.7 V/Ag, AgCl and electrochemical desorption in KOH, giving a measurable reductive peak current (Du et al., 2008).

The majority of the publications report the application of screen-printed electrodes (Alonso et al., 2010; Dondoi et al., 2006; Hildebrandt et al., 2008, Law & Higson, 2005) as transducers. Recent developments in the field of screen-printed electrodes and their related applications

are comprehensively reviewed by Renedo et al. (2007). Disposable screen-printed electrodes are considered as an alternative to the traditional electrodes for "*in situ*" analysis.

As immobilization matrices in electrochemical acetylcholinesterase-based sensors for OP pesticides determination were preferentially used various nanomaterials: nanostructured polymer membranes with integrated multiwall carbon nanotubes (Ivanov et al., 2010) or gold nanoparticles (Marinov et al., 2010), multiwall carbon nanotubes-chitosan composite (Du et al., 2007), etc., to achieve sensitivity increase and sensor stability improvement. Recent trends and challenges in developing nanomaterials-based biosensors for OP pesticides detection are discussed by a number of authors (Balasubramanian & Burghard, 2006; Eftekhari, 2008; Gorton, 2005; Guo & Wang, 2007; Kerman et al., 2008; Kumar, 2007; Liu et al., 2008, Luo et al., 2006; Merkoçi & Alegret, 2005; Merkoçi, 2009; Pumera et al., 2007, Wang & Lin 2009).

The alternative route leading to biosensors sensitivity, selectivity and stability increase involves the incorporation in the biosensing platform of biorecognition elements with tailor designed properties. Genetically modified enzymes are extensively used in inhibition based biosensors for OP pesticides determination (Bucur et al., 2005; Marques et al., 2005; Valdés-Ramírez et al., 2008), allowing attaining LOD as low as 10^{-17} M (Sotiropoulou et al., 2005). Current research efforts are reviewed by Campás et al. (2009).

Another important issue associated with electrochemical biosensors development is that concerning chemometrics. It was demonstrated that artificial neural networks implementation could resolve mixtures of pesticides (Alonso et al., 2010).

Exhaustive reviews on enzyme inhibition-based biosensors, including inhibition determination in organic phase are provided by Amine et al. (2006) and López et al. (2006). The application of various enzymes: acetylcholinesterase, acid phosphatase, alkaline phosphatise, organophosphorus hydrolase, and tyrosinase for the quantification of OP pesticides in the environment is extensively revised by Van Dyk et al. (2011).

Another group of electrochemical biosensors for OP pesticides analysis is that of the microbial sensors. Such sensors, based on Clark dissolved oxygen electrode modified with recombinant p-nitrophenol degrading/oxidizing bacteria endowed with OPH activity were reported by Lei et al. (2005, 2006). The surface-displayed OPH catalyzes the hydrolysis of OP pesticides with nitrophenyl substituent to release products, metabolized by the bacteria while consuming oxygen. The oxygen consumption is measured and correlated to the OP concentration.

A microbial biosensor for direct determination of nitrophenyl-substituted organophosphate nerve agents using genetically engineered *Moraxella* sp. has been proposed by Mulchandani et al. (2006). However, the reached LOD is over the OP concentration in environmental samples and higher than that for acylcholinesterases inhibition-based sensors, immunoassays, and gas, liquid and thin layer chromatography (Mulchandani et al., 2006).

Recently, an electrochemical hybrid biosensor for OP pesticides trace level concentrations determination was developed and characterized (Stoytcheva et al., 2009). It integrates a hybrid biorecognition element consisting of immobilized *Arthrobacter globiformis* and free acetylcholinesterase (ACh) with a Clark type oxygen probe transducer. The bacteria convert the ACh-generated choline to betaine with oxygen consumption measured as a Clark probe current change. This change, representing the sensor response, correlates to the concentration of the OP pesticides inhibiting the Ach catalyzed acetylcholine hydrolysis to choline. Current progress in microbial electrochemical and optical biosensors are reported by Su and al. (2011).

Finally, the few works commenting on optic- and immuno- biosensors development and application to OP pesticides analysis have to be pointed out, too (Llorent-Martínez et al., 2011; Mauriz et al., 2006a, 2006b; Suri et al., 2009). Special attention should be paid to the overview of Jiang and al. (2008), presenting the various transduction systems used in immunosensors: electrochemical, optical, piezoelectric, and nanomechanic, and the immobilization protocols.
Some relevant data are presented in Table 6.

Pesticide	Electrochemical biosensor	LOD	References
Azinphos	Ach/polyaniline carbon/cobalt phtalocyanine	10^{-10} μM	Law et al., 2005
Dichlorvos	Ach/polyaniline carbon/cobalt phtalocyanine	10^{-11} μM	Law et al., 2005
Chlorpyrifos-oxon	Ach/polyvinyl alcohol/TCNQ/C	2 μg L^{-1}	Hildebrandt et al., 2008
Malathion	Ach/AuNPs/chitosan/Au	0.03 μg L^{-1}	Du et al., 2008
Paraoxon	Ach/MWCN/poly-(acrylonitrile-methyl-methacrylate-sodium vinylsulfonate)/Pt	$1.39x10^{-6}$ μg L^{-1}	Ivanov et al., 2010
Paraoxon	Ach/AuNPs poly-(acrylonitrile-methyl-methacrylate-sodium vinylsulfonate)/Pt	$7.39x10^{-5}$ μg L^{-1}	Marinov et al., 2010
Parathion	Ach/polyaniline carbon/cobalt phtalocyanine	10^{-10} μM	Law et al., 2005
Triazophos	Ach/MWCNT-chitosan/GCE	10^{-2} μM	Du et al., 2007

Table 6. Analytical performances of some biosensors-based methods, applied to OP pesticides analysis, namely LOD.

2.5 Chemometrics applied to OP pesticides analysis

Current progress on the application of chemometrics to evaluate the occurrence of organic pollutants, including OP pesticides in the environment are reviewed by Mas et al. (2010). The interpretation of the results of the analytical determination of these substances, applying chemometric approaches is among the addressed issues.
On the improvement of the electroanalytical techniques in particular with the aid of chemometrics (partial least squares, artificial neural networks, and multiple curve resolution methods) comment Ni and Kokot (2008). Some of the suggested methods are successfully applied to OP pesticides analysis.
The strategies for the enhancement of the spectroscopic photochemistry by chemometrics are discussed in the overview presented by Liu et al. (2009). The chemometric methods revealed their efficacy for the simultaneous and selective enzymatic spectrophotometric determination of carbamate (carbofuran, carbaryl) and OP pesticides (chlorpyrifos, dichlorfos, phoxim) (Ni and al., 2007; Rhouati and al., 2010), and for the simultaneous determination of OP pesticides residues: dipterex, dichlorvos and omethoate in vegerable samples by continuous-flow chemiluminescence without any previous separation (Li et al., 2007).

3. Conclusion

The continuous lowering of the maximum residue limits of the OP pesticides in food and in the environment calls for the development of sensitive methods for their determination. Such high effective techniques, well suited for testing complex matrices, are the chromatographic ones. Nevertheless, the review of the advances in OP pesticides analysis

during the period 2005-2011 demonstrated that despite of the efforts to reduce solvent consumption and to simplify sample pretreatment, the chromatographic determinations remain expensive and time consuming. In addition, they require experienced personnel and sophisticated laboratory equipment, inappropriate for "*in field*" application. Therefore, a number of alternative electrochemical and biosensors-based techniques were suggested. Because of the inexpensive instrumentation, the simple operation procedure without or with a minimum sample pretreatment, and the high sensitivity, they gain more and more importance in OP pesticides analyses, as an excellent complement to the classical analytical techniques, for "*in situ*" and "*on line*" determinations.

4. List of abbreviations

Ach: acetylcholinesterase; ASE: accelerated solvent extraction; AuNPs: gold nanoparticles; CFME: carbon fibre microelectrode; DSPE: dispersive solid phase extraction; ELISA: enzyme-linked immunosorbent assay; EPA: U.S. Environmental Protection Agency; FAO: Food and Agricultural Organization of the United Nations; FQPA: Food Quality Protection Act; GC: glassy carbon; GCE: glassy carbon electrode; GC-ECD: gas chromatography using electron-capture-detector; GC–QqQ-MS/MS: chromatography–triple quadrupole mass spectrometry; GC/PFPD: gas chromatography/pulsed flame photometric detector; HPLC-DAD: highperformance liquid chromatography with diode array detection; HS-SPME: solid-phase microextraction in mode headspace; LLE: liquid-liquid extraction; LLSE: liquid-liquid solvent extraction; LOD: limit of detection; M: mediator; MRLs: maximum residue limits; MWCN: multiwall carbon nanoparticles; MWCNT: multiwall carbon nanotubes; NiTSPc: nickel(II) tetrasulfonated phtalocyanine; OGWDW: U.S. Environmental Protection Agency Office of Ground Water and Drinking Water; OP: organophosphorus; OPH: organophosphoro hydrolase; QuEChERS method: quick, easy, cheap, effective, rugged and safe method; SDBS: sodium dodecylbenzene sulfonate; SE: solvent extraction; SPE: solid phase extraction; SPME: solid-phase microextraction; TCNQ: tetracyanoquinodimetane; TOTAD: through oven transfer adsorption desorption; UASEME: ultrasound-assisted surfactant-enhanced emulsification microextraction; WHO: World Health Organization.

5. References

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8

New Methodologies for Assessing the Presence and Ecological Effects of Pesticides in Doñana National Park (SW Spain)

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1. Introduction

Environmental quality can be assessed by a variety of methods (chemical, biological, and ecological) that, when used individually, have multiple limitations. In fact, it is convenient to use different approaches to understand the status of any ecosystem. Actually, in addition to show the presence of pollutants in the environment, their appearance in the organisms and their biological effects on them has to be demonstrated before their possible risks can be evaluated. The chemical analysis of pollutants requires sophisticated tools due to the growing number of compounds, the subsequent transformation of their parent compounds, the need to distinguish between their different chemical forms, and the interest in characterizing the metabolites derived from them. In addition to conventional pollutants (e.g. linear hydrocarbons, PAHs, PCBs, metals, pesticides) there are many others like metal species and persistent organic pollutants (such as aldrin, chlordane, DDT, dieldrin, endrin). Recently further concern has been shown about input of new emerging pollutants. These include pharmaceuticals, alkylphenols, linear alkylsulphonates, new metal species, etc., which, although found in the ecosystems at very low concentrations, are ecologically highly active, even acting as endocrine disruptors with deleterious effects for the reproduction of different species (Strauch et al., 2008). Antiinflamatory drugs (diclophenac, ketoprofen, naproxen, and ibuprofen), antibiotics (penicillins, tetracyclins, sulfonamides, and quinolones), anticonceptives, etc., are used in human and animal health care. Their growing use and increasing presence in the ecosystems has become an important issue, the focus of research being shifted from conventional to emerging pollutants that are highly dangerous since are designed to have biological activity. The amount of pharmaceuticals reaching the environment depends on human consumption and excretion via feces/urine that reach the sewage system. Effluents of wastewater plants are the main source in the aquatic environment, followed by the release of outdated medicines down household (Ruhoy et al., 2007) and pharmaceutical industry waste (Kümmerer, 2004; Larsson et al., 2007).

Homeostasis describes the constant state of the internal environment of living beings. Homeostatic regulation allows an organism to function effectively in a broad range of environmental conditions, and pollution seriously endangers this situation. Biomarkers are used in sentinel organisms (bioindicators) to provide evidence of exposure/effect to one or more pollutants (Livingstone, 1993; Peakall, 1994). Environmental studies usually rely on the assessment of classic bioindicators (bivalve mollusks or fish), but there is a lack of studies on free-living animals, such as *Procambarus clarkii* (red swamp crayfish) or *Mus spretus* (Algerian mouse), from barely polluted ecosystems (Fig. 1).

Fig. 1. *Procambarus clarkii* and *Mus spretus,* and the model laboratory mouse, *Mus musculus*.

P. clarkii is native to Southeastern USA. In Southern Europe, this crustacean is actively colonizing new territory, at the expense of the native crayfish. This organism, of relatively small size (10 cm; 25-30 g) reaches sexual maturity in the marshes of the Guadalquivir River at 6 cm total length. This omnivorous and detritivorous species feeds on plants, rhizomes and small animals like aquatic larvae, insects and cladocerans (Gutiérrez-Yurita & Montes, 1998). *P. clarkii* is an excellent bioindicator of contamination in fresh water ecosystems (Martín Díaz et al., 2006). The mouse is the favorite model organism for generation of knowledge useful to humans. *Mus musculus* is the source of most classical inbred laboratory mouse strains and its genome has been sequenced (Mouse Genome Sequencing Consortium, 2002). *M. spretus* is the best characterized of the aboriginal species. This small animal attains high population densities, typically inhabits marshlands, and feeds on seeds, insects and small invertebrates around its burrow. This free-living rodent has proved to be an excellent bioindicator of contamination in terrestrial ecosystems (Nunes et al., 2001; Ruiz-Laguna et al., 2001; Bonilla-Valverde et al., 2004). The two murine species separated more than one million years ago (Gao & Zhang, 2003) and exhibit a relatively high gene sequence homology. Consequently, molecular studies with the aboriginal species can take advantage of the laboratory species databases (Prieto-Álamo, et al., 2003; Ruiz-Laguna et al., 2005,

2006). Both bioindicators are non-protected species and highly prolific, therefore they are very suitable in environmental studies.

Doñana National Park (DNP, SW Spain, Fig. 2) was settled in 1969 and declared a World Heritage Site in 1981. This area of marsh, shallow streams and sand dunes, in the mouth of the Guadalquivir River, has a biodiversity unique in Europe, contains many ecosystems and shelters wildlife including millions of migratory birds, and endangered species such as the Imperial Eagle and Iberian Lynx (Grimalt et al., 1999). Doñana is watered by the Rocina, Partido and Guadiamar streams and by Guadalquivir River, and is surrounded by areas of intense agricultural activities (citric, strawberry, rice). It is also threatened by industries located at Huelva estuary, 40 Km West, formed by Odiel and Tinto rivers, carrying metals from mining activity, and where the Domingo Rubio stream discharges petrochemical refuses, and pesticides from strawberry crops (Montes-Nieto et al., 2007, 2010). Finally, in 1998 metals released from Aznacóllar pyrite mine, 60 Km north, threatened Doñana through Guadiamar stream (Grimalt et al., 1999).

Fig. 2. South-Atlantic Spanish littoral, between Huelva and Guadalquivir estuaries. The limits of DNP are shown in white, the Odiel, Tinto and Guadalquivir Rivers and the Domingo Rubio (DRS), Rocina (ROC), Partido (PAR) and Guadiamar streams are shown in blue and the Lucio del Palacio (LDP) reference site is indicated in green. The numbers included in the figure show a variety of polluted sites located at: 1, phosphogypsum stacks; 2-4, low, medium and high DRS course, respectively; 5-6, ROC; 7-8, PAR; 9, Matochal; 10, Isla Mayor; 11, Cangrejo Grande; 12, Brazo de la Torre; 13-15 Doñana bank at Guadalquivir Estuary, at the mouth of Guadiamar stream, San Rafael saltworks and across Bonanza harbor, respectively.

While there are many studies in polluted environments there are few in natural ecosystems, and in particular in National Parks around the World. Due to the variety of pollutants, these studies focuse on metals and organic pollutants, in relatively polluted areas (Livingstone et al., 2000; Hobbelen et al., 2004; Hamers et al., 2006; Schwindt et al., 2008). Initial assessment of

contaminants at Doñana area was centered on Guadiamar stream. The high metal levels and low pH detected in its upper course were related to acid-mine draining from Aznalcóllar pyrite mine; metals and herbicides were also found at the rice growing fields East of DNP (Cabrera et al., 1984). A global evaluation of DNP contamination was made by the Scientific Research Council (Albaiges et al., 1987). Streams feeding Doñana marshes were the main source of contaminants. In addition to metals and pesticides, Guadiamar stream contributed organic matter from urban/agro-industrial sources, and petroleum hydrocarbons entered from Guadalquivir River at the South. In addition to water origin, the uniform distribution of PAHs and PCBs at stream-isolated lagoons suggested aeolian transport from industries at Huelva Estuary (Albaiges et al., 1987). After the tailings dam of Aznalcóllar pyrite mine, the effect of this spill in DNP was studied (López-Pamo et al., 1999; Manzano et al., 1999). Although the spill did not have a dramatic effect within Doñana, increasing concern raised about the presence of contaminants at DNP core, not directly related to the spill but possibly derived from the agrochemicals used in nearby areas (Ruiz-Laguna et al., 2001; Bonilla-Valverde et al., 2004, 2006). Actually, the effect of agricultural activity is the most serious gap in the knowledge of Doñana environmental quality, since this type of contamination is more diffuse, difficult to assess and less relevant in the media than a mining spill.

Biomarkers are used in sentinel organisms to provide evidence of exposure, effect or risk to pollutants in biomonitoring studies (Livingstone, 1993; Peakall, 1994; López-Barea, 1995). The so-called "classic" biomarkers are suggested *a priori* by their biological roles but they are somewhat biased in pollution assessment since they concentrate in a small number of proteins but exclude other also altered but whose relationship with pollution is still unknown (López-Barea & Gómez-Ariza, 2006). Table 1 summarizes a number of biomarkers that indicate the responses of organisms to pollutants. They include: 1) Induction (inhibition) of phase I, and 2) phase II biotransformation enzymes (Livingstone, 1993; George, 1994; Goksøyr, 1995; Van der Oost et al., 2003), responsive to PAHs, PCBs, PCDFs, dioxins (inhibited by organotins), that are coordinately regulated by the Ah receptor (Stegeman & Han, 1994). 3) Levels of reduced and oxidized glutathione or non-enzymatic antioxidants, and, 4) activity of primary/ancillary antioxidant enzymes and oxidative damages to biomolecules, responsive to metals and prooxidants. 5) Esterase activities inhibited by organophosphate or carbamate pesticides (Peakall, 1994). 6) Stress proteins, responsive to many organic compounds, such as natural products and anthropogenic contaminants, and transition metals. 7) Reproductive and endocrine effects, sensitive to organotins and endocrine disruptors. 8) Genotoxicity, responsive to genotoxins and carcinogens (López-Barea, 1995). 9) Physiological and morphological parameters.

The cellular genome is in a dynamic equilibrium between processes that damage it and those that maintain its integrity. While genes exert their functions at protein level, the genetic responses to stress are often regulated at the transcriptional level. After completion of the Human Genome Project, an increasing range of post-genomic techniques are available to asses the complete content of genes, proteins or metabolites in a cell under a particular situation. In contrast to the conventional biomarker strategy, these *omic* approaches, supplemented with *in vitro* and *in silico* methods, are becoming a powerful multidisciplinary strategy in environmental studies. Nevertheless, their application is still at an early developmental stage, mainly since most popular bioindicators are poorly represented in gene/protein sequence databases (Ruiz-Laguna et al., 2006; González-Fernández et al., 2008a). The *omic* sciences include *genomics* for study of DNA variations, *transcriptomics* for genome-wide characterization of gene expression via the measurement of mRNAs,

proteomics for measuring cell and tissue-wide expression of proteins, and *metabolomics* for global assessment of metabolite concentrations. These technologies give detailed molecular information that help to identify toxicity pathways and to define pollutant mechanisms and modes of action without requiring previous knowledge.

Category	Biomarker/abbreviation	Reference
Phase I biotransform	CYP 1A protein/mRNA	Goksøyr, 1995
	EROD/AHH activity	Livingstone, 1993
Phase II biotransform	Glut. S-transfer., GSTc,m	Van der Oost et al., 2003
	UDP-glucuronyl transfer	George, 1994
Antioxidants	GSSG/GSH redox status	López-Barea, 1995
	Vitamins C or E	Van der Oost et al., 2003
Primary antiox enzym	Superoxide dismutase, SOD	López-Barea, 1995
	Catalase, CAT	López-Barea, 1995
	Glut. peroxidase, GSHPx	Van der Oost et al., 2003
Auxilary antiox enzym	Glut. reductase, GSSGRase	Van der Oost et al., 2003
	Glucose-6P dHase, G6PDH	López-Barea, 1995
	6Pgluconat. dHase, 6PGDH	López-Barea, 1995
Oxidative damages	Lipid peroxidation, MDA	López-Barea, 1995
	DNA oxidation	López-Barea, 1995
Esterase inhibition	Acetyl cholinesterase, AChE	Peakall, 1994
	Carboxyl esterase, CbE	Galloway et al., 2002
Stress proteins	Heat shock proteins, HSPs	López-Barea, 1995
	Metallothioneins, MTs	López-Barea, 1995
Reproduction	Vitellogenin, VTG	Van der Oost et al., 2003
Genotoxicity	DNA adduct/strand breaks	Van der Oost et al., 2003
	Sister-chromatid exchanges	Van der Oost et al., 2003
	Micronucleous assay	Van der Oost et al., 2003
Physiol/Morphology	Histopathology	Nunes et al., 2001
Other	Promutagens activation	López-Barea, 1995

Table 1. Biochemical pollution biomarkers used in biomonitoring studies

Several technologies are available to analyze the transcriptome, the entire complement of transcripts in a cell, tissue or organism under certain conditions. *Microarrays technology* allows thousands of gene transcripts to be monitored simultaneously, but they are based in the complementarity of sequences between probes and tested samples. Commercial microarrays are not usually applicable for environmental studies, since most bioindicators are poorly represented in databases. Custom cDNA microarrays can be developed for any species of interest but it is labor intensive, time consuming and costly since it requires to previously obtaining expressed sequence tags, cDNA libraries or genome sequence data. Different strategies allow overcoming these inconvenients. One is to choose a bioindicator close to a model organism; in this case, the similitude between their genic sequences allows the use of commercially available microarrays by means of heterologous hybridization. Application of heterologous microarray has gained wide interest to study the response to pollutants and environmental stressors (Bar-Or et al., 2007; Buckley, 2007; Osuna-Jiménez et al., 2009).

When heterologous microarrays cannot be used due to sequence divergence between the studied species and a possible reference, an alternative is *suppressive subtractive hybridization* (SSH) (Williams et al., 2003; Prieto-Álamo et al., 2009). This is a PCR-based technique for generating cDNAs enriched for differentially expressed genes, useful for large-scale gene identification in non-model organisms (Diatchenko et al., 1996). The identification of differentially expressed genes by SSH is shown in a number of papers that analyze the responses to model contaminants under controlled exposure conditions. In comparison, few studies examine individuals from ecosystems with different degrees of pollution, mainly centered in aquatic ecosystems. It is worth mentioning the studies in the black tiger shrimp (*Penaeus monodon*), the grass shrimp (*Palaemonetes pugio*) and the American winter flounder (*Pseudopleuronectes americanus*) (Straub et al., 2004; Griffitt et al., 2006; De la Vega et al., 2007). Hybridization-based approaches provide data that must be confirmed by more refined quantitative methods like real-time reverse transcription (RT) followed by polymerase chain reaction (PCR), the most powerful tool for transcript quantification. Most RT-PCR studies are taken semiquantitatively (fold-variation) and assume that housekeeping genes are stably expressed, or that any changes that might occur are balanced. Absolute quantification (real quantification) gives the molecule copy number of each transcript in each of the samples under study using a calibration curve. Although relative quantification is much easier to perform because a calibration curve is not necessary, relative data are much less informative than absolute data (Jurado et al., 2003; Prieto-Álamo et al., 2003; Jiménez et al., 2005; Ruiz-Laguna et al., 2005, 2006). The commercialization of real-time PCR equipments allows for absolute expression data with precision levels unattainable for those generated by conventional end-time PCR. Real-time RT-PCR is adequate for studies focused in a few number of transcripts, because it is expensive and time-consuming.

Proteomics addresses the post-genomic challenge of examining the entire complement of proteins (proteome) expressed by a genome in a cell, tissue or organ at a given time under defined conditions (James, 1997; Anderson & Anderson, 1998; Blackstock & Weir, 1999). Protein expression is modulated at different levels from transcription to maturation of the polypeptides produced by translation of mature mRNAs. Post-translational modifications are of key importance as they give rise to multiple protein products from a single gene, each of which may have different functions. Proteins were initially separated by *two-dimensional electrophoresis* (2-DE, Wilkins et al., 1996), their expression being analyzed by 2-D softwares (Melanie, etc.). Proteins were identified by mass spectrometry analysis of their peptide mass fingerprint (MALDI-TOF-PMF) or *de novo* sequencing of some peptides (nESI-MS/MS), then contrasting the results with public databases (Simpson, 2003). 2-DE, labor intensive and of a low reproducibility, requires a large amount of sample, and its narrow dynamic range is problematic with proteins of extreme Mr/pI. Fluorescent labeling gave rise to *difference gel electrophoresis* (DIGE), which analyzes two samples in one single gel, facilitating a quantitative assessment of expression (Ünlü et al., 1997). Alternatively, shotgun methods allow the analysis of complex protein mixtures after full digestion, by multidimensional separation coupling tandem liquid chromatography (LC/LC) and MS/MS (Washburn et al., 2001). *Isobaric reagents* (iTRAQ) revolutionized proteomics by allowing the simultaneous quantification and identification of proteins in complex mixtures in one single experiment (Ross et al., 2004). A set of multiplexed isobaric reagents are used to label peptides in their amino groups (Lys and N-terminal). Although the labeled peptides are undistinguishable in MS, they generate low-mass MS/MS signature ions (113-121), supporting their relative and absolute quantification. The application of proteomic technology in environmental studies

(Environmental Proteomics, EP) faces the problem of the lack of genomic information on most non-model sentinel organisms. This makes it difficult to identify differentially expressed proteins by high-throughput methods like MALDI-TOF-PMF analysis (Barrett et al., 2005; López-Barea & Gómez-Ariza, 2006; González-Fernández et al., 2008a).

Within the growing body of Proteomics, issues addressing environmental problems are on the rise. Ecotoxicology uses expression changes of proteins known to be involved in toxicological responses. Unlike directed approaches, proteomics examines how multiple expression changes are associated with a contamination suspected to be harmful. Thus, proteins involved in toxicological responses that have not been described before may be revealed. Following identification of key proteins indicating exposure or effect, Proteomics can be used in risk assessment. To this end, bioinformatics may unveil protein patterns specific to an environmental stress that would constitute a classifier able to distinguish an exposure from a control state. Two main trends are used in expression proteomics: 1) *Pattern-only approaches*, to recognize proteomic patterns and not at protein identification in the first place, initially named PES approach (Shepard & Bradley, 2000; Shepard et al., 2000). 2) *Identity-based approaches*, relying on the identification of proteins with differential expression (Monsinjon & Knigge, 2007). In EP studies the difficulty of protein identification in non-model organisms was initially circumvented by the PES approach. These sets of proteins observed in 2-DE, useful as state markers, signal early pathological stages or stress exposure. Initial studies showed that PES is specific for the type and extent of stress. Since altered PES are used as multimarkers, to diagnose adverse effects there is no need to identify the proteins with altered expression levels (Shepard et al., 2000; Baker 2005), being enough to show the appearance of altered PES patterns to demonstrate pollution effects.

Metal ions play a key role in life chemistry, so that it is essential to identify and quantify the chemical species containing metals in living beings. The corpus of research dealing with metals in biological systems has been named "metallomics" (Haraguchi, 2004). The function of a third of the proteins depends on their interaction with transition metals like Cu, Fe, Zn, or Mo (Hasnain, 2004). Thus, it is vital to find out how an organism generates biomolecules containing metals in response to signals and environmental stress. Therefore, cell chemistry implies knowing, in addition to its genome and proteome, its metallome, i.e. the distribution of metals/metalloids among its different biomolecules and compartments (Szpunar, 2004, 2005; López-Barea & Gómez-Ariza, 2006). There are not analytical procedures to directly determine the chemical forms of the elements in biological matrices at the existing concentrations. To avoid any changes in its original form, it is necessary to study the species in which the element is strongly bound to the biomolecules. As electrospray mass spectrometry does not permit their analysis due to matrix effects, high resolution on-line separations with HPLC or CE have to be combined with sensitive and specific atomic detectors (ICP-MS). These hybrid techniques allow the measurement of metal species in cytosolic extracts using the heteroelement as a marker (Sanz-Medel, 2003). The couplings based on size exclusion (SEC-ICP-MS) permit a rapid screening through the molecular mass of metal-molecules (Szpunar, 2004, 2005; Sanz-Medel et al., 2003; González-Fernández et al., 2008b), although purification of the peaks requires a second chromatographic separation by ionic exchange, reverse phase or hydrophobic interaction, to identify the molecule (Arias-Borrego et al., 2008). Other alternatives use CE-ICP-MS couplings, gel electrophoresis with laser ablation and ICP-MS detection, or detection of the metal by spot digestion and ICP-MS analysis (Sussulini et al., 2007; Monicou & Lobinski, 2008). The potential of isotopic dilution and of the MS in MALDI mode and in the diverse forms of ESI-MS is also used. The basic

work scheme, used with variants in metallomics, incorporates three elements (Fig. 3): i) a separation system; ii) a detector for the element (metal or metalloid) (ICP-MS); and iii) a system for the identification of the molecule (MS) (Gómez-Ariza et al., 2004).

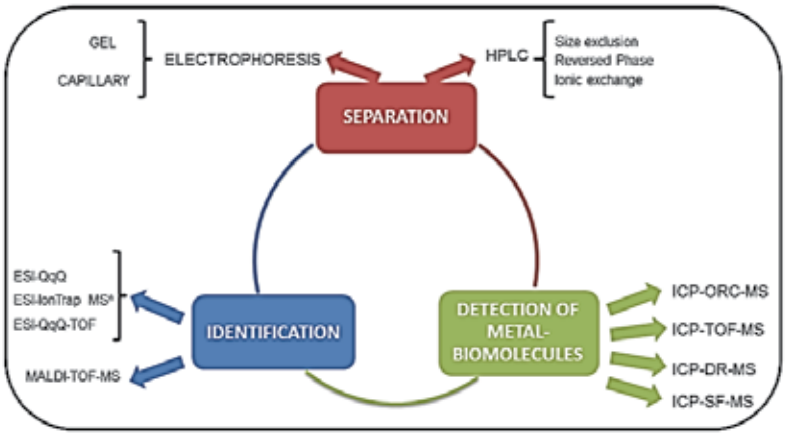

Fig. 3. The basic work scheme of the metallomics approach (Gómez-Ariza et al., 2004).

2. Our previous studies

We will review previous studies of the groups that monitor the pollution status of Doñana and surrounding areas, whose recent results will be summarized in section 3. We only refer to the development of analytical methods for contaminants present at DNP, and the application of conventional biomarkers and new *omic* methods to assess the responses of organisms living in the area. This section is centered in the following aspects: 2.1) New analytical approaches for emerging pollutants determination. 2.2) Developments in metallomics studies for environmental assessment. 2.3) Conventional biomarkers and proteomics in environmental studies. 2. 4) Transcriptomics in environmental studies.

2.1 New analytical approaches for emerging pollutants determination

Procedures for determination of pharmaceuticals and other emerging pollutants in animal tissues have been reviewed, though few exist in fish or bivalves. Most methods imply solid-liquid extraction and further clean up by solid-phase extraction prior to chromatographic analysis, but there is interest in improving detection sensitivity not paying much attention to sample treatment procedures, the most crucial and time consuming step for analyzing the compounds of interest in complex matrices. Additionally, few reports have been published on analysis of bioactive compounds and drugs, in animal tissues.

One of the main problems for quantitative analysis of pharmaceuticals in biological samples is that the analyte is usually bound to proteins or peptides, thus requiring the previous cleavage of these structures. Although enzymatic digestion has been traditionally used, these methods are time consuming and usually require further clean-up (Fernández-Torres et al., 2010a). The use of enzymes combined with ultrasonic energy has been reported as an alternative and since ultrasonic probes provide 100 times more energy than ultrasonic baths, these last are preferred for enzymatic sonication extraction (Fernández-Torres et al., 2010b) in the so called ultrasound probe sonication-assisted extraction. To improve extraction

efficiencies, enzymatic digestion combined to subsequent inhibition of enzyme activity by microwaves (microwave-assisted extraction, MAE) was used finding that at low intensity the enzymatic activity remained stable and recoveries of almost all analytes increased. Thus, a procedure combining enzymes and microwaves was studied further. The parameters to optimize for the enzymatic treatment were: type and quantity of enzyme, and time and power applied. In a bibliography search three enzymes were selected: protease, proteinase-K and a combination protease-lipase. Proteinase-K gave the best recoveries (Fernández-Torres et al., 2011). It is cheap, reliable and environmentally friendly, and shows equal or better extraction efficiency than more conventional techniques. MAE has widespread use in several areas, including environmental analysis, food and clinical determinations.

The presence in carps (*Cyprinus carpio*) of antibiotics was studied (Fernández-Torres et al., 2011) to evaluate MAE approach in fish exposed to these pharmaceutical compounds. Carp was selected since this species live for a long period in aquaria and is obtained in pet shops. Before the assay, fish were maintained in a 1000 L tank (with filtration and aeration systems) for two months to eliminate the antibiotics possibly used in the pet shop for prophylaxis. Four assays were made with different antibiotic mixes to assure that doses and mixes were not lethal: *assay 1*, tianphenicol, trimethoprim and chloramphenicol; *assay 2*, sulfadiazine, sulfamerazine, sulfadiazine and sulfamethoxazole; *assay 3*, sulfadiazine, sulfamethoxazole, oxytetracycline and amoxicillin, and *assay 4*, oxytetracycline, chlortetracycline, amoxicillin and ampicillin. Three animals (~30 g each) were isolated into a 20 L aquarium before antibiotic administration. Following recommended doses (Durborow et al., 1996), 100 mg of each antibiotic were mixed in a small bread ball to assure that animals ingested it fully; a second dose was administered 24 h later. After 1 h, fish were sacrificed and muscle tissue was processed by MAE. Most of the analytes administered were detected, although in some cases their levels were not quantifiable. Several of their metabolites were also detected and/or quantified. In all quantified samples the levels were above maximum recommended limit (MRL, Council Directive 37/2010 EC), highlighting that an adequate time has to elapse from antibiotic administration to commercialization. The viscera of exposed carps were also analyzed by the same procedure, as the presence of the analytes was also expectable. Data obtained confirmed this, although levels in fish viscera are not submitted to legislation, these are higher than the obtained in muscle samples.

To verify whether the presence of pharmaceuticals is significant in aquatic ecosystems, a variety of marine specimens were captured near Aguilas, a town of some importance SW of Murcia (Spain), with an area of 252 km^2 and 28 km coastline at the Mediterranean Sea. It has a stable population of 28,800 inhabitants that reaches 150,000 in summer. The specimens were captured through artisan fishing by net placed 100-200 m from the beach. Specimens of different fish species, *Thrachynotus ovatus, Salpa salpa, Oblada melanura* and *Liza ramada*, were analyzed according to the HPLC-MAE method previously developed. In general, the results obtained show that some specimens had been exposed to antibiotics in some way but levels found were below the MRL fixed by the Council Directive 37/2010 EC and subsequent amendments. To notice the results obtained for oxytetracycline in samples of *T. ovatus* which are really surprising and unexpected, with contents about 3-5 times the corresponding MRL, which could imply a long exposure of the animal to the compound.

Methods for the determination of PAHs and linear alkyl benzene sulfonates (LAS) were also optimized. DNP includes a mosaic of unique ecosystems which are particularly affected by the quality of the incoming flowing water. Characterization of wastewater and sludge from water treatment plants is a key issue due to their high contaminant power if depuration has

not been effective. An analytical procedure was developed using MAE prior to liquid chromatography coupled to diode array (DAD) or fluorescence (FLD) detectors for the determination of PAHs in sewage sludge (Villar et al., 2004). The new method has shown to be suitable for determination of the 16 PAHs recommended by EPA in sewage sludge. The extraction method requires only 0.5 g of sample and no additional clean-up is required prior to the final determination by LC using a FLD or DAD detector. A new method for the extraction and determination of LAS from sewage sludge based on MAE and HPLC coupled to DAD and FLD has been proposed (Villar et al., 2007). The extraction of C_{10}-C_{13} homologues of LAS was carried out by using an extraction time of 10 min and 5 mL of methanol and the determination of LAS by HPLC lasted less than 6 min. The method did not require clean-up or preconcentration steps.

2.2 Developments in metallomics studies for environmental assessment

The concept of metallome and metallomics has evolved to encompass a more complete picture of the role of metals in the living organisms, considering not only the different metal species in a cell but also their identity, quantity and localization, e.g. their presence in a particular tissue or compartment, or a set of metal complexes with a given class of ligands, e.g. metalloproteome (all metalloproteins) or metallometabolome (all metallometabolites) (Monicou et al., 2009). Metallomics is becoming a very multidisciplinary area with impact in the study of biological catalysis, because an important number of enzymes exert their activity through the metal included in the prosthetic groups, such as *heme* for Fe, or covalently bound to the active site, as Se-Cys in GSH peroxidase. Transition metals play an essential role in the metabolism of living organisms and, unlike metabolites, metals are neither produced nor consumed during cell reactions. Thiele & Gitlin (2008) assert that metals and their chemistries act as driving force for the evolution of life, and even more, metals metabolism depend upon their oceanic and atmospheric cycles, so that some living organisms exist due to the presence of metals in an area (Thiele & Gitlin, 2008). This allows to correlate the proteome of sentinel organisms with the metal contamination, the rationale for their use in environmental proteomics and metallomics (Lopez-Barea & Gomez-Ariza, 2006). In addition, the metabolism of plants involves the mobilization of low solubility metals from soils and translocation into the plant and sequestering metal ions in cytosol or in cellular compartments. Therefore, metallomics is a useful tool for the study of plants hyperaccumulation for environmental bioremediation (Shah & Nongkynrih, 2007) and plant defense mechanisms against heavy metal stress (Clemens, 2001).

Several analytical approaches have been proposed in environmental metallomics, mainly focused on detection, identification and analysis of metal-containing biomolecules in the sample under study. The trace levels of metallobiomolecules (proteins and metabolites) require the use of high resolution separation techniques with very sensitive elemental and/or molecular MS detectors. The separation component role is to avoid co-elution of another species of the same element (when ICP-MS detection is used) or that of any easily ionizable species able to suppress the ionization of the analyte (if molecular MS is used). One key point in analytical techniques hyphenation is to consider metal species kinetically inert which are not involved in reactions that provoke replacement of one or more ligands in the coordination sphere by others on the timescale of the analytical procedure.

The specificity of metalloproteomics studies requires the description of metal-binding sites, metal stoichiometry and metal-dependent structure or conformation changes. This is not compatible with many sample treatments used in conventional proteomic methods that

produce proteins denaturation and, thus, loss of non-covalently bound metals (Gomez-Ariza et al., submitted). A valid metallomic protocol requires the preservation of the binding between protein and metal, a suitable system for purification and/or preconcentration of the metal-binding protein of concern, and an instrumental platform for specific detection and identification of metalloproteins from the sample (generally the combined use of HPLC-ICP-MS and HPLC-MS).

Use of size exclusion chromatography hyphenated to inductively coupled plasma mass spectrometry (SEC-ICP-MS) has been recently proposed for pollution assessment (González-Fernández et al., 2011). The approach is based on the use of *M. spretus* as bioindicator in contaminated and non-contaminated DNP areas. Metal SEC-ICP-MS profiles of liver and brain extracts reveal Cu, Mn and Zn-containing proteins and biomolecule-bound non-essential elements, such as Cd, Pb and As. Clear differences existed between the chromatograms corresponding to polluted areas compared to non-polluted. An apparent upregulation of a Cu and Zn-peak at ~7 kDa, which may correspond to metallothionein (induced by pollution), and a downregulation of a ~32 kDa Cu-peak, possibly related to superoxide dismutase (Cu-SOD), are observed in liver extracts from contaminated areas, while opposite results were observed in unpolluted areas. However, in brain extracts the differences between the SEC-ICP-MS chromatograms from polluted and unpolluted areas for Cu and Zn-peaks, related to metallothioneins and superoxide dismutase, are very small, possibly due to the protection of blood–brain barrier.

2.3 Conventional biomarkers and proteomics in environmental studies

2.3.1 Studies in bivalve molluscs

Bivalve mollusks have gained a worldwide importance as bioindicators of marine/estuarine pollution. Different species were used as sentinels at the Huelva and Guadalquivir Estuaries from 1999 to 2003. Since metals and organics that promote oxidative damages are the main contaminants in the area, biomarkers responsive to oxidative stress were selected to monitor their biological effects, paying special attention to antioxidant defenses and oxidative damage to biomolecules. Differences existed in pollution responses of clams (*Chamaelea gallina*) and oysters (*Crassostrea angulata*) in comparison to mussels (*Mytilus galloprovincialis*) (Rodríguez-Ortega et al., 2002; Funes et al., 2006). Clams and oysters were very sensitive bioindicators, since animals from contaminated Huelva sites accumulated high pollutant loads and displayed higher antioxidant defenses, thus containing less oxidative damages. Actually, bivalves chronically exposed to pollutants released by Huelva and Guadalquivir Estuaries had less lipid peroxidation and 8-oxodG level in DNA, and a less oxidized GSSG/GSH status. In contrast, mussels were less adapted to pollution and showed clear increases in oxidized biomolecules, in agreement to their small increases in antioxidant defense mechanisms, in spite of their much lower metal accumulation (Funes et al., 2006).

Its high metal affinity/inducibility and free radical scavenging ability makes of metallothionein (MT) one of the most sensitive biomarkers of pollution in several bivalve species, such as clams (Rodríguez-Ortega et al., 2002), mussels and oysters (Funes et al., 2006), in which MT levels correlated positively with metal load. In search of a more specific, sensitive and reliable assay, a total MT quantification method was developed using reversed-phase HPLC coupled to fluorescence detection (RP-HPLC-FD) after monobromobimane (mBBr) derivatization. The RP-HPLC-FD method was described and successfully applied to assess the MT content in heat-denatured digestive gland extracts of *C. gallina* from different South-Spanish coastal sites (Alhama et al., 2006). The method was later improved, by optimizing mBBr derivatization and

heating treatment, in *Scrobicularia plana* clams (Fig. 4) to assess the pollution status of the Guadalquivir Estuary after the Aznalcóllar spill (Romero-Ruiz et al., 2008). MT content was higher in unheated samples than in heated extracts, and correlated better to metal (Zn, Pb, Ni, Mn and Fe) contents and antioxidant activities. MT correlated negatively to 6PGDH and glyoxalase II activities, inhibited/inactivated at metal polluted sites, and positively to CAT activity and MDA level, increased by metal-promoted oxidative stress. Protein expression profiles were studied also by 2-DE in *S. plana* gills (Romero-Ruiz el al, 2006). The results showed a higher number of upregulated protein spots in animals from sites of Guadalquivir Estuary near its mouth in the Atlantic Ocean, that contained higher content of several redox-active elements (Fig. 4). Taken together, our results suggest that elements detected at Guadalquivir Estuary did not originate from Aznalcóllar spill, but were carried out by Guadalquivir River and deposited at its concave bank at high tide conditions.

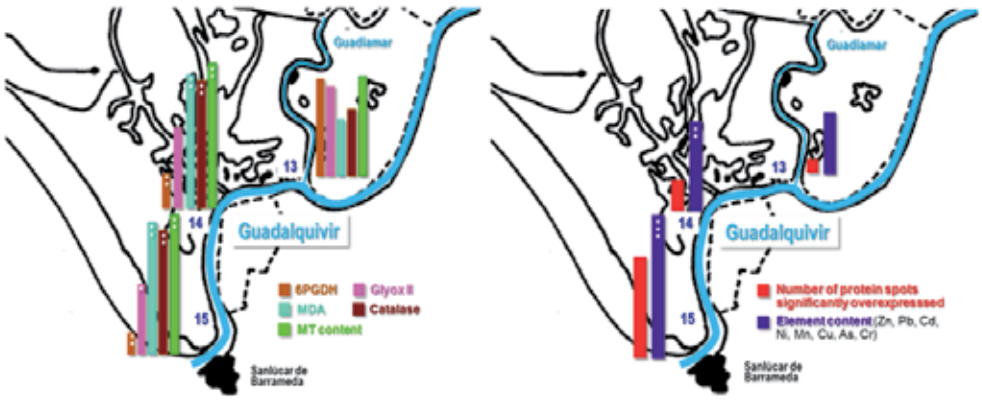

Fig. 4. Pollution of Guadalquivir Estuary studied in *S. plana* in October 2003. A) Responses of "classic" biomarkers, and B) total number of protein spots showing after 2-DE significant upregulated expression levels, compared to content of a number of elements.

2.3.2 Effects of Aznalcóllar spill assessed in *M. spretus*

On April 25, 1998, a tailings dam of the Aznalcóllar pyrite mine partially collapsed releasing to Guadiamar stream acidic water and mud with toxic metals (Fe, Zn, Pb, As, Cu, Sb, Tl, Bi, Cd, Ag, and Sr) that threatened DNP. To assess their possible biological effects, biochemical biomarkers were assessed in *M. spretus* from several Doñana areas and along the Guadiamar course (Ruiz-Laguna et al., 2001). Biomarkers assayed responded to different contaminant types: 1) Metals and prooxidant compounds (CAT, SOD, GSSGRase, GSHPx, G6PDH, MDA, GSSG/GSH status). 2) Aromatic compounds (EROD). 3) Chemicals with both characteristics (cytosolic and microsomal GST). Before the spill mice from the "Brazo de la Torre" (Fig. 2) had GSHPx and EROD activities close to animals of Huelva Industrial Park, suggesting similar levels of oxidants and aromatic contaminants at both sites (Ruiz-Laguna et al., 2001). Six months after the spill, mice from the lower Guadiamar areas, also showed significantly higher GSTc and GSTm activities, altered levels of several antioxidant enzymes, and a highly oxidized GSSG/GSH status. Thus, chemicals spilled from Aznalcóllar dam induced further biological effects in mice from exposed areas.

Monitorization of terrestrial ecosystems of DNP and Guadiamar course continued till 2002 using *M. spretus*. Kidney Pb, Cd and As levels correlated with antioxidative enzymes.

Oxidative stress biomarkers indicated the presence of metals in the upper/medium Guadiamar course, and their response decreased with the distance to the mine. From 1997 to 2002 biomarkers evolved as follows: 1) Antioxidative (CAT, SOD, GSSGRase, G6PDH) and biotransforming (GSTc, GSTm) enzymes and MDA and GSSG levels were lower in LDP mice than in those along Guadiamar course, and decreased in 2001-2002, indicating a clear recovery after dilution of the metals released. 2) Other biomarkers (G6PDH, GSHPx and especially EROD) were also high in LDP mice and increased in 2001-2002, suggesting the presence in the medium/low Guadiamar course, and even in Doñana, of organic pollutants, such as the pesticides used in intensive agriculture of nearby areas, strawberry and citric crops West of DNP, and the rice fields East of Guadiamar (Bonilla-Valverde et al., 2004).

In 2002, the effects of Aznalcóllar spill and those of agrochemicals used around DNP were compared in *M. spretus* from sites along Guadiamar, Rocina ($ROC_{5/6}$) and Partido ($PAR_{7/8}$) streams, within Doñana Biological Reserve (LDP) and Matochal rice fields. In addition to the biomarkers used previously, a PES proteomic approach was undertaken, detecting up-/down-regulation of proteins after 2-DE gels (Bonilla Valverde, 2006). Figure 5A summaryzes the responses of "classic" biomarkers: compared to LDP, mice from areas of intensive agricultural practices, ROC, PAR, Matochal, had increased EROD and GSHPx activities and GSSG levels, indicating that these sites were polluted, even more extensively than sites from Guadiamar course. Near 3,000 proteins were resolved in 2-DE gels (24 cm, pH 4-7), of which 30 showed significant intensity differences when compared to gels of LDP mice. Figure 5B summarizes the conclusions of this PES approach: the intensities of 18 spots were signifycantly altered in mice from intense agriculture ($ROC_{5/6}$, $PAR_{7/8}$, Matochal), and 20 spots in animals collected along Guadiamar course. It should be noticed that the intensity of 8 of the total 30 proteins were simultaneously altered in animals from both sites, suggesting the presence of similar contaminants, probably metals, in areas exposed to Aznalcóllar spill and of intense agricultural practices (Bonilla Valverde, 2006).

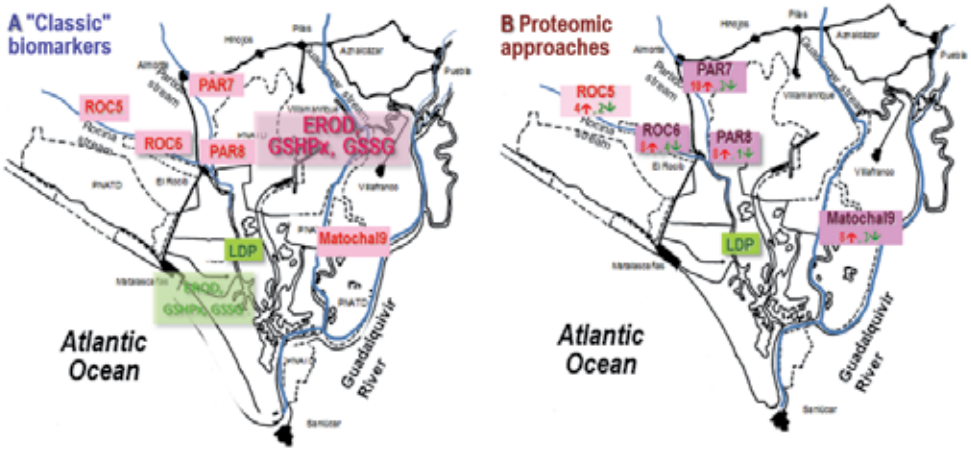

Fig. 5. Pollution of DNP and Guadiamar sites compared to LDP site studied in *M. spretus* liver. A) Responses of "classic" biomarkers. B) Number of proteins with significantly altered expression levels (red up-, green down-regulated).

2.3.3 Conventional biomarkers and PES-proteomic studies in *P. clarkii*

Organophosphate (OP) and carbamate (CM) pesticides replaced organochlorines as the main defense line against agricultural insects. Although less persistent, they affect nontarget organisms and increasing concern exists about their impacts (Scholz & Hopkins, 2006). OPs and CMs are toxic by inhibiting esterases, hydrolases of three types: (i) Acetylcholinesterase (AChE) active at synapses during nerve transmission. (ii) Butyrylcholinesterase (BChE) of uncertain physiological role. (iii) Carboxylesterases (CbE) that hydrolyze fatty acids from xenobiotics (Maxwell, 1992) and play a role in pesticide detoxification and tolerance (Hyne & Maher, 2003). Inhibition of AChE and BChE has been used as an exposure biomarker for CMs and OPs: Peakall (1994) included AChE inhibition in the gold standard biomarkers, useful to diagnose a problem without need for chemical analysis.

AChE, BChE and CbE activities were characterized in different *P. clarkii* tissues, and their sensitivity to inhibitors and model OP and CM pesticides was studied (Vioque-Fernández et al., 2007a). Nervous tissue AChE and digestive gland CbE were proposed as biomarkers; low BChE was found in all tissues studied. Use of TritonX-100 was avoided due to its diverging effects in esterase assays. CMs inhibited AChE 100-fold more strongly than OPs. *In vitro* conditions to assess recovery from inactivation were established for AChE and CbE. The new protocol was proposed as a biomarker of pesticide exposure to differentiate between dilution-reversible inhibitions, indicating CM exposure, from irreversible effects, treating with 2-pyridinealdoxime (2-PAM), attributed to OPs.

Utility of AChE and CbE inhibition and reactivation was assessed in a field study carried out at different freshwater sites of DNP. Esterases were measured in *P. clarkii* from reference or potentially exposed sites, and their possible reactivation was studied after dilution or 2-PAM treatment (Vioque-Fernández et al., 2007b). As summarized in Table 2, crayfish of five potentially polluted sites, $ROC_{5/6}$, $PAR_{7/8}$ and Isla $Mayor_{10}$ (Fig. 2), had lower CbE activity (from -43 to –23%) than those from LDP. Yet, no differences with untreated extracts were found after 50-fold dilution or 2-PAM treatment, indicating that CbE was irreversibly inhibited. Several pesticides were detected in water and/or soil at Matochal and Isla Mayor rice growing sites, and lower levels at agricultural areas near the Rocina and Partido streams. Since no correlation was found between pesticide content and esterase inhibition, other factors, such as the concomitant effects of metals, should affect pesticide response.

Site	CbE activity			Organic compound detected
	% LDP	Dilution	2-PAM	
LDP	**100**	118	109	
ROC 5	**66****	81	86	PCB138
ROC 6	**71****	105	87	Bromacyl, Dimethoate
PAR 7	**77***	100	104	4,4´-DDD, Dichlofluamid, Folpet, Molinate
PAR 8	**57****	98	102	Molinate
Matochal 9	**82**(ns)**	91	97	Malathion, Bromacyl, Dimethoate, Acrynathrin, Chlorpyrifos, Trifluraline, Penconazole, Methidathion
Isla Mayor 10	**57****	106	102	4,4´-DDT, Dichlofluanid, Bupirimate

Table 2. BChE activity in *P. clarkii* and pesticides detected in water/soils from DNP sites. CbE is shown as % of LDP animals, and after 50-fold dilution or 2-PAM treatment, and compared to untreated values. Pesticides are shown in decreasing order of concentration.

Freshwater aquatic DNP ecosystems were monitored using *P. clarkii* in four campaigns carried out from 2003 to 2004 (Vioque-Fernández et al., 2009a). In the "classic" approach, twelve biomarkers, responsive to pesticides, organics and prooxidants were used. Low CAT, G6PDH, CbE and AChE activities existed at $ROC_{5/6}$, $PAR_{7/8}$, Matochal and Isla Mayor, plus high MDA, MT and GSSG levels, suggesting that metals and prooxidants were probably present at sites potentially polluted by agrochemicals. In contrast, high CAT, CbE and AChE activities and GSH levels, and low MDA, MT and GSSG contents existed at LDP (Fig. 6A). The superiority of proteomics was clearly established. 2-DE resolved over 2,500 gill spots, and 35 proteins had significant intensity differences (Vioque-Fernández et al., 2009a). The fold-number of up-/down regulation separated different PES (Fig. 6B). Animals captured within or close to Doñana Biological Reserve (LDP, LD, ROC_6, and PAR_8) showed clean/low polluted PES, with 32 proteins of unaltered intensity compared to LDP. Site PAR_7 had a moderately polluted PES, with 7 proteins up- and other 6 downregulated. Crayfish from the upper "Rocina" course (ROC_5) showed a polluted PES, with 13 proteins up- and other 13 downregulated compared to LDP. The higher proteomic responses at the upper Rocina and Partido courses suggest that non-persistent agrochemicals are used in Doñana surroundings. The highest responses correspond to crayfish from the rice growing areas at Matochal and Isla Mayor, with 30 of the 35 proteins being significantly altered, according to the extended and intensive use of agrochemicals in such areas. Both the "classic" biomarker and the environmental proteomic approach indicate that sites within Doñana Biological Reserve are scarcely polluted, while the agricultural areas around DNP are highly polluted.

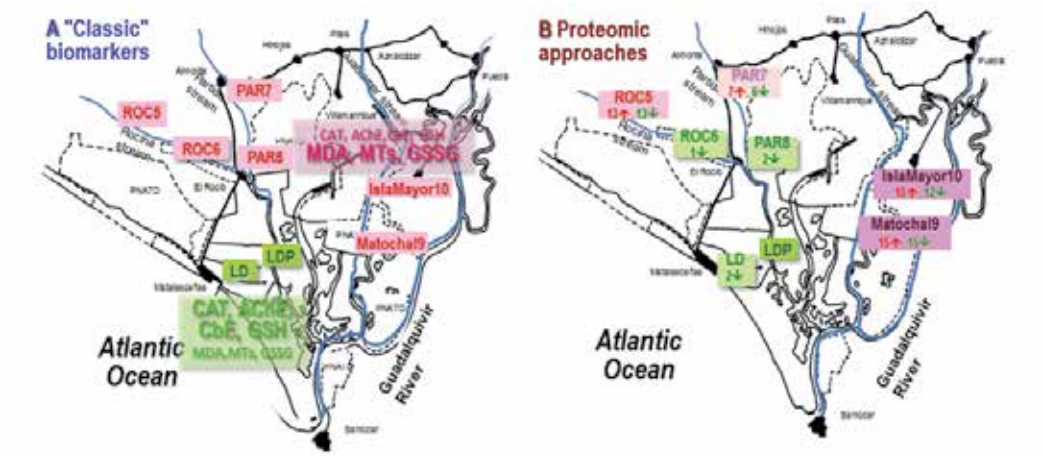

Fig. 6. Pollution of DNP sites compared to LDP assessed in *P. clarkii*. A) Responses of "classic" biomarkers. B) Number of proteins with significantly altered expression levels (red up-, green down-regulated) in gill extracts of crayfish from the different sites studied.

DNP monitoring using *M. spretus* (Bonilla Valverde, 2006) and *P. clarkii* (Vioque-Fernández et al., 2009a) differed to some extent. While the "classic" approach yielded similar results, with higher biomarkers levels being detected at the agricultural areas surrounding Doñana, along the Rocina and Partido streams and the rice growing fields east, the PES approach differed significantly. Actually, while in the *M. spretus* proteomic study the areas close to DNP, ROC_6/PAR_8, were as polluted as Matochal, in the *P. clarkii* PES study these two areas were classified as low or faintly polluted, in view of the small number of proteins whose

intensity was significantly altered when compared to LDP animals. It is worth noticing that mice evaluate the status of terrestrial ecosystems and crayfish that of fresh water aquatic environments, whose pollution could be quite different.

In vivo effects of two model pesticides, chlorpyrifos and carbaryl, were studied under controlled exposure conditions in *P. clarkii* (Vioque-Fernández et al., 2009b). The organophosphate chlorpyrifos inhibited CbE in a concentration-dependent mode, AChE being less sensitive; in contrast, the effects of carbaryl, a carbamate, were less clear. Chlorpyrifos lowered EROD, CAT and GSSG levels but raised GST activity, while carbaryl raised EROD, CAT and GST, but lowered GSHPx and GSH levels. The effects on protein expression were studied also by 2-DE (Vioque-Fernández et al., 2009b). In gill and nervous tissue about 2,000 spots were resolved, with quite different expression patterns. As shown in Figure 7, chlorpyrifos altered 72 proteins, mostly in nervous tissue, and carbaryl 35, distributed evenly between organs. Several spots were selected as specific protein expression signatures for chlorpyrifos or carbaryl exposure in gills and nervous tissue, respectively.

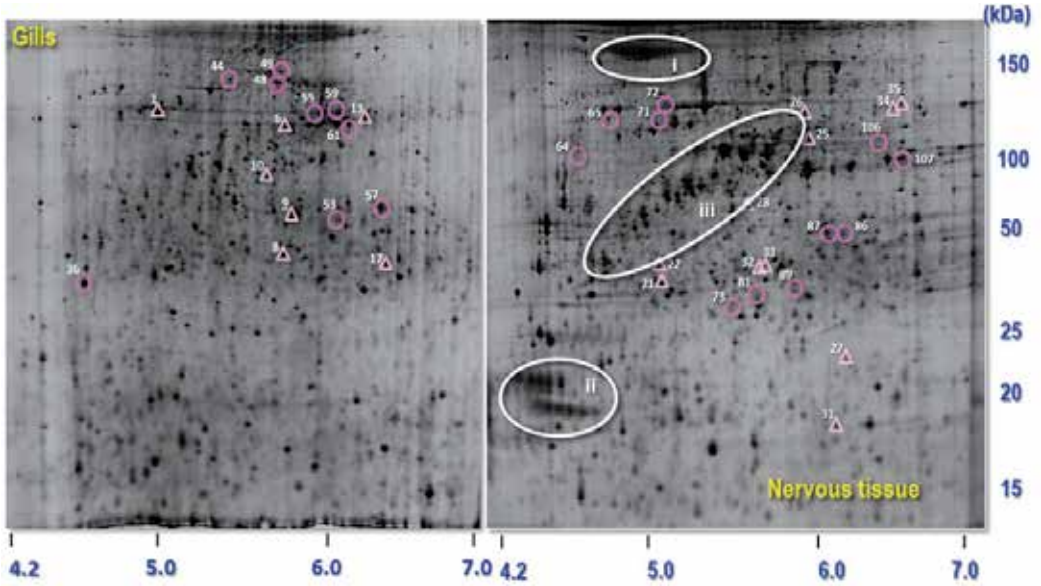

Fig. 7. Representative 2-DE gels of proteins from *P. clarkii* gills and nervous tissue. Symbols show spots with significantly altered intensities after 7 days exposure to chlorpyrifos (O) or carbaryl (Δ) concentrations. Areas i-iii mark clearly different patterns in nervous tissue.

2.3.4 Use of fisrt-generation methods in the environmental proteomic approach

We will now review several studies made by our groups using first-generation proteomic methods for protein separation and identification. These studies were carried out comparing animals from sites within DNP and Doñana surroundings, the Domingo Rubio Stream, and the Guadalquivir Estuary. Bivalves, including *Chamaelea gallina*, *Scrobicularia plana*, the crustacean *Carcinus maenas*, and *M. spretus* mice were used as bioindicators. Proteins showing significantly altered levels were identified by *de novo* nESI-MS/MS sequencing in the non-model species *C. maenas* and *S. plana*, and by MALDI-TOF-PMF in *M. spretus*, due to its homology to model mouse species *Mus musculus*.

Proteomics was used in *C. gallina* clams as a preliminary screening of changes in protein expression caused by pollutants, potentially useful as new biomarkers (Rodríguez-Ortega et al., 2003). Clams were exposed for seven days to four model contaminants, Aroclor 1254, copper (II), tributyltin (TBT), and arsenic (III), and cytosolic fractions were initially analyzed by 2-DE (7 cm, pH 4–7). About 1,000 spots were resolved and altered expression was qualitatively detected in 9–26 spots per treatment. Aroclor 1254, Cu (II) and As (III) had a mainly upregulating effect, in contrast to TBT. Altered protein expression was confirmed in 18 cm gels. The 15 spots more drastically altered were excised and analyzed by MS. Aroclor 1254 and Cu(II) upregulated putative isoforms of tropomyosin and light myosin chain. Actin was downregulated by Aroclor and Cu(II) but upregulated by TBT and As(III), while the opposite behavior was shown by a truncated actin form. The exclusive identification of cytoskeletal proteins could reflect their relative abundance, their prevalence in databases in mollusks, or their role as major targets of pollutant-related oxidative stress.

The utility of proteomics to assess pollutant response of *S. plana* clams from three sites of Guadalquivir Estuary at the southern end of DNP was studied (Romero-Ruiz et al., 2006). Protein expression profiles were analyzed by 2-DE in soluble fractions of *S. plana* gills. Nearly 2,000 well-resolved spots were detected in silver-stained gels, with focused areas in the 4–6.5 pH range. Different protein expression signatures were found at each site, with the highest number of more intense spots in animals with the highest metal content. Nineteen more intense protein spots were analyzed out by nanospray-ion trap tandem MS/MS, *de novo* sequencing and a bioinformatics search for their possible identification. While sequence tags of 16 more intense protein spots were obtained, including several proteins induced by pollutant exposure of model organisms, only 2 proteins were unambiguously identified: hypoxanthine guanine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Both enzymes were significantly higher in animals with the highest metal contents.

The suitability of high-throughput proteomic methods to monitor terrestrial ecosystems was evaluated in *M. spretus* from three sites in the DRS and from LDP, using specimens from an industrial settlement (phosphogypsum stacks) and Matochal rice fields as positive controls (Montes-Nieto et al., 2007). The 2-DE analysis showed 36 spots with significantly altered expression. Sixteen were identified by MALDI-TOF-PMF and peptide matching with *M. musculus* databases. Identified proteins play different roles: cytoskeletal dynamics, proteolysis, biotransformation, oxidative-stress adaptation, and metabolism. Animals from different polluted environments showed contrasting differences in their proteomes, with specific increases and decreases in selected groups of proteins that seem to be coordinately regulated. Proteomic data were consistent with metal biomonitoring and conventional biomarker responses, indicating that DRS animals sustained a heavier pollutant burden than LDP mice and suffered a chronic oxidative stress. Whereas some protein expression differences may protect mice from pollutant toxicity, others should make them more susceptible. Transcript expression signatures agree with the documented lack of correlation between mRNA and protein levels. Nonetheless, there is a positive significant correlation between the gpx1 mRNA molecules and the intensity of one of the two identified GPX1 isospots. It was underlined the usefulness of additional information (element content, biomarker responses and absolute mRNA expression signatures) to assist the biological interpretation of environmental proteomic data.

Element load, "classic" biomarkers and altered protein expression were studied in *C. maenas* crabs, to assess DRS contamination (Montes-Nieto et al., 2010). Lower antioxidative

activities (G6PDH, 6PGDH, CAT) were found in parallel to higher levels of damaged biomolecules (MDA, GSSG), due to oxidative lesions promoted by contaminants, as the increased levels of essential (Zn, Cu, Co) and nonessential (Cr, Ni, Cd) elements. Utility of Proteomics to assess environmental quality was confirmed, especially after considering the six proteins identified by *de novo* sequencing. They include tripartite motif-containing protein 11 and ATF7 transcription factor (upregulated), plus CBR-NHR-218 nuclear hormone receptor, two components of the ABC transporters and aldehyde dehydrogenase (downregulated). These proteins could be used as novel potential biomarkers of the deleterious effects of pollutants present in the area.

Table 3 summarizes a number of proteins with significantly altered expression identified in previous studies of Environmental Proteomics using fisrt-generation methodologies. The Table includes the cellular functions in which these proteins are involved, including: cytoskeleton, axonal transport and cell division, membrane transport, proteolysis and autophagy, biotransformation, adaptation to oxidative stress, central pathways of glucid, fatty acid, aminoacid, methyl and urea metabolism, and transcriptional regulation.

Cellular function	**Protein identified** (reference)
Cytoskeletal dynamics	Tropomyosin[1], light Myosin chain[1], Actin[1], Tubulin beta-3 chain[2]
Membrane transport	Peptide ABC transporter[3], ABC transporter G-family[3]
Proteolysis	Cys-protease ATG4B[2], tripartite motif-containing protein 11[3]
Redox functions	GST ω-1[2], GSHPX 1[2], Peroxiredoxin 1, 2 and 6[2]
Intermediary metabolism	Triose-P isomerase[2], Glyceraldehyde 3-P, DHase[4], Fructose-1,6-bisphosphatase[2], Hypoxanthine P-ribosyl transferase[4], Aldehyde DHase[3], EnoylCoA hidratase[2], HMG-CoA synthase 2[2], L-Asp DHase[2], Met adenosyl transferase[2], Gly-N-methyl transferase[2], Ornithine transcarbamylase[2],
Transcriptional regulation	ATF-7, cAMP-dependent transcription factor, CBR-NHR218 nuclear hormone receptor[3].

Table 3. Proteins with significantly altered expression identified in: [1]Rodriguez-Ortega et al., 2003; [2]Nontes-Nieto et al., 2007; [3]Nontes-Nieto et al., 2010; [4]Romero-Ruiz et al., 2006.

2.4 Transcriptomics in environmental studies

Studies reporting absolute transcript abundance of genes are infrequent, even though it is the only adequate procedure to accurately assess the expression of a gene (Prieto-Alamo et al., 2003). We used this rigorous approach to provide the first comprehensive and absolute quantification analysis of the overall expression patterns of transcript coding for several ecotoxicological interesting protective and detoxificant enzymes in *M. spretus*. A total of 20 transcripts involved in oxidative stress response (heme oxygenase, HO) and biotransformation of electrophilic compounds (CYPs, GSTs) was examined (Prieto-Alamo et al., 2003; Ruiz-Laguna et al., 2005, 2006). Different sites located inside DNP and in Huelva province were chosen due to their proximity to industrial or agricultural areas. Metal biomonitoring indicated that animals from contaminated areas had heavier pollutant burden than those from LDP. Interesting organ-associated differences were found in the expression levels of the transcripts analyzed, being in general the liver the organ with the highest levels, according to its detoxificant role.

The hepatic expression levels of transcripts encoding different CYPs and GSTs showed up-regulation of Cyp1a2, Cyp2a5, Cyp2e1, Cyp4a10, Gsta1, Gsta2, Gstm1, and Gstm2 mRNAs in liver of male mice dwelling polluted areas (Table 4). Hepatic level of *Cyp* and *Gst* mRNAs are collectively activated by many structurally unrelated compounds through a variety of mechanisms. Additionally, CYP proteins usually generate reactive molecules that must be detoxified by GSTs. The concomitant up-regulation of Cyp and Gst genes, that respond to many different classes of chemicals and regulatory mechanisms, seems the logical results of exposure to a complex profile of pollutants, like those associated with the intensive agricultural activity developed close to our studied areas. Important gender-related differrences were found also in the expression of most Cyp and Gst genes, suggesting that absolute amounts of transcripts of biotransformation enzymes are more potent biomarkers in males than in females. Individual quantitation's is also mandatory to prevent biased interpretations by specimens with abnormal expression levels.

Up-regulated genes	**Known inducer mechanisms**
HO1	Induced by oxidants
Cyp1a2	Induced by PAHs/dioxins through the xenobiotic response element (Noda et al., 2003)
Cyp2a5	Induced by N heterocycles non P450 inducers (Su & Ding, 2004)
Cyp2e1	Act on solvents and industrial monomers (González & Kimura, 2003)
Cyp4a10	Induced by peroxisome proliferators via PPAR (cited Kroetz et al., 1998)
Gsta1, Gsta2 Gstm1, Gstm2	Induced by PAHs, phenolic antioxidants, ROS, isothiocyanates, arsenicals, barbiturates, etc., by mechanisms usually involving the antioxidant response element (Chanas et al., 2002).

Table 4. Genes upregulated identified in: Prieto-Álamo et al., 2003; Ruiz-Laguna et al., 2005

3. Ongoing research

The global aim of our current project focused in Doñana and its surroundings is the development and multidisciplinary integration of innovatory analytical tools, that would subsequently be applicable to different types of ecosystems to contribute to a technological updating of environmental evaluation. The following goals are being pursued during the development of our project: 1) Information is being obtained on pollutant presence in Doñana and their biological effects. 2) Analysis methods for the characterization of metals and elements and new species with a toxic potential are being optimized. 3) The presence of pesticides and other pollutants, including some emerging ones, is being evaluated by developing screening and fast analysis methods. 4) Finally, the chemical analyses and the responses of some established biomarkers will be integrated with the expression profiles detected with high-throughput methods at genomic, proteomic and metallomic levels, permitting a global evaluation of environmental quality.

3.1 Novel analytical approaches for emerging pollutants

Analysis of complex samples and detection or quantitation of analytes at very low levels are two of the key analytical problems, whose complexity increases when both problems are

present. The use of clean-up procedures is a traditional tool that has recently undergone very important developments. Liquid-liquid extraction (LLE) used to be a key sample-preparation technique prior to carry out the chemical analysis. Recently, solid phase extraction (SPE), using several sorbent types, has been preferred to extract pharmaceuticals from environmental and biological matrices. Yet, in the last years, high interest exists in developing new extraction and clean-up procedures. Liquid phase microextraction (LPME) is an attractive alternative to the widely used solid phase extraction (SPE). Single, low-cost, disposable, porous, hollow fibers made of polypropylene are used as support for liquid membranes (HF-LPME) as an efficient clean-up procedure that yields also high degree of pre-concentration. Additionally, the low organic solvent consumption makes HF-LPME an interesting and environmental friendly analytical procedure. This extraction procedure has been used for the determination of some pharmaceuticals in environmental and biological samples (Ramos-Payan et al., 2009a,b, 2010, 2011a,b,c) that also decreases matrix effects and ionic suppression when mass spectrometry detection is used.

An electric potential can promote efficient analytical extraction through a supported liquid membrane (SLM). This system, known as electromembrane extraction (EME) produces the analytes to be extracted from an aqueous sample through an organic solvent immobilized as SLM in the wall of a polypropylene porous hollow fiber to an aqueous acceptor solution placed within the lumen of the hollow fiber. Essentially, it is similar to a HF-LPME where the migration through the SLM is forced by the electrical field generated by two electrodes placed out of the fiber and in its lumen, respectively (electromigration, EMI). This extraction procedure has been used to determine six widely used non-steroidal anti-inflammatory drugs. The method was applied to their determination in such a highly complex matrix as urban wastewaters. The EME procedure provides very clean extracts that can be directly injected into the chromatographic system thus producing excellent baselines.

Currently, the efforts are directed to combine enzymatic extraction assisted by ultrasounds probe sonication or microwaves and a subsequent HF-LPME to reach higher selectivity and sensitivities. Extraction procedures are being developed for determination of substituted (chloro-, nitro- and alkyl-) phenols, well known as endocrine disruptors. Procedures that combine microwave and ultrasound energy with use of enzymes followed by a HF-LPME, will be applied to environmental samples and to fish and crustaceans tissues. Work is also being carried out in the design and optimization of exposure experiences to emerging contaminants for *P. clarkii*, an excellent bioindicator in fresh water ecosystems.

3.2 Novel analytical approaches for pesticides

Conventional analytical approaches for pesticides based on gas chromatography with ECD or MS detectors have been improved to enhance sensitivity and selectivity. A coupling based on a two-dimensional detector, electron capture/inductively coupled plasma MS, combines the high sensitivity (ECD) and the selectivity (ICP-MS) for halogens, avoiding interferences from other organic compounds present in organism samples (Gómez-Ariza et al., 2006). Pretreatment is critical to get quantitative recovery and preconcentration from samples in a short time. Polymeric membranes are being used for this purpose since it overcomes well established approaches such as solid phase microextraction with lower cost and no cross contamination. A method based on hollow fiber liquid-phase microextraction has been optimized for extraction of forty COP's, including PCB's, PBDE's and pesticides. Preliminary univariated optimization allowed the selection of more significant variables, subsequently subjected to a central composite rotable design (CCRD) for multivariate

optimization of extraction time, membrane length and temperature. Efficiency and preconceentration factors and recoveries are very high (Manso-Sayago et al., 2010).

3.3 Metallomic analysis in animals from Doñana National Park

The approach based on SEC-ICP-MS coupling is being used in exposure experiments of the laboratory mice, *M. musculus*. Preliminary results show that exposure to a toxic element, Cd, yield clear biological responses, namely the upregulation of Cd-metallothionein in liver and the concomitant downregulation of Cu-superoxide dismutase (González-Fernández et al., 2011). 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) is being applied to extracts of Cd-exposed *M. musculus* to identify all proteins differentially expressed after Cd treatment. Comparison of proteins identified by the massive 2D-DIGE approach and by SEC-ICP-MS will allow to understand the mechanisms involved in Cd response.

The SEC(HPLC)-ICP-MS approach is being optimized and applied to *M. spretus* liver to identify differentially expressed metalloproteins/metallobiomolecules (González-Fernández et al., 2011). Future studies will be focused on experimental exposure to other toxic elements or metal species, such as Hg^{2+}, methyl-Hg, As(V), and organic pollutants (pesticides, pharmaceutical drugs, PCB), involving both mice species, *M. musculus* and *M. spretus*.

3.4 Transcript expression patterns in animals from Doñana National Park

3.4.1 Heterologous microarrays for analysing pollution effects on *M. spretus*

The Domingo Rubio stream (DRS) is a contaminated place near Huelva industrial area that receives elements of pyritic origin from the Tinto River. Tidal changes affect mainly its lower course (Fig. 2, site 2), but also reach its medium course (site 3) that is also affected by nearby chemical plants and a petrochemical complex. The intensive agriculture carried out at adjoining strawberry fields affect the upper DRS course (site 4) and also reach site 3. Previous studies reported that DRS mice accumulate several elements, the metal loads being particularly high in animals from the medium DRS course (Montes-Nieto et al., 2007).

The transcriptomes of *M. spretus* from DRS sites 2, 3 and 4 were studied using heterologous microarrays available for the laboratory mouse, *M. musculus*, and compared to those of LDP reference mice. Although both species diverged ~1-3 million years ago, they conserve a great sequence similitude (one sequence variant every 50 bp) what permit the heterologous hybridization and identification of genes differentially expressed between reference and problem mRNA populations. Microarrays analysis (Fig. 8) identified 1,872 spots as differentially expressed in mice living in at least one DRS site. Expression of 242 genes was significantly altered in mice from the three DRS sites, and 39 genes showed ≥10-fold changes (34 up-/5 down-regulated) in animals from at least one DRS site compared with LDP site. DRS3 animals showed the maximal amplitude and the highest number of changes compared to those from the DRS2 and DRS4 sites (Abril et al., 2011) (Fig. 9).

Many pollutants, including heavy metals and pesticides, modulate the immune function (Galloway & Depledge, 2001). Chemicals in pesticides, mitocides, herbicides and fumigants stimulate, suppress or deregulate the immune system depending on dose and its duration (Rea & Lian, 1991); pesticides generate also reactive oxygen species (Agraval & Sharma, 2010). Genes of the "immune response" and "stress response/DNA repair" categories, such as Chi3l3 or A2m, Gpx3 or Sgk1 genes, are highly induced in DRS mice. CHI3l3 (chitinase 3-like 3 protein), produced by macrophages upon inflammation (Welch et al., 2002), mediates asthma inflammatory response (Shuhui et al., 2009). A2M (α2-macroglobulin), a plasma proteinase inhibitor, binds many biologically important molecules (LaMarre et al., 1991), inhibits the

degradation of matrix proteins and reduce inflammation in liver (Ho et al., 2010). Thus, A2M overexpression in DRS mice might help to the resolution of the inflammatory response and to recover homeostasis. GPX3 (GSH peroxidase 3), a key enzyme in the defense from oxidative stress, induces the Sgk1 gene expression (Leong et al., 2003; Loffing et al., 2006). SGK1 (serum- and glucocorticoid-regulated protein kinase 1) is a protein kinase activated via PI3-kinase to counteract apoptosis (Lutz et al., 2010). Most genes of the "cell cycle/cell differentiation" categories (e. g., Btc) are strongly up-regulated in DRS mice.

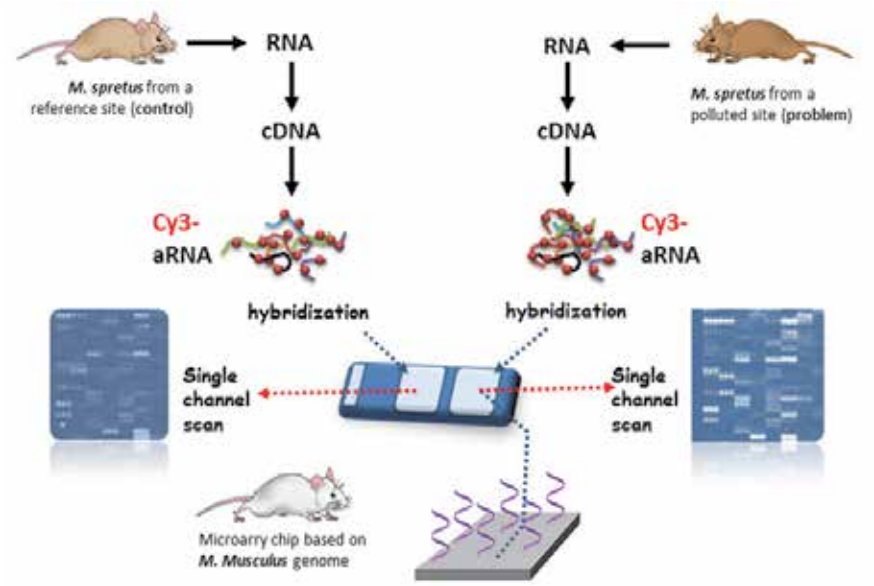

Fig. 8. Workflow for one-color heterologous microarray experiments. RNA is extracted, reverse transcribed into cDNA and labelled with Cy3 while synthetizing aRNA. Each sample is hybridized to a single microarray chip, that is washed and scanned. The relative signal intensity is used to estimate differentially expressed genes in the problem sample. The species for which the microarray was developed, here *M. musculus*, differs from that is hybridized to the microarray, here *M. spretus*.

Pesticide exposure may alter lipid, protein and carbohydrate metabolism (Karami-Mohajeri & Abdollahi, 2010). Interestingly, repression of Inhbe gene, negative regulator of cell growth (Chabicovsky et al., 2003) and inducer of gluconeogenesis (Hashimoto et al., 2009), might be linked to co-induction of genes coding MUP proteins, of the "cell signalling" category. MUPs (major urinary proteins) transport small hydrophobic ligands (pheromones), and inhibit the expression of gluconeogenic and lipogenic genes (Hui et al., 2009; Zhou et al., 2009). The Fgf21 gene was down-regulated in DRS animals (>11 fold), probably explaining their lower gluconeogenesis and lipid metabolism (Tong et al., 2010). Hence, the induction of Mup genes and the repression of Inheb and Fgf21 genes might be considered as a coordinated response to drive energy towards the inflammatory process. As a whole, data from the heterologous microarray study in *M. spretus*, and previous proteomic results (Montes-Nieto et al., 2007), indicate that DRS mice sustained a heavier pollutant burden than LDP animals and, therefore, suffer a chronic stress situation that elicit and maintain immune and an oxidative stress responses.

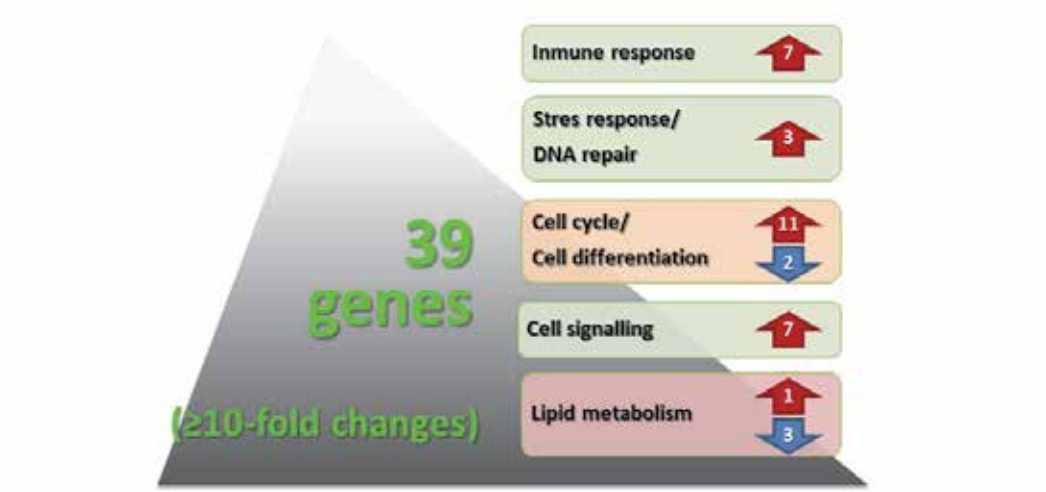

Fig. 9. Summary of genes significantly altered (≥10-fold, 34 up-, 5 down-regulated) in animals from the three DRS sites as compared with the LDP site (Abril et al., 2011).

3.4.2 Substractive suppressive hybridization to analyze pollution effects on *P. clarkii*

The reproductive success of *P. clarkii*, its ability to tolerate environmental changes and to feed on near anything contribute to its huge potential to colonise new areas and exploit natural resources. SSH is used to analyze the pollution response of *P. clarkii*, identifying genes differentially expressed in two populations without previous knowledge of their sequence. Animals from Matochal rice fields were considered as problem, and those from LDP as reference. The analysis was carried out in the digestive gland, main site of xenobiotic biotransformation involved also in key physiological processes (Reddy et al., 1997). Two libraries, one forward (genes up-regulated in MAT) and one reverse (down-regulated in MAT), were obtained. Most of the identified genes are related to the immune response (Abril et al., 2011). One of the up-regulated genes codes for hemocyanin, a Cu-containing protein linked to invertebrate response to PAHs and metals, after activation by Ser-proteases (Lee et al., 2004). The parallel up-regulation of the Ser-proteinase inhibitor-3 gene and the down-regulation of cathepsin-L gene in Matochal crayfish, might decrease the proteolytic generation of immune effectors, seeking for homeostasis recovery and resolution of the inflammatory situation (Jiang et al., 2009). Though the results obtained by SSH have to be accompanied by clone analysis and qRT-PCR validation, the data show that environmental stresses are causing a strong response in crayfish collected at polluted site.

3.5 Protein expression patterns in animals from Doñana National Park

Second generation proteomic methodologies, 2D fluorescence difference gel electrophoresis (2D-DIGE) and quantitative determination after labeling with isobaric reagents (iTRAQ) are being used to identify altered protein expression patterns in animals from sites of DNP with different pollution levels. In *M. spretus*, labeling with isobaric iTRAQ reagents is used to identify hundreds of mouse proteins. To diminish the complexity of mouse liver proteome, after trypsin digestion and iTRAQ labeling the peptides are prefractionated by isoelectrofocusing in IPG strips of 24 cm and 3-10 pH range, that are cut into 1 cm fractions before being extracted and analysed by tandem mass spectrometry in an Orbitrap system.

In *P. clarkii*, 2D-DIGE is being used to identify, by nESI-MS/MS, a high number of proteins with differential expression. Thus, from 2,400 proteins resolved in crabs from a polluted site, BER, 6 proteins are overexpressed in males and 3 in females. The DeCyder software has detected 50 proteins with significantly altered levels of expression when comparing male crabs from two polluted, PAR, MAT, and one reference, LDP, sites in Doñana surroundings. The highest changes have been found in animals from the Partido stream, the changes being lower in crabs from the Matochal rice fields. Pollutant-promoted modifications of proteins, including carbonylation and oxidation of –SH groups, are being studied by electrophoretic methods. Higher modification is being found in extracts from crayfish living in sites potentially affected by pollutants.

4. Conclusion

The difficulty in applying omic methods in field studies with free-living animals is being solved by: (i) a precise experimental design based on a profound knowledge of the area and the bioindicators, (ii) the continuous validation of omic results with more conventional and sensitive methods, (iii) the absolute quantification at the individual level of selected mRNAs, and (iv) the analysis of selected conventional biochemical biomarkers. The integration of results from contaminant analysis and metallomic results with the biological response will allow defining a non-biased set of novel biomarkers for a global biomonitorization of any ecosystem, thus contributing to the technological renewal.

5. Acknowledgment

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Part 2

Chromatographic Pesticides Analysis

9

Modern Sample Preparation Techniques for Pesticide Analysis

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1. Introduction

Sample preparation is applicable to all sample matrices; for example biotechnological, biological, environmental, forensic and pharmaceutical as it presents tremendous benefits. The reduction of interferences helps to prevent an overload on separation columns and subsequently extends the durability of analytical columns. Selective extraction improves the detection sensitivity levels of analytes of interest and simplifies the subsequent interpretation of chromatograms and other analytical data (Fontanals et al., 2007). Automation of the extraction process results in faster analysis and hence reduced overall cost of analysis (Smith, 2003).

Traditional sample preparation techniques such as liquid-liquid extraction (LLE) and soxhlet extraction generated large volumes of solvent, were laborious, had low sample throughput and lacked selectivity (Baltussen et al., 1997; Moreno et al., 2007; Shimelis et al., 2007). Modern sample preparation techniques such as solid phase extraction (SPE) with its various commercially available sorbents and solid phase microextraction (SPME) have been driven by the move towards the consumption of micro-volumes of solvent to techniques that offer solventless extractions, automation, sample throughput, enrichment factor and selectivity (Wells and Lloyd, 2002; Hyötyläinen and Riekkola, 2008). These extraction approaches are usually easy to carry out and call for optimization of several parameters to enhance the performance of the overall analysis.

The key to rational choice of a sample preparation technique for a particular matrix is based on an understanding of the fundamental principles governing the kinetics of mass transfer within the extraction system (Pawliszyn, 2003). This chapter briefly describes the fundamental principles of some of the modern sample preparation techniques employed to liquid and solid matrices for pesticide analysis. Parameters to be considered with each technique such as sample matrix type i.e. liquid or solid and the physicochemical properties of the analyte(s) of interest are discussed. Optimisation parameters for each technique are also discussed.

2. Liquid phase microextraction (LPME)

This technique is a miniaturization of the traditional LLE and employs micro-volumes of solvent instead of the traditional tens or hundreds of milliliters (Mahugo-Santana et al., 2011). LPME is a two-phase system whereby analytes are transferred from an aqueous

sample solution (donor phase) into an organic solvent (acceptor phase). The organic phase is usually a micro-drop of 1-3 µL volume suspended from a syringe (single drop microextraction [SDME]) or present in the pores of a hydrophobic membrane (supported liquid membrane extraction [SLME]).
LPME is an equilibrium process and as such the distribution ratio (K) of an analyte in both the organic and aqueous sample can be described as

$$K = \frac{C_{org,eq}}{C_{aq,eq}} \quad (1)$$

Where
$C_{org,eq}$ = equilibrium concentration of an analyte in the organic phase
$C_{aq,eq}$ = equilibrium concentration of an analyte in the aqueous phase
The mass balance relationship becomes

$$C_{tot}V_{aq} = C_{org,eq}V_{org} + C_{aq,eq}V_{aq} \quad (2)$$

Where
C_{tot} = total concentration of analytes in the aqueous sample
V_{aq} = volume of the aqueous sample
V_{org} = volume of the organic phase
The enrichment factor can be calculated from equations (1) and (2)

$$EF = \frac{1}{\left(\frac{V_{org}}{V_{aq}} + \frac{1}{K}\right)} \quad (3)$$

Equation (3) shows that in order to obtain a high EF, a low organic-aqueous volume ratio and a high distribution coefficient are required. Conditions that are essential for LLME are;

- The immiscibility of the organic and aqueous phases
- Moderately polar and non-polar analytes that will be extracted with ease into the non-polar organic phase

In three-phase LPME, the analyte (X) is extracted from an aqueous sample solution (donor phase) through an organic solvent immobilized in a porous hydrophobic membrane(organic phase) into an aqueous solution (acceptor phase) contained within the membrane. Equation (4) describes the extraction process

$$X_{donor} \leftrightarrow X_{organic} \leftrightarrow X_{acceptor} \quad (4)$$

SLM requires that analytes be ionisable, hence limited to acidic or basic compounds and pH adjustment is critical for the diffusion of analytes through the membrane. SDME and membrane liquid phase microextraction are discussed further in sections 2.1 and 2.2.

2.1 Single drop microextraction (SDME)

The possibility of performing LLE in a micro-scale was introduced by Liu and Dasgupta (1996) when they extracted sodium dodecyl sulphate as an ion pair into a 1.3 µL drop of chloroform enclosed inside the aqueous drop. Later in the same year, the technique was termed solvent microextraction by Jeannot and Cantwell (1996). An 8 µL droplet of 1-octanol was suspended at the end of a Teflon rod in a stirred aqueous solution and after a fixed time, the Teflon rod was withdrawn from the aqueous solution and the octanol drop sampled with a micro-syringe for injection into the GC. Jeannot and Cantwell (1997) as well

as He and Lee (1997) independently improved the technique by the use of a micro-syringe, hence the name – single drop microextraction. The SDME syringe can be fully immersed into a solution (static SDME) (Fig. 1).

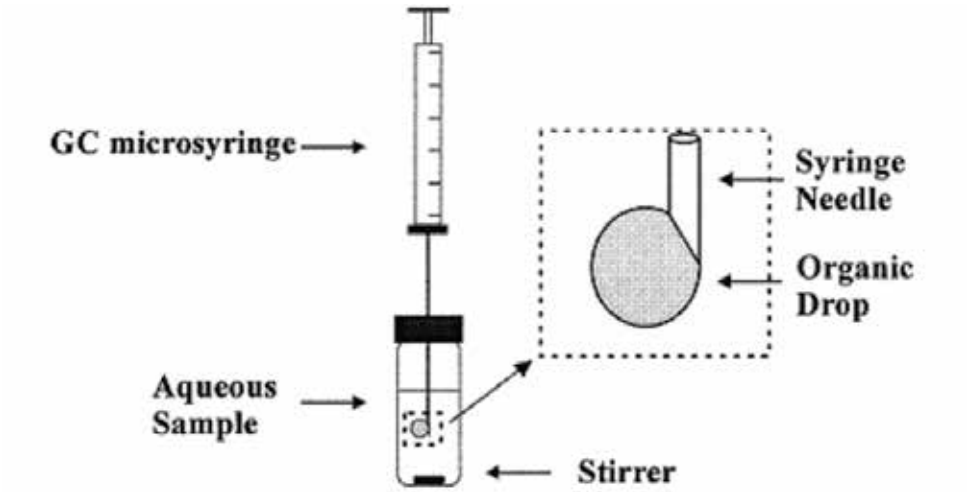


Fig. 1. Schematic representation of static single drop microextraction (static SDME) (Xu et al., 2007).

Alternatively, dynamic SDME can be carried out with a repeated movement of the syringe plunger (Fig. 2).

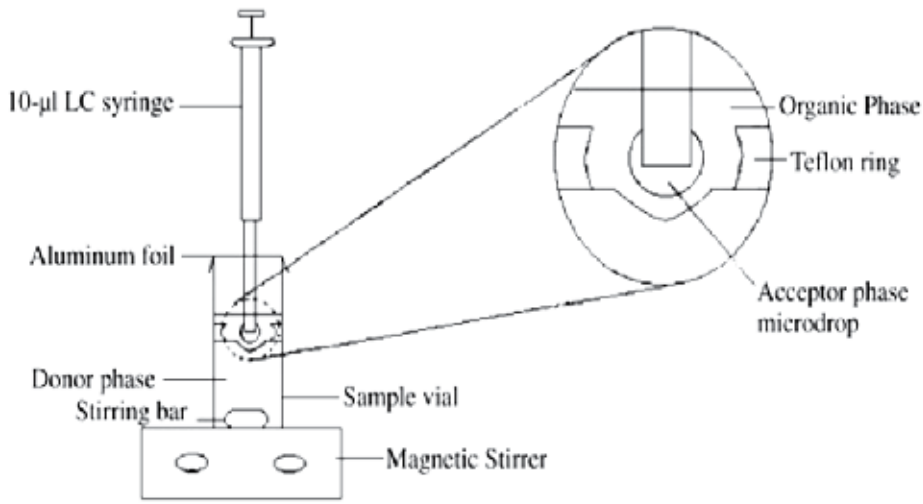


Fig. 2. Schematic representation of dynamic single drop microextraction (DSDME) (Lambropoulou, 2007).

Liu and Lee (2000) introduced a continuous flow microextraction in which an aqueous sample solution flows continuously past a microdrop of solvent (1-5 µL) suspended from a microsyringe. The organic microdrop interacts continuously with the sample solution and

hence allows enrichment factors much higher (1000-fold) than those obtained in static SDME. Table 1 lists the advantages and limitations of SDME.

Advantages	Limitations
Simple to operate	Requires high manual dexterity
Requires inexpensive apparatus	The organic solvent has to have a high surface tension to allow it to hang at the end of a microsyringe
Uses micro-quantities of organic solvent	Long extraction times due to low stirring rates
Allows high throughput sampling and pre-concentration	Instability of the droplet i.e. the microdrop is affected by matrix components such as humic acids and suspended solids causing dissolution of the microdroplet

Table 1. Advantages and limitations of SDME (Psillakis and Kalogerakis, 2002).

Parameters to consider for optimization in SDME

- *Duration of extraction* – extended sample extraction times may cause swelling of the solvent drop size as the solvent absorbs analytes and water and may result in the instability of the drop.
- *Physical and chemical properties of the solvent* – the extracting solvent should be immiscible with water and not be volatile so as to remain stable during the extraction and give good reproducibility. The analytes should be more soluble in the extracting solvent than in the sample solution.
- *Drop size* – Solvent drop volumes between 1 and 2 μL are preferred as those larger than 2 μL are less stable and result in poor reproducibility (Wardencki et al., 2007).
- *Stirring rate* – The stirring rate directly affects mass transfer kinetics of the analytes from the sample solution to the extracting drop but high stirring rates may de-stabilize the solvent drop.
- *Temperature during extraction* – higher temperatures result in higher extraction efficiencies but may also cause solvent drop instability hence most extractions are carried out at room temperature (Lopez-Blanco et al., 2005).

SDME has been widely employed as a sample preparation technique for pesticide analysis and Table 2 shows some examples.

2.2 Membrane liquid phase microextraction

Further improvements of SDME were aimed at minimizing the instability of the microdrop by supporting it with a polymeric membrane (Jönsson and Mathiasson, 2001). This acts as a barrier between the aqueous and organic phases and allows the use of larger solvent volumes. Hollow fiber liquid phase microextraction membranes (HF-LPMEs) can be in the form of flat, rod or U-shaped formats and involve the use of a membrane placed between stagnant aqueous and organic phases (Fig. 3).

Supported liquid membrane (SLM) is a three-phase system and consists of two flat membranes sandwiched by an organic solvent-impregnated membrane (Fig. 4).

This format requires a pump to drive the donor phase through one membrane and the acceptor phase through the other membrane and the extraction process is described by equation (4).

Sample	Analyte	Solvent	Final analysis	Reference
Water	Organochlorines	Hexane	GC/ECD	Jager and Andrews, 2000.
Water	Organophosphorus pesticides	Isooctane	GC/NPD	Lopez-Blanco et al., 2003.
Water	α- & β- Endosulfan	Isooctane	GC/ECD	Lopez-Blanco et al., 2005.
Water	Organophosphorus pesticides	Toluene	GC/MS	Lambropoulou et al., 2004.
Water	Chloroacetanilide pesticides	Toluene	GC/μECD	Zhao et al., 2006.
Water, fruit juice	Organophosphorus pesticides	Toluene	GC/FPD	Ciao et al., 2006.
Orange juice	Organophosphorus	Toluene	GC/FPD	Ahmadi et al., 2006.

Table 2. Examples of SDME applications in pesticide analysis

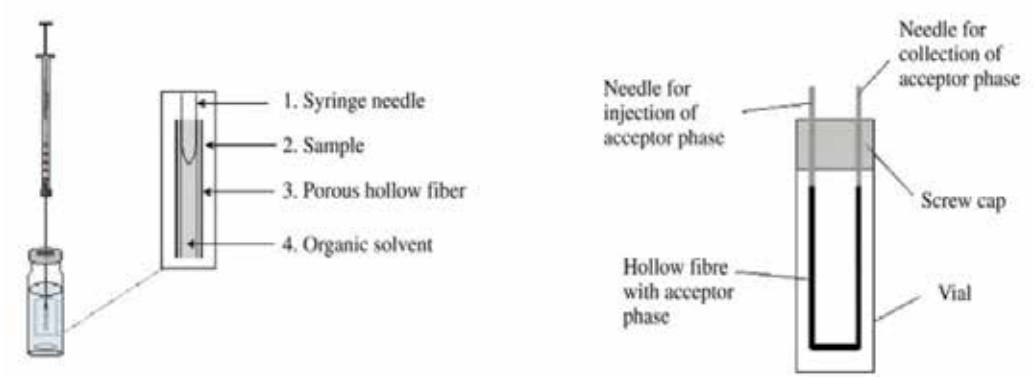


Fig. 3. Schematic representation of rod and U-shaped porous hollow fiber membranes.

Parameters to be optimized in SLME

- *Extracting solvent* – the extracting solvent should be immiscible with water and strongly immobilized in the pores of the membrane. In addition, the solvent should not be volatile as that would result in poor reproducibilities.
- *Pore size of the membrane* – This affects selectivity as some degree of size exclusion also occurs during extraction (Hyötyläinen and Riekkola, 2008).

3. Solid phase extraction (SPE)

Solid phase extraction (SPE) is based on selective retention of analytes on a sorbent and subsequent elution with a suitable solvent (Jakubowska et al., 2009). SPE generally involves four steps i.e.

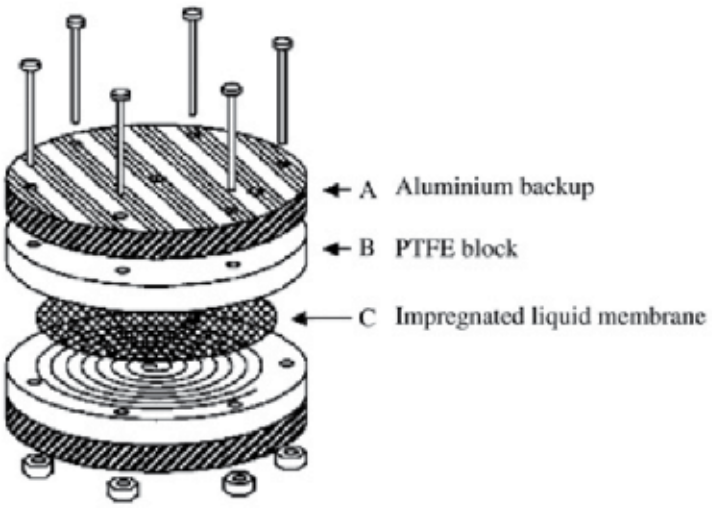


Fig. 4. Schematic diagram of a supported liquid membrane extraction (SLME).

1. *Sorbent conditioning step* – functions to activate or 'wet' the sorbent to prepare for its interaction with the analyte. This is especially necessary for hydrophobic sorbents that would not be activated by an aqueous sample. If the sorbent is not adequately conditioned, poor reproducibility and analyte recoveries may be obtained. If pH is critical for retention, then the conditioning solvent has to be adjusted to match the sample pH. The organic strength of the conditioning solvent also has to be matched to that of the sample to prepare for maximum retention of the analyte during the loading step.
2. *Sample loading step* – when the sample is added to the sorbent, sufficient residence time should be allowed for maximum interaction and avoid breakthrough. This is especially critical when employing ion exchange to provide adequate residence time of the sample solution in the sorbent since the analyte has to achieve an appropriate orientation for electrostatic retention with the sorbent functional groups.
3. *Washing step* – this serves to remove/minimize interferences retained on the sorbent and should not affect the retained analytes. The elution strength of the wash solvent should be higher than that of the sample solution but should be less than that of the elution solvent. This may entail employing a solvent with a higher organic phase content, ionic strength or different pH in comparison to the sample solution. However, a wash solvent that is too weak will not remove interferences but will result in co-elution of interferences with the analytes in the elution step. Hence optimization of this step is aimed at identifying the strongest solvent to ensure the highest recovery of analytes and minimal interferences. In mechanisms employing ion exchange, the pH of the wash solvent should be sufficient enough to disrupt the charged sites of interferences but not affect the analyte.
4. *Elution step* – the elution solvent should be strong enough to disrupt all analyte-sorbent interactions in order to obtain the highest recoveries. However the use of harsh solvents will not only strip analytes from the solvent but will elute strongly adsorbed interferences as well.

Things to consider before SPE;

- Log P – this is the octanol-water partition coefficient and is an indicator of the hydrophobicity of an analyte. If the Log P is positive, the analyte is hydrophobic and hence hydrophobic sorbents such as C_8 and C_{18} should be employed to obtain sufficient analyte retention
- pKa – this describes the extent of ionisability of the analyte. When analytes contain acidic or basic functional groups with low (<1.5) or high (>9.5) pKa values, then weak anion or cation exchange sorbents are required. The charge states of weak ion exchange sorbents are more easily altered by solution pH to strengthen or disrupt the analyte-sorbent interaction. When pka values are between 2 and 8, strong or weak ion exchange sorbents can be employed and it is possible to alter the charge state of either the analyte or sorbent through solvent pH. Mixed mode sorbents, consisting of a polymeric hydrophobic backbone with ionisable functional moieties make use of both hydrophobic and ionic interactions and are popular in modern SPE especially in the pharmaceutical industry. In the environmental industry, mixed mode sorbents are employed to selectively extract both acidic and basic pesticides. The solution pH should be adjusted such that both the analyte and sorbent are ionized during sample loading and then re-adjusted to neutralize either the analyte or sorbent for the elution step.

In addition to the 4 basic steps of SPE protocol, extra steps such as soaking and sorbent drying can be included in the SPE protocol to help improve recoveries. The soaking step may be employed to enhance interaction between the analyte and the sorbent and can be applied to the conditioning or loading steps. The drying step may be necessary after the wash step especially if the elution step involves the use of an organic solvent that is immiscible with the aqueous sample solution. The drying step would serve to eliminate aqueous films on the sorbent surface and allow interaction between the organic elution solvent and the analyte-sorbent bonds. Even if the elution solvent is miscible with the sample solution, a drying step may be necessary to reduce the duration of subsequent solvent evaporation of the eluate. This is necessary when the eluate has to be concentrated down by evaporation or if the analytes have to be re-constituted in a different solvent that may be more compatible with the analytical instrument. SPE is widely popular with liquid samples and boasts of several advantages listed in Table 3.

Advantages	Limitations
Less time consuming than traditional LLE	Considerable variation of the performance of the same type of sorbent e.g. C_{18}, amongst the different manufacturers
Eliminates the formation of emulsions characteristic of LLE	Lot-to-lot variation of sorbents from the same manufacturer
Low volumes of solvent used	Sample clean-up of large sample volumes is not always possible
High sample throughput with SPE manifolds or 96-well plates	SPE cartridge materials may absorb analytes and increase interferences in the analysis.
Can be automated	
Availability of several commercial SPE sorbents in various formats	

Table 3. Advantages and limitations of SPE

There are several published applications of SPE in pesticide analysis employing mostly C_{18} and polymeric sorbents (Martel and Zeggane, 2002; Jimenez et al., 2001). New types of sorbents such as graphitised carbon sorbents have demonstrated high recoveries for polar pesticides that were poorly recovered with the classical C_{18} (Hennion, 2000). Magnetic nanoparticles have also found applications in environmental analysis (Ding et al., 2010). Carbon ferromagnetic nanocomposites containing a hydrophobic sublayer and a hydrophilic surface are employed in dispersive SPE. This eliminates packing columns and the magnetic particles can easily be removed from the sample matrix by the use of a magnet (Khajeh, 2009; Zhao et al., 2008; Liu et al., 2009; Ji et al., 2009). Adeyemi and co-workers (2011) have employed electrospun nanofiber sorbents for the pre-concentration of DDE with subsequent desorption by hot water extraction, thus eliminating the use of organic solvent for elution. The wide applicability of SPE in sample preparation has led to the development of more specialized SPE sorbent materials such as molecularly imprinted polymers (MIPs), immunosorbents (ISs) and restricted access materials (RAMs) (Hennion, 1999) that are discussed in sections 3.1, 3.2 and 3.3.

3.1 Molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers (MIPs) possess recognition sites that are adapted to the 3-dimensional shape and functionalities of the analyte of interest. They have demonstrated high specificity by being capable of repeated binding and re-binding to analytes of interest in the presence of other closely related compounds hence their incorporation as SPE sorbents (Caro et al., 2006; Kandimalla, 2004; Qiao et al., 2006). In aqueous mobile phases, MIPs display reversed phase interactions. MIP sorbents are robust, resistant to a wide range of pH, solvents and temperatures (Yin et al., 2006). The greatest limitation of MIPS is the incomplete removal of analytes during the elution step hence they are susceptible to poor recoveries and carry-over after repeated use.

3.2 Immunosorbents (ISs)

Immunosorbents contain covalently bonded immobilized antibodies or antigens that have a strong affinity for their corresponding antigens or antibodies (Majors, 2007). ISs have been widely employed for sample preparation in the pharmaceutical and food industries and have subsequently found applications in the environmental industry (Delaunay et al., 2000; Stevenson, 2000; Delaunay-Bertoncini et al., 2001). Class specific ISs are commercially available for herbicides. In a comparison of three anti-atrazine ISs by Delaunay-Bertoncini and co-workers (2003), anti-ametryn IS bound to all atrazines in the study while anti-atrazine IS was found to be specific to the chloroatrazines and anti-dichloroatrazine IS was specific to tertbutylatrazine and cyanazine. Finding the appropriate solvent for elution of analytes from immunoaffinity sorbents can be a challenge since organic solvents denature antibodies hence the use of competitive binding agents, pH and temperature variation (Hennion and Pichon, 2003). An elution solvent of MeOH:Water (1:1 v/v) gave the highest recovery of diazinon (93%) on an anti-diazinon IS (Prince et al., 2001).

3.3 Restricted access materials (RAMs)

These were initially employed for the extraction of low molecular drugs from biological fluids but have since found use in the extraction of herbicides from surface waters (Simpson, 2000; Boos and Grimm, 1999; Hogendoorn and van Zoonen, 2000). RAMs function by

preventing/restricting access of macro molecules such as proteins to regions of the sorbent surface where analyte retention occurs (Fig. 5)

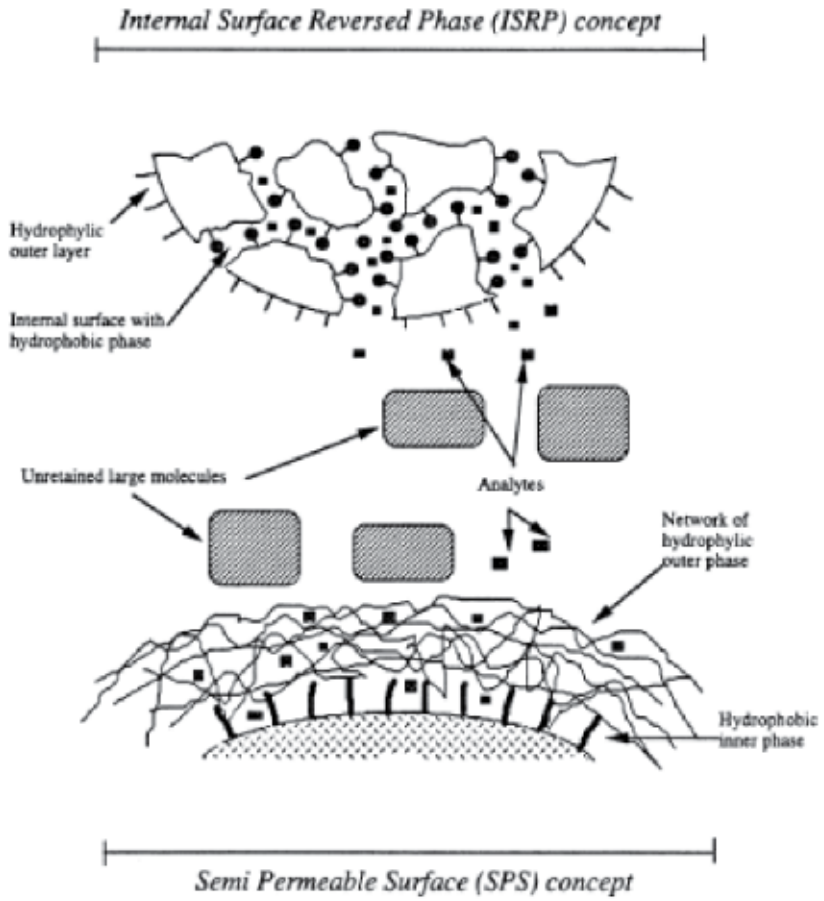
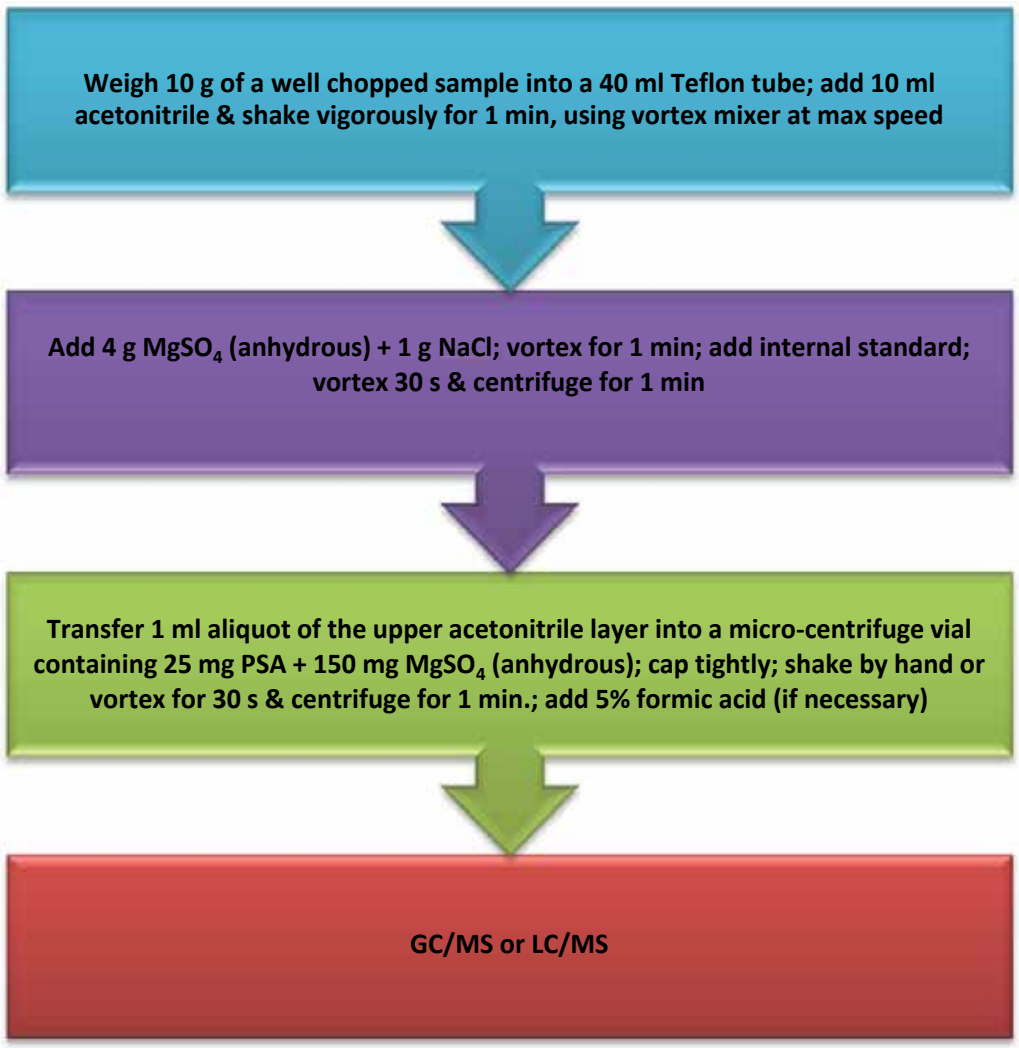


Fig. 5. Schematic representation of the principle of restricted access materials (Poole, 2003).

Restricted access to the retentive part of the sorbent is achieved by either size exclusion in internal surface reversed sorbents or a chemical diffusion barrier such as polymer network on the outer sorbent surface in semi-permeable surface materials. 60-90% recoveries were obtained for acidic pesticides in organic matter - rich soil cleaned online with RAM-C_{18} internal reverse phase GFF-II columns (Hogendoorn et al., 2001).

4. QuEChERS

The quick, easy, cheap, effective, rugged and safe (QuEChERS) technique was developed by Anastassiades and colleagues (2003) for the simultaneous extraction/isolation and clean-up of pesticides in food matrices. Since its inception, the technique has gained popularity as a multi-class pesticide extraction method that combines several steps of sample preparation into basically three steps involving extraction, dispersive SPE clean-up and solvent exchange. Scheme 1 shows the general steps in the QuEChERS technique;



Scheme 1. The main steps in the QuEChERS protocol.

- *Extraction* – a chopped sample is extracted with acetonitrile. The sample must be thoroughly homogenized & sufficient water must be added to dry samples & this volume should reflect in the final calculations. $MgSO_4$ and NaCl are added to ensure that water is separated from acetonitrile.
- *Cleanup* – dispersive SPE is carried out to remove a majority of the matrix. C_{18} & primary secondary amine (PSA) help to remove a fatty matrix – more may be added to remove fatty acid co-extractives in cereals and grains at the expense of ~20% loss of certain polar pesticides (Lehotay et al., 2005). The inclusion of graphitic carbon black (GCB) in the d-SPE assists to remove pigments such as chlorophyll from dark green vegetables even though it reduces recoveries of some planar pesticides (Anastassiades et al., 2003). Both column based SPE and d-SPE were compared using C_{18}, PSA and GCB and d-SPE was found to be more effective and flexible (Table 4).

	Traditional column based SPE	Dispersive SPE
Advantages	• Ensures better sample clean-up	• Ensures larger and more reproducible recoveries of analytes with acidic or basic properties (e.g. acephate, carbendazim, imazalil, methamidophos, pymetrozine and thiabendazol), • Does not require SPE apparatus, cartridges, vacuum, pretreatment of sorbent, channelling, drying out, collection tube, flow control, elution solvent, dilution of extract or solvent evaporation steps, d-SPE is therefore quicker and cheaper • Uses less sorbent, smaller amounts of sample and less equipment; d-SPE is thus a cheaper and easier technique • Provides better interaction with the extract for clean-up
Disadvantages	• Requires plastic cartridges containing 250–2000 mg of a sorbent material and vacuum manifolds, • Requires a larger sample, • Requires column preconditioning, solvent evaporation steps, manual operation and multiple solvents, • Generates solvent waste fractions	• Can only be used when the SPE sorbent removes matrix components and not the analytes

Table 4. Comparison of column based SPE with dispersive SPE (Wilkowska and Biziuk, 2011).

- *Solvent exchange* - ensures that the final solvent is compatible with the analytical instrument and may be used to concentrate analytes in order to reach lower detection limits. Solvent exchange may also function to discriminate against undesired compounds in the final extract eg changing the solvent from acetonitrile to hexane:acetone (9:1) in green tea extracts excluded caffeine & polyphenols (due to their poor solubility in the final solvent system) (Anastassiades et al., 2003).

After the introduction of the multiclass - multiresidue QuEChERS method, it was realised that recoveries of some "problematic" pesticides such as chlorothalonil, folpert and tolylfluanid were not improved. These pesticides are base-sensitive, unstable and were poorly recovered by any existing multiresidue method (Maštovská and Lehotay, 2004). This led to modifications of the original method to an acetate-buffering version adopted as the AOAC Official Method 2007.01 (Lehotay, 2007) and the citrate-buffering version adopted as the European Committee for Standardisation (CEN) Standard Method EN 15662 (Lehotay et al., 2010). When the acetate and citrate buffered versions were compared alongside the unbuffered original method on apple-blueberry sauce (mixture of fruits), peas (a green vegetable) and limes (acidic fruit), all gave good recoveries with the acetate buffered method giving the highest recoveries. However, recoveries for the "problematic" pesticides were consistently low amongst the three methods but better with the acetate buffered method (Lehotay, 2010).

QuEChERS has not only proved to be a useful sample preparation technique for pesticides in different classes of fruits and vegetables including those with a high fat and pigment content, but has found applications in the determination of pesticide residues in other food types such as fish (Mpofu et al., 2011), veterinary drugs in animal tissue (Stubbings and Bigwood, 2009), hormone esters in muscle tissue (Costain et al., 2008). A version of QuEChERS has been suggested by Pinto and colleagues (2010) for the extraction of organochlorines in soils. The flexibility of QuEChERS to various matrices will continue to open its potential to many more applications beyond pesticides (Wilkowska and Biziuk, 2011).

5. Solid phase microextraction (SPME)

Solid phase microextraction (SPME) was introduced by Arthur and Pawliszyn (1990) and has found applications in the environmental, food and medical industries have since followed its commercialization in 1993. The SPME device (Fig. 6) is based on a fused – silica fiber, coated with a thin layer of polymeric sorbent or immobilised liquid that is encased in a steel needle within a syringe-like arrangement.

The SPME device can be immersed directly into gaseous or liquid samples or suspended in the headspace above liquid or solid samples (HS-SPME). The principle behind SPME is based on a partition mechanism and the establishment of an equilibrium between the analyte adsorbed on the fiber and analyte in the sample matrix. The partition coefficient, K, is described as;

$$K = \frac{C_e}{C_s} \tag{5}$$

Where

C_e *and* C_s are the equilibrium concentrations of the analyte in the extracting sorbent and sample matrix, respectively.

At equilibrium, a linear relationship exists between the number of moles of an analyte adsorbed on the fiber and the concentration of the analyte in the aqueous phase. The relationship is represented by the equation;

$$n_{e} = \frac{KV_eV_sC_s}{KV_e + V_s} \quad (6)$$

Where
n_e = the number of moles of the analyte extracted into the extracting sorbent
V_e = volume of analyte in the extracting sorbent
V_s = volume of analyte in the sample solution (Arthur and Pawliszyn, 1990; Lord and Pawliszyn, 2000).

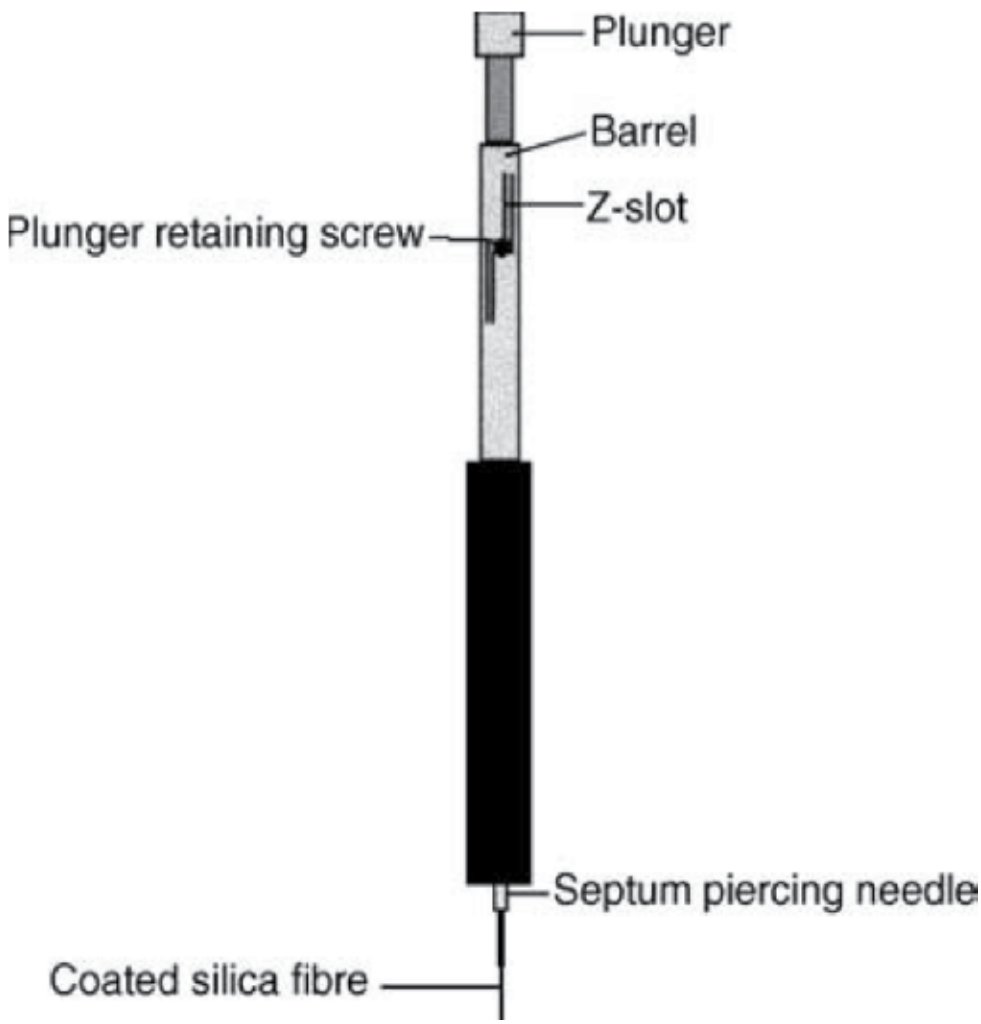
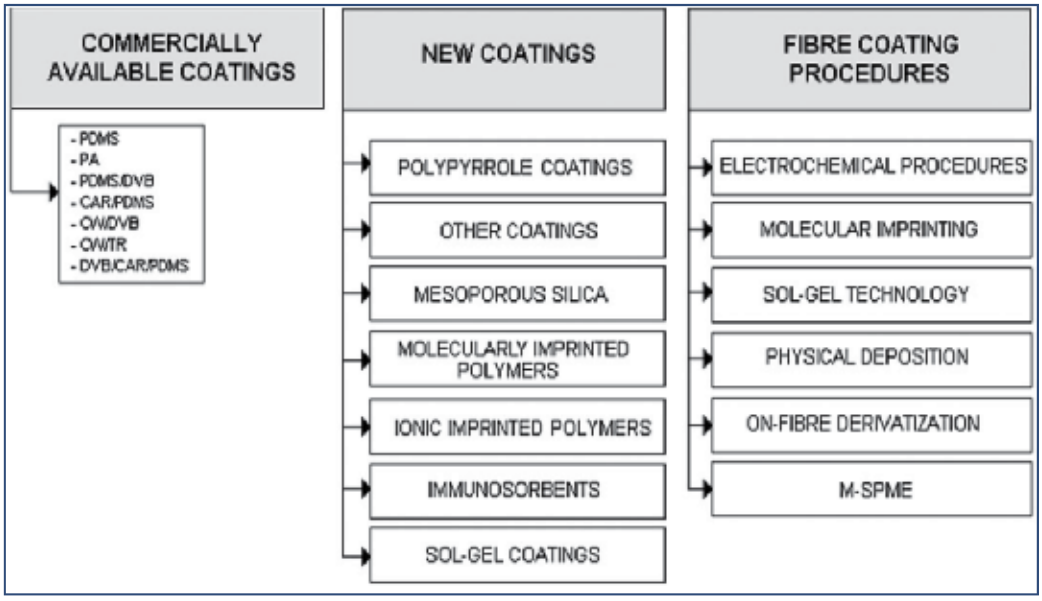


Fig. 6. Schematic diagram of a SPME device (King et al., 2003).

Advantages	Limitations
Ease of operation	Fragility of the fibers
Solventless extraction	Poor extraction efficiency of polar analytes contained in polar matrices
Short extraction time	Limited choice of commercially available fibers
Compatibility with GC	
Possibility of automation	

Table 5. Advantages and limitations of SPME (Zhang et al., 1994).

The limited choice of commercially available fibers has led to the in-house fabrication of various fiber types. Scheme 2 shows the different types of commercially available fibers, in-house fabricated fibers and the techniques employed for their synthesis.



Scheme 2. A diagrammatic representation of the various SPME sorbent and the techniques employed for their synthesis (Spietelun et al., 2010).

Parameters to consider during the optimization of SPME

- *Mode of extraction* – volatile analytes are often sampled in the headspace mode as this eliminates simultaneous adsorption of interferences and safe-guards against deterioration of the fiber coating experienced during direct immersion (Kataoka et al., 2000).
- *Sample volume* – lower sample volumes have been found to favour higher extraction efficiencies since equilibrium is reached faster without overloading the fiber coating (Pawliszyn, 1997; Yang and Peppard, 1994).

- *Temperature and extraction time* – A higher extraction temperature facilitates transport of analytes from the sample solution to the headspace, however excessive temperature may result in premature desorption of analytes from the fiber. Longer extraction times allows more analyte adsorption on the fiber but when all sites on the fiber are occupied, longer extraction times will not improve extraction efficiency but may result in desorption (Zhang and Pawliszyn, 1999).
- *Salting out effect* – Increasing the ionic strength of the sample solution suppresses the solubility of hydrophobic analytes in the sample solution hence promoting their adsorption onto the fiber. This approach has been widely employed in the extraction of pesticides (Wu et al., 2000; Hwang and Lee, 2000; Fernandez et a., 2001).

SPME sorbent type	Sorbent thickness (μm)	Final analysis	Type of pesticides	Reference
PDMS/DVB	65	GC	Organochlorines	Mmualefe et al., 2009.
PDMS	7, 30, 100	GC/HPLC	Organochlorines & organophosphorus	Batlle et al., 1999; Lipinski, 2000.
PA	85	GC	Triazines & organophosphorus Nitrogen containing & herbicides	Batlle et al., 1999; Eisert andLevsen, 1995. Boyd-Boland and Pawliszyn, 1995.
CW/DVB	65, 70	GC	Herbicides	Batlle et al., 1999; Hernandez et al., 2000.

Table 6. Applications of commercially available SPME sorbent types in pesticide analysis.

6. Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE), also referred to as pressurized fluid extraction (PFE), pressurized solvent extraction (PSE) or accelerated solvent extraction (ASE), is a technique that was introduced by Dionex corporation in 1995 (Richter et al., 1996). The principle of the technique is based on using elevated temperatures (50 – 200 °C) and pressures (50-150 atm) to extract analytes from solid or semi-solid samples within short periods of time (5 – 15 min).

The Dionex ASE® 200 system consists of a solvent delivery component controlled by an HPLC pump, nitrogen gas purge valve, a carousel for extraction cells and collection vials as well as a waste vial (Fig. 7).

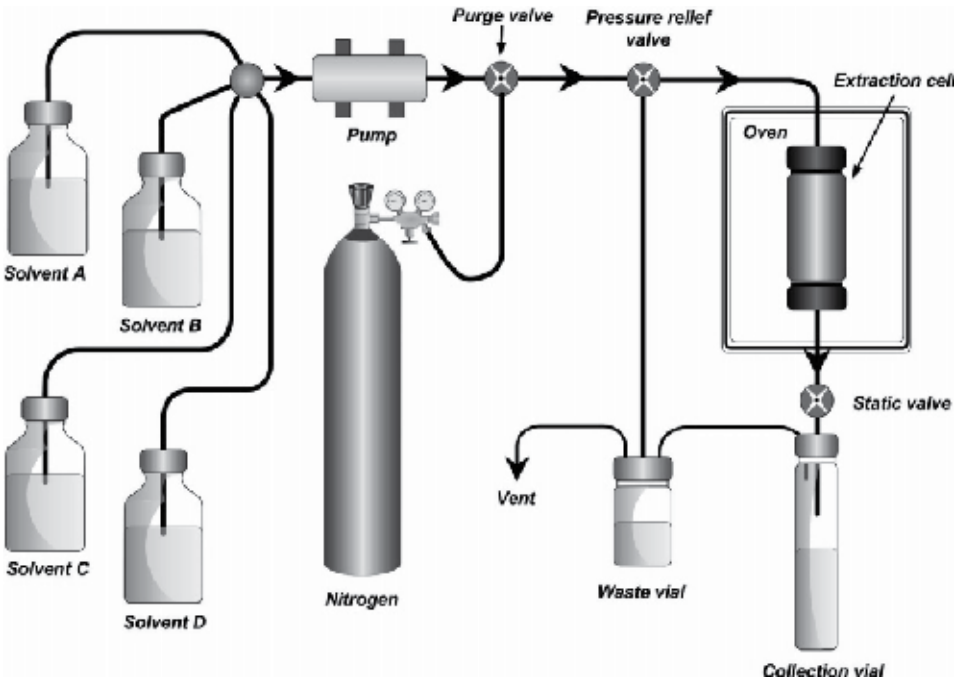


Fig. 7. Diagram of a Dionex ASE® 200 pressurised liquid extraction system

Prior to extraction, a solid/ semi-solid sample is placed into a stainless steel extraction cell lined with a filter paper disk on the outlet end to prevent passage of solid matter from the cell into the collection vial. The extraction cell is then placed onto a carousel and automatically drawn into the oven and filled with solvent. During extraction, the cell is heated, causing thermal expansion of the solvent and hence an increase of pressure inside the cell. The static and pressure relief valves function to regulate pressure inside the cell during static extraction by adding more solvent or opening the static valve to let solvent out of the extraction cell, whichever one is needed to maintain the desired pressure.

After static extraction, some of the solvent inside the extraction cell can be replaced by fresh solvent for a subsequent extraction cycle. This flush volume can vary from 5 to 150 % of the extraction cell. The introduction of fresh solvent increases the concentration gradient between the extraction solvent in the cell and the surface of the sample matrix resulting in improved mass transfer and consequently better extraction efficiency compared to a single cycle extraction (Richter et al., 1996). Finally, pressurized nitrogen purges the remaining solvent from the cell and lines to a collection vial. Parameters to optimize in PLE include;

- *Temperature (60 – 200 °C)* - the increased temperatures disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bonding and dipole interactions of the solute molecules and active sites on the matrix. When the solvent is in contact with the matrix, the thermal energy in the heated solvent assists to desorb analytes from the matrix by overcoming cohesive (solute-solute) and adhesive (solute-matrix) interactions. This decreases the activation energy required for the desorption process (Richter et al., 1996; Mockel et al., 1987).

- *Pressure (50 – 100 bar)* - The high pressures employed in PLE maintain the solvent in its liquid state even at temperatures above its atmospheric boiling point. The high pressure increases the solvation power and speeds up the extraction kinetics of solvents by forcing solvent into the pores of the matrix that normally would not be in contact with solvent at atmospheric pressure. This helps solvate analytes trapped in matrix pores that have been "sealed" with water or air bubbles. The pressurized flow in PLE also assists to solubilize air bubbles surrounding analytes that are found on the surface of the matrix as well (Richter et al., 1996).

For polar solvents such as water, increasing the temperature lowers the dielectric constant thus making it suitable for the extraction of less polar compounds (Turner et al., 2006). The dielectric constant of water at 25 °C is ~80, making it an extremely polar solvent. Increasing the temperature of water to 250 °C while applying sufficient pressure to maintain it in its liquid state reduces the dielectric constant to 27 which is midway between those of methanol (ε = 33) and ethanol (ε = 24) at 25 °C (Miller and Hawthorne, 1998). As a result, water at higher temperatures is more "miscible" or "soluble" in organic solvents and is often referred to as pressurised hot water extraction (PHWE). Table 7 shows some examples of applications of PLE in the extraction of pesticides.

Sample	Analyte	Extraction solvent	Reference
Baby food	Malathion, chlorpyrifos, 4,4'-DDE, 4,4'-DDT.	Acetonitrile or ethyl acetate	Chuang et al., 2001.
Oranges and peaches	Benzimidazoles and azoles, organophosphorus, carbamates, neonicotinoids & acaricides	Ethyl acetate	Blasco et al., 2005.
Fresh vegetables	Wide range of pesticides	Ethyl acetate/acetone (3:1 v/v)	Garrido et al., 2005.
Fresh pear, cantaloupe, potato and cabbage	Wide range of pesticdes	Acetone/ dichloromethan e (3:1 v/v)	Adou et al., 2001.

Table 7. Examples of PLE in sample preparation for the determination of pesticides.

7. Supercritical fluid extraction (SFE)

This technique employs fluids in their supercritical states for the extraction of solid samples. Supercritical fluids behave like gases although they have the density of liquids and as a result, they have a high diffusivity, low viscosity, good penetration capability and adjustable density (Goncalves et al., 2006). In comparison to soxhlet extraction, supercritical fluid extraction (SFE) offers several advantages such as shorter extraction times, lower solvent consumption (hence environmentally friendly), suitability for thermally labile compounds and reduced working temperature (Brachet et al., 2000).

In SFE, a solid or semi-solid sample is placed in a pressure vessel (Fig. 8) and extracted with a re-circulated stream of supercritical fluid which is well mixed with the sample matrix to allow analytes to transfer to the fluid. At the end of the extraction, the extract is collected in a vial or cartridge.

SFE involves five sequential steps;

1. Wetting of the matrix with supercritical fluid.
2. Partitioning of the analyte from the matrix into the supercritical fluid
3. Diffusion of analytes from the matrix.
4. Elution of the analyte from the extraction cell
5. Collection of the analytes.

Wetting of the sample with supercritical fluid is especially important when the sample matrix contains water. Partitioning of non-polar analytes from the matrix is a relatively fast process if supercritical carbon dioxide is employed. For analytes that are strongly bound to the matrix, a higher temperature or addition of an organic solvent is required. The second step depends on factors such as diffusion of the analyte between the matrix active sites and the ability of the supercritical fluid to displace the analyte from these sites. This initial desorption step is often the rate determining step in the SFE of most environmental samples (Bowadt and Hawthorne, 1995). All three steps contribute to the overall extraction efficiency.

SFE has been widely employed as a sample preparation tool for pesticide analysis such as in sediments (Mmualefe et al., 2008), wheat and maize (Norman and Panton, 2001) and in vegetables (Ono et al., 2006).

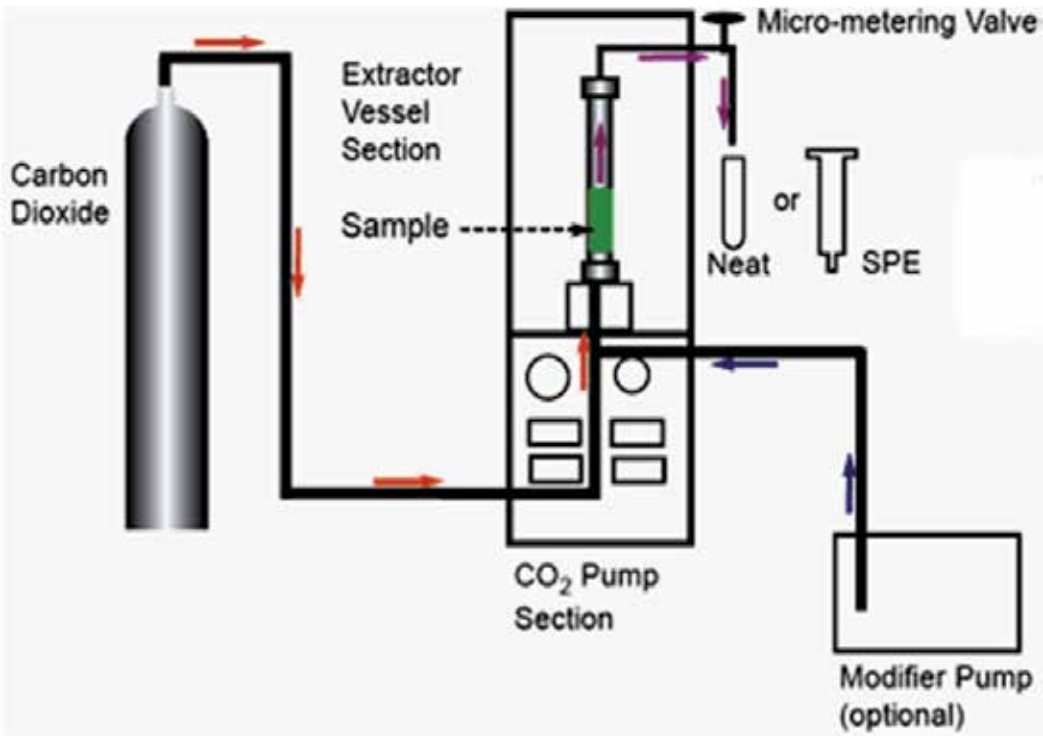


Fig. 8. Diagram of a Spe-ed™ Prime SFE by Applied Separations Inc. (2008)

8. Conclusions

Sample preparation continues to evolve with the search for techniques that offer higher selectivity to handle highly complex matrices and hence improve recoveries and reproducibility of results. Sample throughput, ease of operation and cost of analysis are critical parameters when choosing sample preparation techniques in the industry. Limitations of any technique open opportunities for analytical chemists to come up with alternative strategies or invent completely new techniques to conquer challenges of the existing technique.

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10

Modern Extraction Techniques for Pesticide Residues Determination in Plant and Soil Samples

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1. Introduction

Due to intensive use of pesticides, their residues have become an unavoidable part of the environment, and they are often detected in all environmental segments and therefore their monitoring has been frequently performed throughout the world.
As the presence of trace amounts of both pesticide residues and their degradation products could be potential health hazards, UN organization has formed specialized groups: World Health Organization (WHO) and Food Agriculture Organization (FAO), with the aim to establish restrictive measures to protect the environment against pollution. These organizations and their experts groups on annual meetings summarize international achievements in pesticides domain, establish legislation and make recommendations obligating member states to act in accordance with international standards.
As pesticides are a very heterogeneous group of compounds with different biological and physicochemical properties, the current trend in pesticide residues analysis is developing multi-residual methods that not only provide simultaneous determination of large number of pesticides, but also can be applicable to large numbers of samples of different origin.
Although separation chemical analysis involves several stages (sample preparation, analyte separation i.e. quantification and data analysis), sample preparation step can be marked as "the most critical" one. Traditional sample preparation methods (liquid-liquid extraction, Soxhlet extraction, etc.) are laborious, time consuming, expensive, requires large amounts of organic solvents and usually involve many steps, leading to loss of some analyte quantity. Additionally, consequences of hydrocarbon solvents use, such as ozone depletion and generation of considerable cancer waste, lead to reduction of not only their use but also their manufacture. As a result, modern sample preparation procedures, such as accelerated solvent extraction (ASE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE), solid phase extraction (SPE), solid phase microextraction (SPME), matrix solid phase dispersion (MSPD) extraction and QuEChERS (quick, easy, cheap, effective, rugged and safe), have been developed to overcome the drawbacks of the traditional approaches. It should be indicated that SFE, ASE and MAE are instrumental techniques, and often use SPE and SPME for purification of obtained extracts, and also its concentration in case of SPME.
Overall, a comprehensive analytical procedure is carried out in the way that obtained results can be found within prior established concentration range. Whether the measurement will

be performed at the desired concentration range is determined by the instrument sensitivity and the choice of sample preparation method. Multiple factors, related to the physical properties of not only tested active ingredients (volatility, solubility in water and organic solvents, stability, acid-base properties, etc.), but also sample matrix (water, lipids, pigments content etc.), must be considered during an experiment planning. The choice of sample treatment depends directly on mentioned factors, but also on analysis purpose i.e. on required detection method sensitivity (limits of detection) and quantification accuracy (whether the aim is to establish the exact concentration value regardless maximum residues limits (MRLs), or just to establish if result is above or under MRLs). Considering complexity of these issues, analytical procedure for each specific case should be chosen in order to minimize problems relating to analysis duration, consumption of solvents and other necessary reagents, and also to reduce number of involved analytical steps, which would minimize potential sources of errors. Additionally, it is desirable that chosen analytical procedure allows the simultaneous determination of large number of pesticides.
This paper describes the basic principles of modern extraction techniques, comparing their advantages and drawbacks, and their ability and applicability for pesticide residues determination, with special emphases on plant material and soil samples.

2. Supercritical fluid extraction (SFE)

This technique uses supercritical fluid (SF)[1] as an extraction tool for "drawing out" the organic compounds from solid matrices. Commonly used for this purpose is CO_2, as it has relatively low critical temperature (31^0 C) and low critical pressure (73 kPa) (Atkins & De Paula, 2002), it is not reactive and is accessible in a high degree of purity at low cost. Changes in temperature and pressure at which the supercritical CO_2 is held will increase or decrease the "strength" of solvent and thus the selectivity of extraction performed. At constant temperature which exceeds critical temperature, the supercritical CO_2 will be able to extract analytes of low polarity at low pressure, and high polarity analytes at high pressure. SFE with CO_2 is usually performed at pressures that are not high enough to achieve efficient extraction of polar compounds. In such conditions, the supercritical CO_2 is a good extraction medium for non-polar compounds and moderately polar ones, such as PAHs, PCBs, organochlorine (OCPs) and organophosphorus (OPPs) pesticides, etc.. The efficiency of supercritical CO_2 can be improved by adding small amounts of modifiers, which identity is often more important than their concentration, since the major role of a modifier is to interact with the sample matrix to promote desorption into the fluid (Langenfeld et al., 1994). Some of the common solvents such as acetone (Valverde-García et al., 1996; Kaihara et al., 2002; Ono et al., 2006) and methanol (Valverde-García et al., 1996; Rissato et al., 2005a, 2005b) are now mostly used as

[1] Supercritical fluid is a fluid held at temperature and pressure close to but somewhat larger than its critical values. Under these conditions, the fluid shows rapid diffusivity and viscosity that are typical for the gas phase, but also solvation properties (moistening) that is characteristic of a liquid. Properties similar to gas phase allow the fluid to penetrate into the sample and to establish contact with the analytes much faster than the liquid solvent does. When the mentioned contact is established, the extraction process depends on the solvation abilities of the fluid to release the analytes from the adsorption centers of the sample.

modifiers. Besides CO_2, supercritical N_2O has been much in use as well, and it could be used both with and without modifiers.

The basic principles and possibilities of applying the SFE technique for determining pesticide residues in samples of different origin can be found in the reports generated by Gilbert-Lopez et al. (2009); Sunarso & Ismadji (2009); Rissato et al. (2005a, 2005b); Motohashi et al. (2000) and Torres et al. (1996).

In general, SFE usually lasts less than two hours, and the further analysis can be accomplished in several ways. According to one, SF with analytes is passed through a capillary that is immersed in an appropriate solvent. While in the capillary, SF exists, but after leaving the capillary it becomes a gas (the pressure falls below the critical pressure). The largest part of this gas passes through the solvent, while the extracted analytes are retained in the solvent (the degree of retention depends on the solvent, i.e. the solubility of the analyte in it). Read phoneticallyAlso, the flow of SF can be directed to a solid sorbent, which will then bind analytes, and its elution by appropriate solvent, analysts translate into a solution suitable for further analysis. Also, the flow of SF could be directed directly to capillary column of the gas chromatograph (GC), thus obtaining the "on-line" SFE. This approach enables analytical scheme with the highest sensitivity for a limited amount of sample available for analysis.

The recent studies have shown that SFE methods, followed by additional purification of the obtained extracts, meet the strict criteria of the pesticide residues analysis. Thus, Ono et al. (2006) showed that SFE method combined with purification on the SPE columns (C18 and Envicarb/NH2), could be used for determination of 242 pesticides in spinach, 245 in green beans and 263 in orange. Rissato et al. (2005a) concluded that the SFE preparation of potato, tomato, lettuce and apple samples, combined with the extracts cleanup on amino propylene columns, could be used for determination of 37 pesticides. Kaihara et al. (2000, 2002) have developed the SFE method that combined with the purification on PSA and florisil columns may be used for determination of 27 pesticides in cucumber, potato, radish, apple, banana and rice samples. Norman & Panton (2001) have developed the SFE method for determination of OPPs in wheat and maize using GCB (graphitized carbon black), and Quan et al. (2004) found out that SFE of ginseng followed by purification on C18, gave satisfactory results in determining OCPs. The same sorbent was shown to be the best choice for determination of 32 pesticides in soil using SFE sample preparation (Rissato et al., 2005b).

3. Solid phase extraction (SPE)

SPE is one of the most commonly used sorbent techniques in analyzing pesticide residues. This method is based on the omission of extracts containing target analytes through a column filled with the appropriate sorbent (which was previously conditioned by an appropriate solvent or solvent mixture), or passing of an appropriate solvent through the SPE column to which a suitable amount of sample was previously added. Using selective solvents, first the coextractants from the SPE column can be successfully eluted, and then the target analytes (Figure 1, A), or the elution of analytes can be direct, where undesirable coextractants derived from the sample matrix remain in the SPE column (Figure 1, B).

Compared with the traditional methods, SPE has many attractive features. It is easy to operate, costs less, it has been automated and uses small amounts of solvent. SPE is the multifunctional techniques, since the purification and the concentration occur in the same step. Unfortunately, SPE has certain limitations, primarily related to lower yields (recovery),

i.e. slightly lower sensitivity, in situations where there is "clogging" of the SPE column (blocking of the sorption centers by solid and oily components originating from the sample).

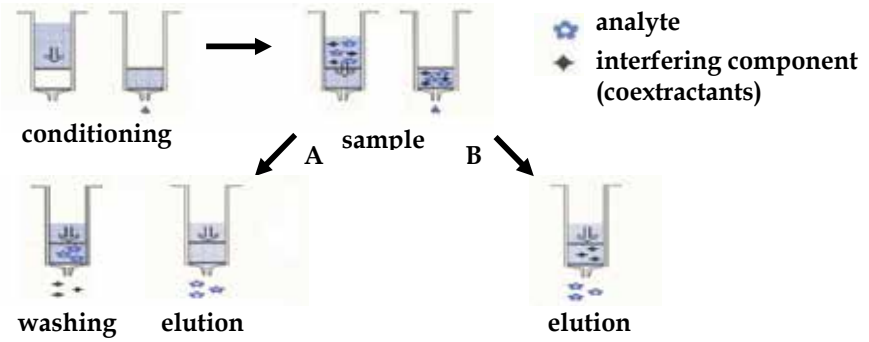

Fig. 1. The basic principle of SPE technique

The most commonly used SPE sorbents in pesticide residues determination are: reverse-phase octadecyl (C18), normal-phase aminopropyl (-NH2) and primary-secondary amine (PSA), anion-exchanger three-methyl ammonium (SAX) and adsorbents such as graphitized carbon black (GCB). Normal-phase sorbents such as florisil ($MgSiO_3$), aluminum oxide (Al_2O_3) and silica (SiO_2) are usually used in combination with the previously mentioned sorbents. The SPE cartridge should be chosen depending on the physicochemical properties of pesticides that are searched for in a particular sample, and the nature of the sample matrix. For instance, the researches indicate that the GCB sorbent is suitable for extraction of compounds of different polarity, and that it causes retention of pigments (carotenoids and chlorophylls) and sterols, and now are widely used for purification of plant extracts (Schenck et al., 2002; Anastassiades et al., 2003). On the other side, PSA sorbent proved to be effective in removing polar compounds and fatty acids, while the SAX is suitable for the removal of fatty and other organic acids and sugars (Okihashi et al., 2005). Activated charcoal proved to be a good solution for removal of pigments from tomato extracts (Kaipper et al., 2001), and cabbage and carrots (Wang et al., 2008), while the florisil gave good results in pyrethroids determination in lettuce, cabbage, cauliflower, carrot, green pepper and green beans (T. Chen & G. Chen, 2007), and metribuzin and quizalofop-p-ethyl in potato and soil samples (Hu et al., 2010). Diatomaceous earth was a good solution for neonicotinoids determination in apricot, peach, pear, celery and courgette (Di Muccio et al., 2006), and C18 cartridges for determination of various pesticides in strawberry, grape, lettuce, tomato (Juan-García et al., 2005) and carbamates in soil (Santalad et al., 2010). Silica gel is effective in determination of OCPs in soil samples (Lehnik-Habrink et al., 2010).

The most of the SPE methods are based on a combination of two or three cartridges. As GCB is suitable for removal of lipids, waxes and other nonvolatile, non-polar coextractants with high molecular weight, it is usually used in combination with other sorbent. Thus, Pang et al. (2006) used SPE for determination of 446 pesticides in cabbage, tomato, cucumber, spinach, cauliflower, celery, peas, carrots, potatoes, lettuce, onion and leek, and found out that the C18/GCB/-NH2 combination was the best. Except for slightly lower recoveries obtained for onion and leeks, the method was shown as good choice for all other samples. Okihashi et al. (2005) tested SPE methods by determining 180 pesticides in tomato, lettuce,

pepper, broccoli, spinach, orange, apple and banana, and found out that the PSA/GCB combination was the most effective. The same combination proved to be a good solution for determination of 23 pesticides in lettuce, chard and spinach (González-Rodríguez et al., 2008), while Tao et al. (2009) used a GCB/Al_2O_3 combination in the analysis of cucumber and cabbage, and obtained satisfactory results for 39 pesticides (carbamates, pyrethroids, OCPs and OPPs). The SAX/PSA combination was a good solution for pyrethroids and OCPs determination in grape, orange, tomato, carrot and green mustard (Sharif et al., 2006).

4. Solid phase microextraction (SPME)

Solid phase microextraction, one of the newest extraction techniques, is widely used in the pesticide residues analysis in samples of different origin, due to the fact that purification and concentration of the sample extract (analytes of interest) are running simultaneously.
The basic part of the SPME system is SPME syringe that visually resembles on the chromatographic, except for the fact that it contains a 1 cm long fiber located within a syringe needle, which is made of an appropriate polymer deposited on the holder of fused silica. Microextraction process is based on the redistribution of analytes between microextraction fiber and sample matrix, i.e. on the selective sorption of target analytes in the active layer of the fiber and direct desorption in the chromatograph injector (thermal in the case of the GC, i.e. by solvent elution in the case of LC - liquid chromatography). The basic principle of analytes microextraction from the solution is shown in Figure 2.

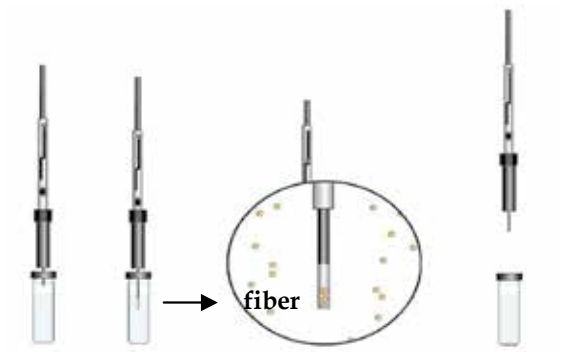

Fig. 2. Procedure for microextraction of analytes from solution

Before the analysis, the fiber is drawn into a metal tube of the SPME syringe. After breaking through the vial septum in which a certain sample amount was previously inserted, the fiber is pulled out from the syringe i.e. it is exposed to the sample by lowering the syringe plunger. After specific time, the fiber with the sorbed analytes is drawn into the needle, which is then pulled out from the vial. Analytes desorption from the fiber is performed by introducing a SPME syringe needle into the injector of the chromatographic system.
SPME is an equilibrium technique, where analytes are distributed between the three phases: sample, gas phase and fiber. The fiber does not extracts all analytes present in the sample, but by the proper calibration, this technique can be used for successful quantification (Đurović et al., 2007a; Pawliszyn, 1997). The amount of analytes that would be adsorbed on the fiber will depend on the thickness and polarity of the active fiber layer, sampling mode (direct sampling - microextraction from solution, »DM-SPME« and headspace sampling -

microextraction from gas phase, »HS-SPME«), the nature of the sample and the analyte (analyte polarity, its molecular weight, pH value, nature of matrix), the mode and speed of the sample mixing, the SPME duration, the temperature at which it is performed, and so on.
Today, about thirty different fiber types are in use (different types of polymers and their thickness), so when selecting the fiber it is necessary to take into consideration several factors: molecular weight, structure and polarity of the analyte molecules, the polarity of fibers, the mechanism of extraction (used sampling mode), the detection limit and range of linearity that is desired to be achieved. In order for a fiber to extract specific compounds from a given matrix, it must have a much higher affinity for the given analytes than a matrix, where the general rule applies: non-polar analytes are more efficiently extracted by non-polar active fiber layer, i.e. polar by polar. The research in the field of pesticide residues has indicated that, in the most of the cases, fibers with extremely non-polar polydimethyl-siloxane (PDMS) and highly polar polyacrilate (PA) active layers are most effective in the analysis of samples of different origin (Doong & Liao, 2001; Sakamoto & Tsutsumi, 2004; Đurović et al., 2007b, 2007c, 2010b; Fernandez-Alvarez et al., 2008). After fiber selection, it is necessary to determine optimal conditions for analytes transfer in the chromatographic system. Adsorbed analytes are desorbed from the fiber by introducing the SPME syringe needle into the injector. Defining the parameters of desorption involves determination of the optimal injector temperature, flow of the carrier gas and desorption time in the case of GC, i.e. proper choice of elution solvent, its flow rate and desorption time, in the case of HPLC.
Although the maximum of SPME sensitivity is achieved at equilibrium times, for practical reasons, extraction time can be shortened (Đurović et al., 2007a, 2010a, 2010b; Pawliszyn, 1997). The most effective ways to overcome the kinetics restrictions are heating and efficient sample mixing. The temperature has two opposite effects. On one side, it increase increases the analytes transfer from the sample to the fiber, while on the other side, due to the simultaneous heating of the fiber during extraction, there is enhanced desorption of analytes from it. Therefore, the necessary step in method development is optimization of the extraction temperature. The speed of extraction is also determined by the sample stirring efficiency. Intensive stirring increases the analytes mobility, and therefore reduces the equilibrium time and increases the analytes amount adsorbed on the fiber. However, in method developing it should be noted that the sample stirring leads to its warming, which may also have non-preferred effects, especially in the case of direct mode.
The matrix nature greatly influences the SPME efficiency, too. Since the analytes distribution coefficients are partially determined by analytes/matrix interaction, appropriate matrix modification can increase the analytes partition coefficients. Thus, for example, the presence of chloride and sulfate ion increases the ionic strength of the solution, which makes a large number of compounds less soluble. In this way, by weakening the matrix/analyte interaction, distribution coefficients can be significantly increased (Arthur et al., 1992).
Considering the fact that SPME is a single-stage method that does not require additional purification and concentration of the sample, the problems related to the matrix occur in the analysis of samples with complex matrices. The researches have shown that the negative effect of the matrix could be significantly reduced by adequate dilution of the sample with the distilled water (Simplicio & Boas, 1999; Đurović et al., 2005, 2007c, 2008).
There are a significant number of SPME applications in pesticide residues analysis. In most cases, SPME method was based on the analysis of sample mixture with water, regardless of whether the extraction medium was placed directly into the formed suspension (Berrada et al., 2004; Beltran et al., 2003; Đurović et al., 2007c, 2010b), or the sampling was done from the

gaseous phase (Chai & Tan, 2009; Kin & Huat, 2010; Zhao et al., 2006; Lambropoulou & Albanis, 2003; Fytianos et al., 2006). However, quite a number of researchers opted for methods based on SPME determination of pesticides combined with the extraction of samples with organic solvent or solvent mixture, and the corresponding dilution of extract obtained with water (Wennrich et al., 2001; Ravelo-Pérez et al., 2008; Parrilla Vázquez et al., 2008; Đurović et al., 2007c, 2010a, 2010b; Herbert et al., 2006; Vega Moreno et al., 2006). Majority of these methods include adjustment of the ionic strength of solution (Berrada et al., 2004; Beltran et al., 2003; Kin & Huat, 2010; Chai & Tan, 2009; Đurović et al., 2010a, 2010b; Zhao et al., 2006; Filho et al., 2010; Lambropoulou & Albanis, 2003; Fytianos et al., 2006; Đurović et al., 2009), and some of them adjustment of the pH value (Berrada et al., 2004; Ravelo-Pérez et al., 2008; Parrilla Vázquez et al., 2008; Filho et al., 2010;) prior to SPME analysis.

The research results indicate that the most often used SPME fibers in the pesticide residues analysis (PDMS and PA) are a good choice for determination of: phenyl urea in carrot, onion and potato (Berrada et al., 2004); OCPs in soils (Zhao et al., 2006; Herbert et al., 2006); OPPs in strawberries and cherries (Lambropoulou i Albanis, 2003) and samples of banana, apple, grapefruit, orange, lemon, kiwi, pineapple, pear, peach, apricot, grapes, melon, lotus, plum, mango, cherry and coconut (Fytianos et al., 2006); OCPs and OPPs in cucumber and strawberry (Kin & Huat, 2010); pesticides belonging to different chemical groups in tomato and cucumber (Chai & Tan, 2009), mango (Filho et al., 2010), soil (Đurović et al., 2010a, 2010b), i.e. in samples of potato, tomato, onion, cabbage and pepper (Marković et al., 2010).

Considering that in the SPME analysis only 1 cm of fiber is exposed to the sample, not only the nature, but also the size of the active surface layer will significantly affect the microextraction efficiency. Thus, by adding an additional material into the active layer of the fiber, its outer surface may increase, and therefore often the SPME efficiency, too. On the other side, the added material can significantly change the polarity of the fiber (similar to the GC stationary phase). Thus, for example, by using mixed PDMS/DVB (polydimethyl-siloxane/divinyl-benzene) fiber, Beltran et al. (2003) provided satisfactory analytical parameters for SPME determination of pyrethroids in tomato samples; Wennrich et al. (2001) for determination of OCPs in kohlrabi, lettuce and tomato; Parrilla Vázquez et al. (2008) for determination of pyrethroids in cucumber; Vega Moreno et al. (2006) for determination of OCPs in soil, while Ravelo-Pérezet et al. (2008) developed a method for determination of pesticides belonging to different chemical groups in tomatoes.

5. Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is a technique based on usage of the microwave energy, and where compounds can be extracted more selectively and rapidly, with similar or better recovery compared to conventional extraction processes.

The MAE effects a direct migration of the desired components out of the matrix, as a result of selective energy application into the matrix. High method efficiency is a result of the matrix macrostructure destruction (Lambropoulou et al., 2007). During the MAE of plant material, microwave rays travel freely through the solvent and interact selectively with the free matrix water causing localized heating. The result is non-uniform temperature rise with more pronounced effects where the free water is in larger proportions. The result is a volume expansion within the systems. The walls of these systems cannot accommodate the high internal pressures and rupture spontaneously, allowing the organic contents to flow freely toward the relatively cool surrounding solvent that solubilizes them rapidly (Paré &

Belanger, 1994). Considering the complexity of plant material and its non-uniformity regarding different amount of free water, the MAE advantage can be noticed. Particularly, by providing different microwave energy levels, it is possible to selectively rupture some systems over others, thus it is possible to develop schemes that will effect the selective extraction of the given systems contents. MAE is also a promising technique for soil samples, particularly owing its possibility to control temperature, pressure and microwave energy, as well as to perform a few extractions simultaneously (Ranz et al., 2008).

For method optimization, several variables such as volume and solvent composition, extraction temperature and time, are usually studied. In order to heat a solvent, part of it must be polar with high dielectric constant to absorb microwave energy efficiently. Non-polar solvents with low dielectric constants can be also used, by adding certain amount of polar solvent that absorbs the microwave radiation and passes it on to other molecules (Caddick, 1995). For example, hexane and toluene can be modulated by the addition of small amounts of acetone or methanol (Ericsson & Colmsjö, 2000).

The first use of MAE technique for pesticide residues determination (parathion and bromophos in maize, soya bean, fava bean, walnut, cotton seed and soil), was reported by Ganzler et al. (1986). In 1993, Onuska & Terry evaluated the extractability of various pesticides in sediment samples, and in the same year Steinheimer (1993) extracted atrazine from soil samples. These works were followed by an extensive paper by the group of Lopez-Avila (1994), presenting procedures for extraction of organic compounds such as OCPs, among others, from soils and sediments. In 1997, Pylypiw et al. used a MAE for determination of several pesticides in beet, cucumber, lettuce, pepper and tomato. The results show that MAE is a viable alternative for determination of atrazine and OPPs in orange peel (Bouaid et al., 2000), carbendazim, diethofencarb, azoxystrobine, napropamide and bupirimate in strawberries (Falqui-cao et al., 2001), fenitrothion in beans (Diagne et al., 2002) and pyrethroids in strawberries (Sanusi et al., 2004). Application of MAE for extraction of acetamiprid from cabbage (Pritam & Mukherjee, 2010) and thiophanate methyl and carbendazim from cabbage and tomatoes (S.B. Singh et al., 2007) are also reported. MAE was also carried out for the determination of thiamethoxam, imidacloprid and carbendazim in cabbage, tomatoes, chilies, potatoes and peppers (S. B. Singh et al., 2004). Barriada-Pereira et al. (2005, 2007) described it as a satisfactory method for OCPs determination in legume vegetation and horticultural samples (lettuce, tomato, spinach, potato, turnip leaf and green bean). Likewise, Paíga et al. (2009) used the MAE method for quality control vegetable samples of zucchini, cucumber, lettuce and pepper, i.e. for determination of carbamates and urea pesticides, while El-Saeid & Al-Dosari (2010) applied MAE technique in monitoring of insecticide (OCPs, OPPs, pyrethroids), fungicides, acaricides and herbicides in Riyadh dates. In 1994, 20 OCPs were extracted from six marine sediments and soils (Lopez-Avila et al., 1994), and in a subsequent study the list of compounds was expanded to nearly a hundred OCPs and OPPs (Lopez-Avila et al., 1995). Investigations on MAE extractions of OCPs and OPPs from soil, optimization and comparison of method, was performed by numerous authors in years to come (Concha-Graña et al. 2003; Fuentes et al., 2007; W. Wang et al., 2007). MAE determination of triazines in soils was first reported in 1993 (Steinheimer), followed by Hoogerbrugge et al. (1997) work. MAE determination of imidazolinone herbicides have been reported by Stout et al. (1996, 1997). Other investigated herbicides are sulfonyl- and phenylurea herbicides (Font et al., 1998; Molins et al., 2000). The investigated fungicides were hexaconazole (Frost et al., 1997), and dimethomorph (Stout et al., 1998), both extracted from soils. De Andréa et al. (2001) applied MAE for determination of methyl

parathion, p,p'-DDE, HCB, simazine and paraquat dichoride in soil, Sun & Lee (2003) for carbamates in soil, and Paíga et al. (2008) for 8 pesticides in the same matrix type.
From economical and practical aspects, MAE is a strong competitor to other recent sample preparation techniques. The main MAE advantages are the low temperature requirement, high extraction efficiency, complete automation, and the possibility of extracting different samples at the same time without interference. The main disadvantage of MAE seems to be the lack of selectivity resulting in the co-extraction of significant amounts of interfering compounds. Additional cleanup is therefore needed before chromatographic analysis. Apart from that, the poor efficiency of microwaves when either the target compounds or the solvents are non-polar, or when they are volatile, can be regarded as another disadvantage. Besides, it is important to notice that the application of microwave energy to flammable organic compounds, such as solvents, can pose serious hazards in inexperienced hands, thus an extraordinary level of safety and attention to details when planning and performing experiments must be used by all personnel dealing with microwaves.

6. Accelerated solvent extraction (ASE)

Accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE), is relatively new sample preparation technique, that uses small amounts of water and organic solvents, and is based on the extraction under elevated temperature (up to 200 ^{0}C) and pressure (up to 20 MPa) for short time periods, resulting in better extraction efficiency.
In practice, a used solvent is pumped into an extraction cell containing the sample, which is then brought to an elevated temperature and pressure. Later, the extract is transferred to a collection vial for cleanup and analysis. At high temperatures, viscosity and the surface tension of the solvent decrease, resulting in a substantial extraction rate increase (Anastassiades et al., 2003). The solvent is kept below its boiling point by applying high pressure that forces its penetration into the sampleores. The combination of high temperature and pressure results in better extraction efficiency, thus minimizing solvent use. The extraction efficiency is almost independent of sample mass, i.e. is mainly dependent on temperature (Richter et al., 1996; Smith, 2002). Besides the type of the solvent used, the main parameters which influencing ASE efficiency are extraction temperature and time (Luo et al., 2010). Although high temperatures increase the efficiency, it may lead to degradation of thermally labile compounds, and to the co-extraction of interfering species. Hence, a compromise between the extraction efficiency and minimization of interfering compounds must be performed carefully, and in addition, usually a further clean-up step involves.
ASE has been successfully used for pesticides determination in samples of different origin. Thus, Di Muccio et al. (2006) developed ASE method for neonicotinoids determination in apricot, peach, pear, celery and zucchini. Richter et al. (2001) developed ASE for *OPPs* determination *in carrot,* Wennrich et al. (2001) for OCPs in strawberry, apple, lettuce, tomato and kohlrabi, while Cho et al. (2008) tested ASE for determination of iprodione, chlorpyrifos-methyl and endosulfan in kiwi. In 2007, Barriada-Pereira et al. described it as a satisfactory method for determining 21 OCPs in lettuce, tomato, spinach, potato, tomato and bean, and in the same year Carabias-Martínez et al. (2007) used ASE to determine triazines in potato, carrot, lettuce, bean, zucchini and orange. ASE was also used for determination of 28 pesticides in pear, potato and cabbage (Adou et al., 2001). In subsequent studies, this list was expanded to 74 pesticides extracted from cucumber, lettuce and tomato (Garrido

Frenich et al., 2005), and up to 130 pesticides extracted from oranges, nectarines and spinach (Cervera et al, 2010). A group of authors managed to extract 29 carbamates from dried fruits (raisin, prune and mango), spices (turmeric, masala, sage, thyme and red pepper) and from soybean paste and soy sauce (Terada et al., 2008), while Gilbert-López et al. (2009) indicated ASE application for pesticide from fatty vegetable matrices. Lehotay et al. (2005, 2005a) reported ASE method for 229 pesticides determination in various fruits and vegetables. ASE efficiency was also confirmed for atrazine and alachlor determination in soil (Gan et al., 1999), as for the arylphenoxypropionic herbicides (Marchese et al., 2001). ASE was carried out for determination of DDT and its metabolites (Tao et al., 2004), i.e. abamectin in soil samples (Brewer et al., 2004). ASE methods for soil samples were reported for OCPs (W. Wang et al., 2007), for bromacil and diuron (Pinto & Lanças, 2009), and dichlorvos, dimethoate, parathion, malathion and chlorpyrifos determination (J. S. Zhang et al., 2010).
Obvious ASE advantage is that it requires much less solvent and shorter extraction times than conventional techniques. Using elevated temperatures and pressures with organic solvents, an enhanced analytes extraction can be achieved. Additionally, ASE is reduced both, waste levels and analysts exposure to harmful solvents. However, samples with high moisture contents require desiccation before the extraction step (Cervera et al, 2010).

7. QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method

Despite mentioned disadvantages related to conventional solvent extraction (SE) methods, they are still the most popular methods for routine analysis. To overcome the SE drawbacks, new trends in pesticide residues analysis have appeared. A good example of this is the QuEChERS method (Anastassiades et al., 2003). The authors questioned the conditions previously used for pesticide residues analysis, and through extensive experiments and novel use of MgSO4 for salting out extraction/partitioning and dispersive solid-phase extraction (d-SPE) for cleanup, they devised a highly streamlined sample preparation method with excellent results for a wide range of pesticides in many types of samples.
The original procedure consists in the sample extraction by hand-shaking or vortex mixing with the 10 mL of acetonitrile (MeCN). Gram quantities of salts (4 g of MgSO4 and 2 g of NaCl) are then added to the sample by mixing, to drive analytes partitioning between the aqueous residue and the solvent. After vortex mixing and centrifugation, clean-up and removal of residual water is performed using a d-SPE procedure (PSA adsorbent and anhydrous MgSO4 are mixed with the sample extract), that requires less time than the traditional SPE and simultaneously removes residual water and many polar matrix components, such as organic acids, some polar pigments, and sugars.
As a polar solvent, miscible with water, with sufficient dispersive (hydrophobic) properties to extract effectively both polar and non-polar pesticides, MeCN is chosen as the QuEChERS solvent. Use of this solvent in the QuEChERS method proved to be successful for extraction of several pesticides classes from different matrices (Anastassiades et al., 2003; Asensio-Ramos et al., 2010; Drozdzyński & Kowalska, 2009; Lehotay et al., 2005, 2005a, 2005b; X. Li et al., 2010; Rashid et al., 2010; Shi et al., 2010; Yang et al., 2010; Đurović & Đorđević, 2010c, 2010d).
Studies showed that some pesticides gave lower recoveries depending on pH of the matrix (Anastassiades et al., 2007; Lehotay et al., 2005, 2005a). Anastassiades et al. (2007) realized that buffering at pH=5 during extraction gave the optimum balance to achieve acceptably recoveries (>70%) for pH-dependent pesticides, independent of the matrix. On the other hand, Lehotay (2007) modified the method to use even stronger acetate buffering conditions.

Both versions of methods went through extensive laboratory trials and successfully met statistical criteria for acceptability by independent scientific standards organizations. So the acetate-buffering version becomes AOAC Official Method 2007.01 (Lehotay, 2007) and the citrate-buffering version being named as Standard EN 15662 Method (www.cen.eu).
There is an abundance of the QuEChERS applications for pesticides determination in different plant samples. Thus, for example, QuEChERS provides satisfactory results for determination of 229 pesticides in lettuce and orange (Lehotay et al., 2005), 109 in rice (Thanh et al., 2007), 160 in tomato, pear and orange (Kmellár et al., 2008), 140 in cucumber and orange (Fernández Moreno et al., 2008), 118 in vegetables juice (Nguyen et al., 2009), 138 in apples, bananas, pears, apple juice, peas, creamed corn, squash and carrots (J. Wang & Leung, 2009), 150 in tomato, strawberry, potato, orange, and lettuce (Koesukwiwat et al., 2010), 300 in tomato, apple, lettuce, cucumber, carrot, mushroom, grapes, lemon, pepper, pear, potato and cabbage (Kmellár et al., 2010), 69 in zucchini, melon, cucumber, tomato, garlic, lettuce and pepper (Camino-Sánchez et al., 2010), 46 in onion, spinach, potato, carrot, cucumber, cabbage and tomato (Srivastava et al., 2010), 150 in grapes (Afify et al., 2010), 148 in onion, spinach, potato, carrot, peas and tomato (J. Wang et al., 2010), 73 OPPs and carbamates in rice, tree nuts and citric fruits (Chung & Chan, 2010) and 14 OCPs in apricot, plum, cherry, nectarine, pear and apple (Cieślik et al., 2011). Besides, QuEChERS has been successfully used for determination of metaflumizone (Dong et al., 2009), azadyrachtin, spinosad, rotenone (Drozdzyński & Kowalska, 2009), oxadiargyl (Shi et al., 2010) and 38 pesticides (Yang et al., 2010) in soil samples. As a modified version, it was applied for OCPs (Rashid et al., 2010) and OPPs determination in soil samples (Asensio-Ramos et al., 2010).
The QuEChERS advantages are the high recovery, accurate results, low solvent and glassware usage, less labor and bench space, lower reagent costs, and ruggedness. The main QuEChERS disadvantage is that the final extract must be concentrated to furnish the necessary sensitivity i.e. to achieve the desired limits of quantification (LOQ).

8. Matrix solid phase dispersion (MSPD)

Matrix solid-phase dispersion (MSPD) is a new SPE-based extraction and clean-up technique developed for pesticide multi-residue analysis (Kristenson et al., 2006). The MSPD method is based on the homogenization of a viscous, solid or semi-solid sample with an abrasive solid support material in a glass mortar, in order to perform the complete disruption and dispersal of the sample. After blending, the sample is transferred into a column and analytes are eluted with appropriate solvent. Complete disruption of the sample and its dispersion over the support surface greatly enhance surface area for the sample extraction. Furthermore, interferences are retained on the adsorbent and in that way, extraction and clean-up are performed simultaneously, reducing the analysis time and the amount of solvent used (Barker, 2000; Kristenson et al., 2006).
Reversed-phase materials such as C8 and C18-bonded silica are the most commonly used adsorbents, because their lipophilic properties enable good disruption, dispersion and retention of lipophilic species (Lambropoulou & Albanis, 2007). Several methods based on use of these adsorbents have been used for determination of several pesticides classes including OPPs and OCPs in tomato, lettuce, orange, lemon, grapefruit, pear and plum (Torres et al., 1995), carbamates in orange, onion, grape and tomato (Fernández et al., 2000), eight fungicides in orange, apple, tomato, carrot and zucchini (Navarro et al., 2002), thirteen fungicides and insecticides in pepper, lettuce, tomato, orange, apple and pear (Torres et al.,

1997), five fungicides in orange, lemon, banana, pepper, chard and onion (Blasco et al., 2002), five pesticides in strawberry, orange, potato, pear and melon (Pous et al., 2001), five pesticides in onion (Rodrigues et al., 2010), and 14 pesticides in tomato, pepper, orange, raspberry, banana, cucumber, lemon, peach, grape, apple, grapefruit, pear, red currant and leek (Kruve et al., 2008). Besides C8 and C18-bonded silica, florisil has been successfully used for determination of OCPs in tomato juice (Albero et al., 2003), lemon, orange, apple, mango, grapes, banana, carrot, potato, onion, cucumber, tomato, cabbage, spinach and wheat (Abhilash i sar., 2007; 2009), pyrethroids in cucumber, eggplant, cabbage and peas (Ling & Huang, 1995), nine pesticides in artichoke, lettuce and tomato (Viana et al., 1996), phenthoate (Z-Y. Li et al., 2002), OCPs (Z. Shen et al., 2005; X. Shen et al., 2006) and five pesticides in soil (X. Shen et al., 2007). Also, Al_2O_3 has been used as an adsorbent in determining of 15 pesticides in corn, potato and cabbage (Lin et al., 2010), while Bogialli et al. (2004) used sand for carbamates determination in lettuce, zucchini, spinach, tomato, apple and pear. Basically, the adsorbent choice depends on analyte polarity and interferences which could be co-extracted from sample matrix.

Also, the nature of the elution solvent is crucial for efficient pesticides elution from the adsorbent (Albero et al., 2003; Blasco et al., 2002; 2002a; Bogialli et al., 2004; Chu et al., 2005).

The MSPD expansion in the last decade is evidenced not only by over 360 papers dealing with its applications, but also by reviews that have been published (Barker, 2000, 2000a, 2007; Bogialli & Di Corcia, 2007; Garciá-López et al., 2008; Kristenson et al., 2006; Moreda-Piñeiro et al., 2009). Compared with other extraction techniques, the MSPD performance was usually similar or superior, and results depending on the target analyte and sample matrix (Abhilash & N. Singh, 2009a; Gilbert-López et al., 2010; Montes et al., 2009).

In comparison to traditional extraction methods, MSPD aproach has several advantages, including simplified and faster sample-treatment, reduced use of toxic solvents, eliminated emulsion formation and increased selectivity and sensitivity. In MSPD, the sample extraction and clean-up are performed in the same step by use of small amounts of adsorbent and solvent, thus reducing the cost and analysis time. As a drawback, a number of applications still use large volumes of solvents for extraction and clean-up, which requires solvent evaporation. There is every reason to believe that solving of this problem will make MSPD more useful in the near future.

9. Conclusion

The sample extraction step, which accounts for about two-thirds of the total analysis time, is still the weakest link and the time-determining step in the whole analytical procedure. It is also the primary cause of errors and discrepancy between laboratories. However, in the recent past, improvements in the sample preparation techniques for different environmental samples have led to modification of the existing methods and development of new techniques, in order to save time and reduce use of chemicals and thus improve the overall performance of analytical process. As a result, several rapid, low cost, environmentally friendly, and readily automated methods of extraction are now available. Besides, because of the complexity of the matrices, extraction is usually followed by very specific clean-up procedures to achieve accurate sample quantification, so the new methods are modified in order to achieve a compromise between cost, selectivity, and sensitivity.

Reduced solvent methods, including supercritical fluid extraction (SFE), solid phase extraction (SPE), solid phase microextraction (SPME), microwave assisted extraction (MAE),

accelerated solvent extraction (ASE), QuEChERS and matrix solid phase dispersion (MSPD) have grown in their maturity, which increased application of these techniques in pesticide analysis of plant and soil matrices. Although the composition of environmental matrices such as plants and soil varies from place to place, which requires application of different approaches and strategies, the development of a uniform procedure is highly encouraged. Future developments in all areas of analytical sample preparation are expected to continue to be application-driven in a quest for improved recovery, higher sample throughput, and reduced consumption of organic solvent with capability to provide accurate results.

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Cloud Point Extraction of Pesticide Residues

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1. Introduction

Pesticides including organochlorine pesticides (OCPs), organophosphorus pesticides (OPPs), and nitrogen-containing herbicides are types of well-known environmental contaminants. Pesticides are generally categorized based upon their persistence in the environment. Organochlorine pesticides are considered persistent pesticides. These pesticides have long environmental half-lives and tend to bioaccumulate in humans and other animals. The contemporary pesticides include organophosphates, carbamates, triazines, chloroacetanilides, synthetic pyrethroids, and others and are considered nonpersistent. These pesticides have much shorter environmental half-lives and tend not to bioaccumulate (Barr & Needham, 2002). Table 1 provides a very abbreviated synopsis of major families, or chemical classes, of pesticides grouped by their uses. Among the pesticides, organophosphate and carbamate compounds are the most widely used due to their high insecticidal activity and relatively low persistence (Dyson et al., 2002). These pesticides are toxic because they act as inhibitors of acetylcholinesterase, an enzyme that catalysis in a very efficient way the hydrolysis of the neurotransmitter acetylcholine. This enzyme is present in vertebrates and insects and its inhibition can disrupt the transmission of nerve impulses (Hassal, 1983; Simonian et al., 1997). The increasing production and application of pesticides for agricultural and non-agricultural purposes has caused the pollution of air, soil, ground, and surface water which involves a serious risk to the environment and as well as human health due to either direct exposure or through residues in food and drinking water. In the world, alarming levels of pesticides have been reported in air, water, soil, as well as in foods and biological materials (Pico´et al., 2003; Lambropoulou & Albanis, 2007; Guzzella et al., 2006; Konstantinou et al., 2006; Núñez et al., 2005; Harner et al., 2006; Chang & Dong, 2006; Barr & Needham, 2002). Nowadays, approximately 300000 tonnes of pesticides per year are used for agricultural production in Europe and their residues can be found in soil, water, foods, etc. For environmental and drinking waters, the maximum admissible concentration of a single compound established by the European Union (EU) is 0.1, and 0.5 μg L^{-1} is the maximum allowed for the total concentration of all pesticides (http://ec.europa.eu/environment/water). The WHO threshold values for concentrations of pesticides in drinking water, based on toxicological considerations, are less strict than the maximum concentrations allowed by EU (Hamilton et al., 2003). Pesticide residues are highly mobile in soil and can leach into ground-water, so may be a potential health hazard.

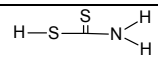
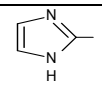
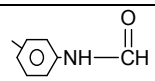
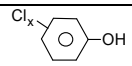
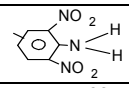
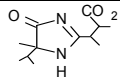
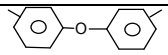
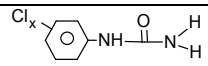
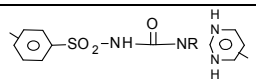
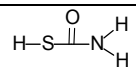
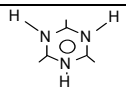
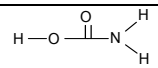
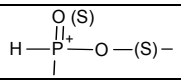
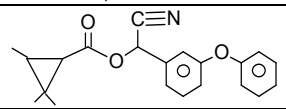
Use: Family	Examples	Basic Structure
Fungicides:		
Dithiocarbamate	mancozeb, maneb, metiram, zineb	
Imidazole	carbendazim, imazalil,thiabendazole	
Phthalimide	procymidone, captan, captafol, folpet	
Triazole	myclobutanil, propiconazole	
Herbicides:		
Acetamide	alachlor, dichormid, metolachlor	
Chlorophenoxy	2,4-D, 2,4,5-T, MCPA, silvex	
Dinitroaniline	pendimethalin, trifluralin, dinitramine	
Imidazolinone	imazaquin, imazapyr, imazethapyr	
Phenylphenoxy	fomesafen, bifenox, fluazifop-butyl	
Phenylurea	linuron, diuron, thidazuron, neburon	
Sulfonylurea	chlorsulfuron, chlorimuron-ethyl	
Thiocarbamate	vernolate, asulam, butylate, thiobencarb	
Triazine	amine, ametryn, simazine, prometon	
Insecticides:		
Carbamate	carbofuran, aldicarb, propoxur, oxamyl	
Organochlorine	methoxychlor, DDE, lindane, endosulfan	insecticides containing chlorine
Organophosphorus	diazinon, chlorpyrifos, acephate, ethion	
Pyrethroid	permethrin, eyfluthrin, fenvalerate, bifenthrin	

Table 1. Major types of pesticides (Lehotay, 1997).

Since over 900 pesticid substances are used throughout the world, screening approaches are being developed to analyze as many pesticides as possible (Garcia-Reyes et al.,2008; Fernández-Alba, 2005).

The identification and determination of trace and ultra-trace pesticides in complex matrices still remains a challenge to analytical chemists. A number of spectrophotometric and flouorimetric methods have been developed in recent years for the determination of pesticides. The majority published spectrophotometric methods are based on coupling of a diazonium ion with the phenols obtained by hydrolysis of the carbamates in alkaline medium (Khalaf et al., 1993; Zanella et al.,1999; Alvarez-Rodríguez et al., 1997; Coly & Aeron, 1998). Colorimetric and flouorimetric methods are sensitive, but not highly specific in general. The determination of pesticide residues is an intricate problem because of the large number of chemicals involved. The ideal method for the analysis of pesticide residues should have high sensitivity, selectivity, accuracy, high precision, and low cost and should be applicable to a wide range of sample matrices. Thus, several chromatographic techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) and thin layer chromatography (TLC), can be applied for the determination of pesticides residues (Xie et al., 2010; Lambropoulou & Albanis, 2007). Nowadays, hyphenated techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) is becoming popular and fast gaining grounds for pesticide residues analysis (Balinova & Balinov, 1991; Bernal et al., 2009). Gas chromatography (GC) has been used widely for analysis of pesticide residues in plants tissues, soils and water samples (Yeboah et al., 2001; Roseboom & Herbold, 1980; Balinova, 1996.). However, the thermal instability of some pesticides make them necessary to first prepare stable derivatives, and indirectly determine them by GC in the form of these derivatives, or to use other techniques such as liquid chromatographic (LC) or capillary electrophoresis (CE). Pesticide concentrations in real samples (water, food, etc.) are frequently very low and their direct determination is not possible; it is therefore necessary to perform previous pesticide enrichment and separation. Separation and preconcentration are areas of increasing interest, particularly for enhancing the inherent capabilities of analytical signals and lowering the detection limits in analytical chemistry. There are several different enrichment and pre-separation techniques for pesticides reported in literature, but each has its own limitations. Pesticide samples are usually enriched by liquid–liquid extraction (LLE), solid-phase extraction (SPE). LLE, based on the transfer of analyte from the aqueous sample to a water-immiscible solvent, is widely employed for sample preparation. The efficiency of this process depends on the affinity of analytes with the extracting solvent, ratio between the phases and number of extractions. Nevertheless, some shortcomings such as emulsion formation, use of large sample volumes and toxic organic solvents and hence, generation of large amounts of pollutants make LLE labour to be intensive, expensive, time-consuming and environmentally unfriendly. In addition, polar pesticides cannot be extracted sufficiently quantitative with nonpolar solvents. Another popular sample-preparation approach is solid-phase extraction (SPE). Although it uses much less solvent than LLE, the usage can still be considered significant, and normally an extra step of concentrating the extract down to a small volume is needed. SPE can be automated but this entails complexity and additional cost (Xu et al., 2007; Pena-Pereira et al., 2009; Rezaee et al., 2010).

The most frequently used methods for the extraction of organic compounds from soils are Soxhlet or ultrasonic extraction. Soxhlet extraction is the most widely used extraction method

for organic pollutants strongly adsorbed in solid matrices. To extract soil samples with Soxhlet extractors takes a long time; the analyte is held at high temperature and temperature-sensitive pesticides may be destroyed. Moreover, large quantities of solvents are wasted and additional concentration and clean-up steps are necessary (Antunes et al., 2003; Luque de Castro & García-Ayuso, 1998). Sonication is faster than Soxhlet extraction and allows extraction of large amounts of sample, but it still uses about as much solvent as the Soxhlet extraction (Ferrera et al., 2004). Chemical quantification in soil is particularly difficult since it is highly heterogeneous, and very efficient extraction methods are required. During the last years, several fast extraction techniques were developed to overcome the limitations of conventional methods. Pressurised liquid extraction (PLE), also named accelerated solvent extraction (ASE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and subcritical water extraction (SWE) are techniques that can be used instead of Soxhlet for the extraction of organic compounds, because they are rapid compared to the several hours needed for Soxhlet extraction and, in turn, much less solvent is required (Ferrera et al., 2004 ; Tadeo et al., 2010; Xie et al., 2010, Eskilsson & Mathiasson, 2000). Table 2 summarizes and compares the characteristics, advantages and disadvantages of each extraction technique (Xie et al., 2010; Carabias-Martínez et al., 2000c). Modern trends in analytical chemistry are towards the simplification and miniaturization of sample preparation procedures as they lead inherently to a minimum solvent and reagent consumption and drastic reduction of laboratory wastes. In view of this aspect, several micro-extraction techniques are being developed in order to reduce the analysis step, increase the sample throughput and to improve the quality and the sensitivity of analytical methods. Unconventional LLE methodologies have been arisen like: single drop microextraction (SDME), wetting film extraction (WFE), cloud point extraction (CPE), homogeneous liquid–liquid extraction (HLLE), dispersive liquid–liquid microextraction (DLLME) and dispersive liquid–liquid microextraction based on solidification of a floating organic drop (DLLME-SFO) (Miro et al., 2005; Anthemidis & Ioannou, 2009; Lambropoulou &Albanis, 2007).

Because of the advantages mentioned below cloud point extraction has an increasing interest recent years. Separation and preconcentration based on cloud point extraction (CPE) are becoming an important and practical application of surfactants in analytical chemistry. The CPE has been used successfully for the pre-concentration of species of widely differing character and nature, such as metal ions, proteins and other biomaterials or organic compounds of strongly differing polarity. Historically, the first application of CPE for the extraction of metal ions forming complexes sparingly soluble in water was introduced by Watanabe & Tanaka (1978). The technique is based on the property of most nonionic surfactants in aqueous solutions to form micelles and to separate into a surfactant-rich phase of a small volume and a diluted aqueous phase when heated to a temperature known as the cloud point temperature. The small volume of the surfactant-rich phase obtained with this methodology permits the design of extraction schemes that are simple, cheap, highly efficient, speedy, and of lower toxicity to the environment than those extractions that use organic solvents. CPE has been used to separate and preconcentrate pesticides as a step prior to their determination in hydrodynamic analytical systems such as liquid chromatography (LC), capillary electrophoresis (CE) and gas chromatography (GC). The theory and main findings of these procedures have been summarized in well-documented reviews (Paleologos et al., 2005; Madej, 2009; Silva et al., 2006; de Almeida Bezerra et al., 2005; Carabias-Martínez et al., 2000; Stalikas, 2002; Saitoh & Hinze, 1991; Quina & Hinze, 1999), there are no detailed reports on the applicability of the above-mentioned cloud point

extraction techniques in pesticide analysis. The applications addressed in this review cover aqueous and solid environmental samples and food samples. Finally, possible future trends and developments of CPE in this area were briefly discussed.

Extraction technique	Solvent type /Extraction Time/Solvent consumption	Temperatur/ Pressure/ Cost	Disadvantages	Advantages
Soxhlet	Organic solvent/ 6–24 h/ 60–500 mL	Boiling point of Solvent/ Atm.pressure/ Low cost	Long extraction time, large consumption of organic solvent, exhaustive extraction, preconcentration of sample required after extraction.	Large amount of sample, filtration not required, not matrix dependent, and easy to operate
Supercritical fluid extraction (SFE)	CO_2 / 30–60 min / 10–40 mL	70–150 ^{0}C / 15–50 MPa/ High cost	Limited sample size, extraction efficiency depends on matrix and analyte	Fast extraction, non-toxic, environmental friendly, small amount of solvent, filtration not required,
Ultrasonic-assisted extraction (UEA)	Organic solvent / 30–60 min/ 30–100 mL	30–35 ^{0}C / Atm. pressure/ Low cost	Large amount of organic solvent, labor intensive, filtration required, risk of exposure to solvent vapor.	Fast method, large amount of sample, not matrix dependent, easy to operate
Microwave-assisted extraction (MAE)	Organic solvent / 20–30 min/ 10–40 mL	100–150 ^{0}C / Atm. Pressure/ Moderate cost	Extracts must be filtered, polar solvent needed, exhaustive extraction,	Fast extraction, small amount of solvent, and full control of extraction parameters
Pressurized liquid extraction (PLE)	Organic solvent / 10–60 min / 10–60 mL	100–150 ^{0}C/ 7–15 MPa/ High cost	Extraction efficiency is more matrix dependent	Fast technique, small solvent usage, no filtration needed and easy to use.
Subcritical water extraction (SWE)	Water/ 30–60 min/ 30–60 mL	200–300 ^{0}C/ 5MPa/ Moderate cost	Required optimization of operating conditions	Fast method, water is non-toxic, environmental friendly, small amount of solvent.
cloud point extraction (CPE)	Surfactant solution /10–20 min / 5–10 mL CP of surfactant	Atm. Pressure/ Low cost	Required optimization of operating conditions	Fast extraction, surfactant is non-toxic, environmental friendly, small amount of solvent.

Table 2. Comparison of various extraction techniques for pesticides in solid samples (Xie et al., 2010; Carabias-Martínez et al., 2000c).

2. Principle of cloud-point extraction (CPE)

The extraction method using surfactants, termed ''cloud point extraction or micelle-mediated extraction '' provides an alternative to the conventional extraction systems due to its easy steps and lack of requirement for organic solvents. CPE is a new promising environmentally benign extraction technique which is based upon phase separation behavior exhibited by aqueous solutions of certain surfactant micelles. Surfactants are amphiphilic organic substances. Their molecules present a long hydrophobic hydrocarbon chain and a small charged group or polar hydrophilic. The combination of hydrophilic and hydrophobic groups in the same molecule provides to the surfactant a property of dissolution in water and others solvents. The hydrophobic groups tend to form aggregates called micelles. The concentration at which surfactants begin to form micelle is known as the critical micelle concentration (CMC). The CMC of a surfactant depends on several factors, such as its molecular structure, and experimental conditions such as ionic strength, counterions, temperature, etc. Upon appropriate alteration of the conditions such as temperature or pressure, addition of salt or other additives, the solution becomes turbid at a temperature known as cloud point (CP) due to the diminished solubility of the surfactant in water. CP varies widely with temperature from one surfactant to another. When the temperature reaches the cloud point, the solution containing the surfactant becomes turbid and is separated into two phases: the small volume 'surfactant-rich phase" and the large volume of "aqueous phase". This phenomenon is reversible and upon cooling, a single isotropic phase is obtained again. However, the mechanism by which separation occurs is poorly understood. Some authors have proposed that it would be due to an increase in the micellar aggregation number when temperature is increased (Lindman & Wennerstrom, 1991; Corti et al., 1984). And some others have suggested that the phase separation at the lower consolution point is driven by the effective inter-micellar interaction potential which is repulsive at low temperature but becomes attractive at high temperature (DeGiorgio et al., 1984). Other authors have proposed that the phase separation behavior is a result of the competition between the internal-energy effects which promote separation of micelles from water and entropic effects together with the miscibility of micelles in water (Blankschtein et al., 1986; Liu et al., 1996). Kjellander & Florin (1981) and Claesson et al. (1986) have also proposed that the phase separation results from the competition between entropies (Shariati & Yamini., 2006)

In aqueous solution, the unique structure of surfactant allows sparingly soluble or water-insoluble substances to be solubilized because they can associate and bind to the micellar assembly (Quina & Hinze, 1999). Aqueous solutions of some surfactants have been used in CPE of different species prior to their determination by several techniques (Paleologos et al., 2005; Silva et al., 2006; de Almeida Bezerra et al., 2005; Madej, 2009; Stalikas, 2002). The interaction between surfactant and analyte may be electrostatic, hydrophobic or a combination of both (Ferrera et al., 2004). CPE mainly depends on the solubilization of surfactant solution and phase separation for the extraction and preconcentration of analytes (Pramauro & Prevot, 1995). The use of micellar systems as an alternative to other techniques of separation offers several advantages including low cost, safety and high capacity to concentrate a wide variety of analytes of widely varying nature with high recoveries and very high concentration factors. The extraction efficiency for the target analyte by CPE is influenced by many factors, such as pH of a sample solution, surfactant type and concentration, temperature and duration of reaching equilibrium and ionic strength. The

effect of these factors on the percentage extraction of the analytes studied therefore needs to be established. The steps of the cloud point extraction process are shown in Fig. 1 .

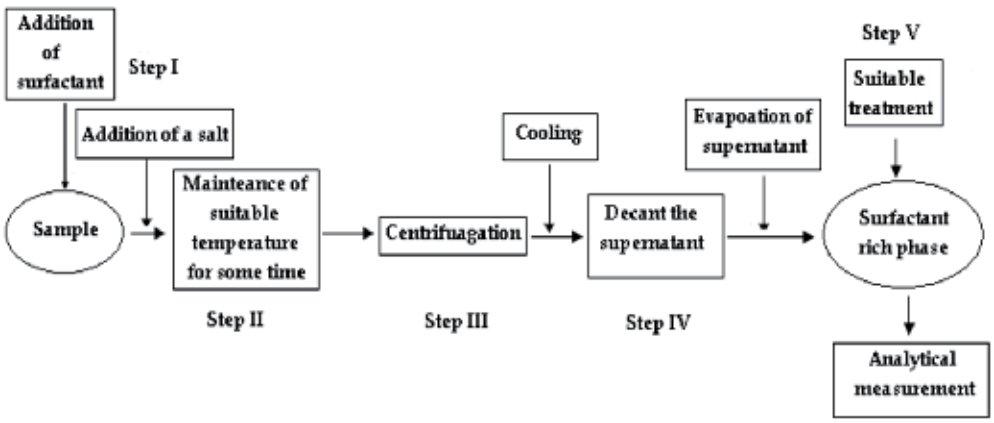


Fig. 1. Five key steps in cloud-point extraction (CPE) (Madej, 2009).

2.1 Effect of pH

The pH effect on CPE depends on the characteristics of both surfactants and analytes. Solution pH is an important factor during CPE process involving analytes that possess an acidic or basic moiety. For organic molecules, especially for ionizable species, maximum extraction efficiency is achieved at pH values where the uncharged form of the analyte prevails, and therefore, target analyte is favored to be partitioned into the micellar phase. The ionic form of a neutral molecule formed upon deprotonation of a weak acid or protonation of a weak base normally does not interact with and bind the micellar aggregate as strongly as its neutral form does. However, changing the pH will change the ionization form of certain analytes and will thereby affect their water solubility and extractability. Thus, pH appears to be also an important factor for the cloud point extraction of pesticides from water samples. Generally, the relationship between pH and extraction efficiency has not been studied extensively, and contradictory results have been reported (Zhang et al., 2009). In most of the CPE studies in pesticide analysis, the pH of the samples is not adjusted. Furthermore, a wide range of pH values between 2 and 10 has been reported for the analysis of organochlorine and organophosphorus pesticides (de Almeida Bezerra et al, 2005; Madej, 2009; Xie et al., 2010).

2.2 Properties of surfactant

Surfactants are amphiphilic organic substances. Their molecules present a long hydrophobic hydrocarbon chain and a small charged group or polar hydrophilic. A typical surfactant has a R-X structure, where R is a hydrocarbon chain, which can have between 8 and 18 atoms of carbon, and X is the polar or ionic head group. The most usual chemical classification of surfactant is based on the hydrophilic group nature. A surfactant can be classified by the presence of formally charged groups in its head. The four general groups of surfactants are defined as non-ionic, cationic, anionic, and amphoteric (or zwitterionic) (de Almeida Bezerra et al., 2005). A non-ionic surfactant has no charge groups in its head. The head of an ionic surfactant carries a net charge. If the charge is negative, the surfactant is more specifically

called anionic; if the charge is positive, it is called cationic. If a surfactant contains a head with two oppositely charged groups, it is termed zwitterionic. However, the application of cationic surfactants in CPE is scarce. A correct choice of surfactant is fundamental for obtaining an optimal extraction process. When selecting the extractant, consideration should be given to its interaction with the matrix, as well as the solubility of the analyte. To date, non-ionic surfactants (mainly polyoxyethylenated alkylphenols, from the Triton series, Igepal series and PONPE series) are those most widely employed for CPE pesticide analysis. They are all commercially available of high purity grade, stable, non-volatile, non-toxic and environmentally friendly. The extraction efficiency typically increases with a surfactant concentration up to a maximum value, with essentially quantitative recovery often being observed. Thus, the minimum concentration that produces quantitative extraction should be chosen in order to obtain the best aqueous phase volume/surfactant-rich phase volume ratio. As a general principle, CPE will be more efficient when more hydrophobic surfactants and more hydrophobic analytes are used (Paleologos et al., 2005; Silva et al., 2006; de Almeida Bezerra et al., 2005; Madej, 2009; Stalikas, 2002).

2.3 Effect of concentration

It is important to discuss the effect of surfactant concentration on CPE. The surfactant concentration affects both the extraction and theoretical preconcentration factor. During CPE, the recoveries and theoretical maximum enrichment depended mainly upon the concentration of surfactant. Thus, it is necessary to optimize the surfactant concentration for sufficient extraction of the target analytes. There is a narrow range within easy phase separation, maximum extraction efficiency and accomplished analytical signal. Increasingly, outside this optimal range, the analytical signal is observed to deteriorate due to the increase in the final volume of the surfactant that causes the preconcentration factor (phase–volume ratio) to decrease. However, if surfactant concentration is decreased from that recommended, accuracy and reproducibility would probably suffer because the resultant surfactant-rich phase would not be sufficient to make reproducible measurements of extraction and separation. The surfactants, which have too high or too low cloud point, are not suitable for the CPE separation/preconcentration of trace pesticide residues.

2.4 Ionic strenght

The cloud point of micellar solutions can be altered by salt addition, presence of alcohol, other surfactants, polymers, and some organic or inorganic compounds, which can cause an increase or decrease on the phase micellar solubility (de Almeida Bezerra et al., 2005). It was observed that the presence of electrolytes decreases the cloud point (salting-out effect), resulting in low extraction efficiency. The salt concentration is also a key parameter in CPE. The addition of inert salt to the solution can influence the extraction/preconcentration process since it can alter the density of the aqueous phase for most non-ionic surfactants and remarkably facilitate phase separation. Also, it can change the CP temperature of non-ionic surfactant (Saitoh & Hinze, 1991; Hinze et. al. 1984). When the salt concentration is increased, the micelle size and the aggregation number are increased and the critical micellar concentration remains constant (Fröschl et al.,1997). The recovery increases with the inert salt concentration up to saturation. In addition, non-polar analytes may become less soluble in the solution at higher salt concentrations and thus contribute to higher recoveries. The results obtained indicate that the addition of salt produces an increase in the extraction

of the more polar solutes while the recoveries of the less polar compounds are not affected (Carabias-Martinez et al., 2000; Eiguren-Fernández et al., 1998, 1999). According to Komaromy-Hiller et al. (1996) the salting-out phenomenon is directly related to desorption of ions to the hydrophilic parts of the micelles, increasing interaction between micelles and consequently leading to the precipitation of surfactant molecules.

2.5 Effect of equilibration temperature and time

The cloud-point temperature depends on the structure of the surfactant and on its concentration. Thus, optimal equilibration temperature and incubation time are necessary to complete reactions, and to achieve easy phase separation and preconcentration as efficient as possible. If the temperature is lower than the cloud-point, two phases cannot be formed. But too high temperature may lead to the decomposition of analytes. The greatest analyte preconcentration factor is reached when the CPE process is conducted with equilibration temperatures well above the cloud point temperature of the system. Moreover, by increasing the equilibration temperature, a reduction in the surfactant rich phase has been observed (Okada, 1992). Thereby, the preconcentration factor increases with increasing temperature depending on the surfactant concentration. As the temperature, or the equilibration time, increases, the amount of water in a surfactant-rich phase decreases and hence the volume of that phase decreases. Optimal equilibration temperature surfactant is important, since the temperature corresponding to cloud point is correlated with the hydrophilic property of surfactants.

2.6 Effect of of centrifugation

In general, centrifugation time hardly ever affects micelle formation but accelerates phase separation in the same sense as in conventional separations of a precipitate from its original aqueous environment. Centrifugation times around 5–10 min are usually efficient for most micelle-mediated extraction (MME) procedures. If the temperature is lower than the cloud point, the phase separation is difficult to be formed (Paleologos et al., 2005).

3. Analytical applications

The identification and determination of very low levels of pesticides in complex matrices is extremely difficult. Recently a promising environmentally benign extraction and preconcentration methodology based on cloud point extraction (CPE) has emerged as an efficient sample pretreatment technique for the determination of trace/ultra-trace pesticides in complex matrices. Here we address the most recent analytical applications of this methodology when used as an isolation and trace enrichment step prior to the analysis of pesticides via spectrophotometry, liquid and gas chromatography or capillary electrophoresis. Table 3 summarizes some recent applications in this topic along with fundamental features of the methods developed.

3.1 Spectrophotometry

A procedure for CPE and spectrophotometric determination of carbaryl in natural waters is described for the first time by Melchert & Rocha (2009). Carbaryl is hydrolysed in alkaline medium to 1-naphthol, which reacts with the oxidised form of *p*-aminophenol (PAP), generated by reaction with molecular oxygen or other oxidising agent. Addition of oxidising

agents is usually required to convert PAP to benzoquinoneimine that reacts with 1-naphthol. After extraction of the reaction product with a nonionic surfactant, the indophenol blue species in the surfactant-rich phase is measured by spectrophotometry at λ=630 nm. The clean up step was carried out only with TX-114 in alkaline medium in order to avoid the use of toxic organic solvents as well as to minimise waste generation. Cloud point preconcentration of the product of the reaction of the analyte with PAP and cetyltrimethylammonium bromide (CTAB) was explored to increase sensitivity and improve the detection limit.

Extraction of analytes from sample matrices is a challenging task. Non-ionic surfactants such as TX-100 and TX-114, have been widely used as extractant for various organic compounds. However, high temperature (> 70 °C) is required for CPE, so it may affect on the stability of the compounds especially carbamate insecticides. Acid-induced anionic surfactant micelle-mediated extraction (acid-induced CPE) has been demonstrated to be a powerful method for the extraction of carbaryl residues in water and vegetable matrices prior to spectrophotometric detection. An acid-induced CPE is employed for extraction of thermally-labile carbaryl. In acid-induced CPE, anionic surfactants such as sodium dodecyl sulfate, sodium dodecyl sulfonate and sodium decyl sulfate are used as extractants. The main advantages of this approach are the absence of UV chromophores in alkylsulfate or alkylsulfonate molecules, the lack of time and temperature dependence in the extraction step, the speed of extraction and the ability to extract thermally labile and polar compounds. Santalad et al. (2008) demonstrated a method for the determination of carbaryl based on acid-induced anionic surfactant micelle-mediated extraction (acid-induced-CPE) coupled to derivatization with 2-naphthylamine-1-sulfonic acid (ANSA) reagent. In this method, an anionic surfactant, sodium dodecyl sulfate (SDoS) and concentrated HCl were used as extractants at room temperature. ANSA derivatization was directly reacted with carbaryl without alkali hydrolysis. The conditions for both extraction and derivatization are optimized before applying to spectrophotometric determination of carbaryl residues in waters and vegetables. The proposed method shows good analytical features with low detection limit (50 μg L^{-1}) as well as linearity covered a wide range up to 7.0 mg L^{-1}, good precision with the RSD of 2.3% , and high recoveries in the samples (> 85%).

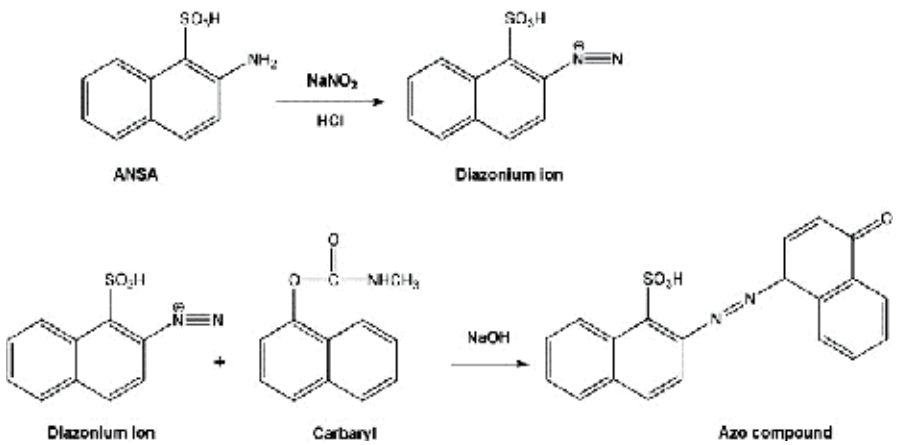


Fig. 2. Proposed reaction mechanism of carbaryl with 2-naphthylamine-1-sulfonic acid by means of diazotization reaction (Santalad et al. , 2008).

Stangl & Niessner (1994) presented a CPE-spectrofluorimetric method for determination of the herbicide napropamide and thiabendazole in water and soil samples. The analytes could be quantitatively extracted to the phase rich in the surfactant Genopol X-080 and be concentrated, then determined by spectrofluorimetry. The detection of thiabendazole and napropamide were performed by excitation at 297 nm and 290 nm, respectively. The use of Genapol X-080 and Genapol 150 combined with fluorimetric detection has been applied (Stangl et al. 1995). The results obtained in this study indicate that the use of Genapol X-080 provides better results than Genapol 150 for the extraction-preconcentration of the herbicide napropamide using CPE methodology. Genapol X-080 should be prefered due to a shorter extraction time. One advantageous feature of micellar systems (CPE) is the enhanced fluorescence sensitivity due to diminished quenching.

3.2 High-performance liquid chromatography

The CPE technique has been successfully exploited for the extraction/preconcentration of pesticides as a sample pretreatment step using a variety of non-ionic surfactants, such as TX 114 prior to their determination by HPLC (Carabias-Martínez et al., 1996; Zhou et al., 2009 a, b), TX-100 (Zhang et al., 2009, 2011; Chen et al., 2009), polyoxyethylene 10 lauryl ether (POLE) (Sanz et al., 2004), oligoethylene glycol monoalkyl ether (Genapol X-080) (Sanz et al., 2004), polyethylene glycol 600 monooleate (PEG600MO) (Tang et al., 2010). A general problem encountered by both zwitterionic and non-ionic surfactants in CPE is that the surfactant-rich phase is too viscous for convenient sampling by a HPLC micro-syringe. Thus, in some applications, a relatively small volume of an appropriate solvent (diluent) has been added to dilute the surfactant-rich phase (Saitoh & Hinze 1991). When cloud point extraction prior to HPLC analysis is used, two important disadvantages arise: a high background absorbance at UV detection and the lengthy operating time required for total elution of the surfactant injected. These two drawbacks clearly affect the use of this methodology in chromatographic determinations with optical detection (Pinto et al., 1992). The surfactant-rich phase obtained in the extraction process is compatible with the hydro-organic phase which is usually employed in HPLC. However, one of the greatest limitations to this methodology is the high absorbance shown by many surfactants in the UV region; in most cases, this prevents their use in a step prior to chromatographic separation when a system of spectrophotometric detection is to be used later unless the mobile phase used contains a high methanol content, in which case elution of the surfactant occurs in a short period of time and does not hinder detection of the analyte (Pinto et. al., 1995). Several ways to overcome this problem have been proposed: Saitoh & Hinze (1991) used the zwitterionic surfactants 3-(nonyldimethylammonium) propyl sulfate (C_9APSO_4) and 3-(decyldimethylammonium) propyl sulfate ($C_{10}APSO_4$) which do not absorb at the customary working wavelengths in HPLC, Pinto et al., (1992) and Moreno-Cordero et al. (1993) used electrochemical detection owing to the electrodic inactivity of commercially available surfactants such as TX-114, and Ferrer et al. (1996) used a clean-up step with a silica gel column to remove the surfactant before sample injection. When injecting the surfactant-rich phase (neat or diluted) into a HPLC system, it is often necessary to wash the nonionic surfactant from the analytical column with a strong hydroorganic mobile phase between the chromatographic runs (Quina & Hinze, 1999).

A soil washing/CPE technique has been used for the decontamination of soil polluted with DDT. Evdokimov & von Wandruszka (1998) proposed a mixture of two surfactants–Igepal

CO-630 (ICO-630) and TX-114 –for studying the possible elimination of DDT from polluted soils; the recovery percentage obtained proved to be greater than 83% when polluted soil in question was treated for 2 h with a 3% mixture of the surfactants. Soil samples spiked with DDT were washed with 3% and 5% nonionic surfactant solutions consisting of a mixture of ICO-630 and TX-114. The extraction of DDT from the soil matrix was monitored by HPLC of the washing solution.

Recently the use of microwave-assisted extraction (MAE) technique in the CPE process has been developed. Microwave-assisted extraction (MAE) has became a viable alternative to the conventional techniques. It has been reported that the combination of MA with micellar media as extractants (microwave-assisted cloud point extraction) (MA-CPE) allows the extraction of different organic compounds from solid samples (Ferrera et al., 2004). In conventional extraction techniques, a higher volume of solvent will generally increase the recovery, but, in MA-CPE, a higher surfactant volume does not influence the extraction efficiency. MA-CPE in combination with HPLC for the determination of organochlorine pesticides (OCPs) has also been reported (Moreno et al., 2006). OCPs such as DDT, dieldrin and aldrin have been determined in agricultural soils by MA-CPE with two non-ionic surfactant mixtures (POLE/polyoxyethylene 10 cetyl ether and POLE/polyoxyethylene 10 stearyl ether) prior to their separation by HPLC with UV detection. An experimental design was applied for the determination of variables which affect to recovery and to optimize the extraction parameters, surfactant concentration and volume, microwave time and power. The optimized method was used to determine the extraction of the pesticides from five different types of agricultural soils spiked with the mixture of OCPs. The recoveries largely depend on the type of surfactant mixture used and soil characteristics. The soils with high organic matter have good recoveries because the surfactant can also extract humic substances which are linked to the pesticides. But these recoveries decreases when the temperature is too high. On the other hand, the recoveries decrease with the aging time for all compounds which could be explained for the sorption process. The former phenomenon occurs at the early stages of sorption, where H-bonding and Van der Waals forces prevail. Only in the case of dieldrin using Stearyl mixture, the recovery remains practically constant with the time.

Carbamate pesticides are polar compounds and can be extracted with the CPE method. However, they cannot be directly determined with the CPE-HPLC-UV method due to the intense absorption of surfactant in the UV region. This problem can be possibly solved by using surfactants that do not absorb at the working wavelengths used in chromatography (Saítoh & Hinze, 1991) or employing cleanup procedures (Carabías-Martínez et al., 2000). However, these methods are somewhat inconvenient. Zhou et al. (2009b) proposed another simple way to overcome this drawback. This method is to make the working wavelength red shift, which is based on the formation of colored products derived from the pesticides. The method is applied to determine the four pesticides (Arprocarb (AC), carbofuran (CF), isoprocarb (IC), and fenobucarb (FC)) in corn samples. First the pesticides are hydrolyzed into different phenols in alkaline solution. The resultant hydrolysis products (*i.e.*,phenols) are reacted with 4-aminoantipyrene (AP) to form intensely red colored compounds in the presence of an alkaline oxidizing agent. The colored compounds are enriched and separated by CPE method, and the coacervate phase containing the compounds is determined with a HPLC system in the visible region. The CPE-HPLC-Vis method has been shown to be very attractive for the detection of carbamate pesticides. Compared with the absorbance maximum of the four carbamate pesticides (λ=230 nm), the wavelength positions of

derivants are in the visible region (λ=510 nm). In this case, the background absorbance of TX-100 does not overlap with the peak of targets. Therefore, the surfactant-rich phase was directly analyzed with the HPLC system in the visible region. The derivatization method could also increase the detectability of carbamate detection in HPLC analysis.

Prometryne [2,4-bis(isopropylamino)-6-(methylthio)-s-triazine], a selective herbicide of the s-triazine chemical family, has been extensively used as a pre- or post-emergence controller of annual grasses and broadleaf weeds in modern agriculture. Prometryne is a ubiquitous environmental pollutant in water and soil. It is frequently detected in groundwater, surface water, and even breast milk (Albanis et al., 1994; Papadopoulou-Mourkidou et al., 2004). Based on one classification scheme (Swann et al., 1983), the soil organic-carbon adsorption coefficient (K_{oc}) value is within 311–614, indicating that Prometryne is expected to have moderate to low mobility in soil and may be adsorbed to solids and sediment suspended in water. K_{oc} values are useful in predicting the mobility of organic soil contaminants; higher K_{oc} values correlate to less mobile organic chemicals while lower K_{oc} values correlate to more mobile organic chemicals. The K_{oc} value is relatively constant for a particular compoud among soil samples from different origins. Zhou et al. (2009a) reported the quantification of Prometryne in water and soil samples by CPE using TX-114 as the surfactant coupled with HPLC–UV detection. In this method, nonionic surfactant TX-114 was first used to extract and pre-concentrate Prometryne from water and soil samples. The separation and determination of Prometryne were then carried out in an HPLC–UV system with isocratic elution using a detector set at λ=254 nm wavelength. Under optimize conditions, the recovery rates of prometryne ranged from 92.84% to 99.23% in water and 85.48% to 93.67% in soil, respectively.

Tang et al. (2010) developed a CPE method for the determination of trace levels of triazole fungicides (tricyclazole, triadimefon, tebuconazole and diniconazole) in environmental waters. The triazole fungicides were extracted and preconcentrated using polyethylene glycol 600 monooleate (PEG600MO) as a low toxic and environmentally benign nonionic surfactant, and determined by HPLC–UV detection. The triazole fungicides were well separated on a reversed-phase kromasil ODS C_{18} column with gradient elution at ambient temperature and detected at 225 nm. Since the surfactant-rich phase was compatible with the mobile phase, no additional washing step was required to remove the surfactant from the kromasil ODS C_{18} column. This study demonstrates that the nonionic surfactant PEG600MO is very effective for the extraction and separation of the triazole fungicides from environmental waters.

Organophosphorus *pesticides* (OPPs) are widely found in water resources. They are released into the environment from manufacturing, transportation and agriculture applications. OPPs, such as methyl and ethyl parathion, paraoxon and fenitrothion have been determined in river water samples by using CPE with the TX-114 prior to their separation by liquid chromatography; electrochemical detection permits suitable detection and quantification of these pesticides (Moreno-Cordero et al., 1998). Upon preconcentrating 15.0 ml of water with a 1% concentration of TX-114, the detection limit is 0.5 ng mL^{-1}. This sensitivity can be increased by preconcentrating 200 mL with 0.25% TX-114; under these conditions, the detection limits range between 0.03 for fenitrothion and 0.08 ng mL^{-1} in the case of paraoxon. The method could also be used in the determination of these analytes in drinking water, in which the maximum concentration permitted by the EU is 0.1 ppb / individual substance.

Sanz and his coworkers (2004) developed to extract, preconcentrate and determine a mixture of eight organophosphorus pesticides (OPPs) (Chlorpyrifos, Diazinon, Dimethoate, Ethoprophos, Malathion, Methidathion, Parathion methyl and Paration ethyl) by using the POLE and Genapol X-080 as extractants with LC-UV detection. The results obtained in this study indicate that the use of Genapol X-080 provides better results than POLE for the extraction of OPPs using CPE. One problem with the UV detection is that pesticides absorb appreciably at wavelengths below 250 nm (DiCorcia & Marchetti, 1991; Ellington et al.,2001), the same spectral region where many reactives and matrix-derived interferences absorb. For this reason, LC–UV analysis is generally more applicable in high-concentration formulations (Cho et al., 1997) or very clean environmental substrates. The extract is compatible with the mobile phase used in LC and provides overall satisfactory results for non-polar pesticides. It is faster than solid phase microextraction and since it is not necessary to evaporate the solvents, no analyte is lost as a result of the process. Recoveries between 70 and 100% were obtained for the majority of cases. Only Dimethoate and Ethoprophos were extracted with recoveries <50%.

Benomyl (BN), carbendazim (MBC), thiabendazole (TBZ) and fuberidazole (FB) are benzimidazole fungicides (BIFs). Such BIFs and their derivatives are used to protect several crops, both during field and post-harvest treatments. The majority of such substances are either applied directly to the soil, or they are sprayed the over crop fields and hence released to the environment. In this manner the fungicides can get into natural water as contaminants either directly or through agricultural land drainage. In order to estimate the environmental impact, the original substances and their metabolites need to be measured at very low concentration levels. An analytical method has been developed to determine the Benzimidazole fungicides and their residues (BN, MCB, TBZ and FB) in real water samples. The CPE methodology for these fungicides using non-ionic surfactant Genapol X-080 and POLE was used in combination with their analysis by RP-HPLC with direct fluorescence detection. The recoveries of fungicides obtained in spiked water samples ranged from 68% to 94% for Genapol and from 68% to 96% for POLE (Halko et al., 2004). CPE using non-ionic surfactant such as POLE and Genapol X-080 provides good extraction efficiency of the studied fungicides, as compared to the conventional extraction method, such as solid-phase extraction (SPE).

CPE of herbicides, including chlortoluron, metoxuron, chloridazon, simazine, propazine and atrazine, using Genapol-X-080 was previously employed as a preconcentration step prior to HPLC using micellar liquid chromatography and an aqueous mobile phase containing that same surfactant (Halko & Hutta, 2002). In that work, no detection limits were reported, but the enrichment factors achieved ranged from 8 to 65 depending upon the specific herbicide.

Zhu et al. (2007) described a CPE methodology for the indirect determination of trichlorfon residue. Trichlorfon is one of the most widely used insecticides in rice, cotton, fruit tree, vegetables and tea tree. When trichlorfon was extracted by CPE method, its recovery was poor due to the fact that it is highly soluble in water. On the other hand, the surfactant TX-100 absorbs in UV region and would interfere with the determination of trichlorfon by HPLC-UV method. Therefore, trichlorfon was directly extracted using the aqueous, followed by addition of benzidine and sodium perborate, 4-amino-4′-nitrobiphenyl was formed based on its catalytic effect. Then 4-amino-4′-nitrobiphenyl was separated and preconcentrated by CPE method, and then detected using a HPLC with UV detection. In

addition, for the maximum absorption wavelengh of 4-amino-4'-nitrobiphenyl is at 365 nm, Triton X-100 could not interfere with its determination.

Simultaneous determination by HPLC with electrochemical detection of Captan, Folpet and Captafol in river water samples has been described by Carabias-Martínez et al. (1996). To concentrate the fungicide residues, a CPE step employing the Triton X-114 was applied. Electrochemical detection with single and dual glassy-carbon electrodes was evaluated for possible amperometric detection of these fungicides; the reductive-oxidative detection mode with a dual electrode in the series configuration proved to be more appropriate than direct reductive detection with a single working electrode. Chromatographic elution of these fungicides requires a mobile phase with a relatively low organic solvent content (45%, v/v, acetonitrile–water). Under experimental conditions, not all the TX-114 is eluted from the chromatographic column: to remove the surfactant remaining in the stationary phase, a washing cycle with 100% acetonitrile 10 min was performed. In addition to the extraction preconcentration of the fungicides, the presence of TX-114 stabilises the fungicides and prevents their hydrolysis in aqueous medium. The addition of surfactant at the time of sample collection is a simple way to avoid losses of fungicide during the period of sample storage. Also in this case, electrochemical detection permits the simultaneous quantification of all three fungicides since spectrophotometric detection only allows the quantification of the fungicide Folpet.

Another method proposed by Pinto et al. (1995) was depends on a dual electrochemical (reductive-oxidative) detection. The presence of nitro and azo groups in the structure of organophosphorus compounds would allow their determination by reductive electrochemical detection as long as the dissolved oxygen is completely eliminated in order to avoid high residual current. This drawback can be readily overcome by oxidative electrochemical detection after transformation, by reduction of the analytes, in derivatives susceptible to later oxidation. Dual electrochemical detection (reductive-oxidative mode) was used for the liquid chromatographic analysis of OPPs (paraoxon (diethyl 4-nitrophenyl phosphate), methyl parathion *(o, o*-diethyl-*o*-(4-nitrophenyl) phosphorothioate), fenitrothion [*o,o*-dimethyl-*o-(*3-methyl-4-nitrophenyl)], and ethylparathion (*o,o*-diethyl-*o*-p-nitrophenylthiophosphate) after CPE with the TX-114 (Pinto et al., 1995). Because the surfactant does not have electroactive groups in its structure, these electrochemical signals could be due to impurities in the TX-114 itself, arising in its synthesis; these can be detected directly or after reduction on the working electrodes.

Since many pesticides are colourless, a technique for yielding of coloured derivative of pesticide has been applied in the determination of pesticides in visible region. This technique is based on the derivative reaction of pesticide where the analytes are detected in visible region which is transparent to surfactants (Chen et al., 2008). Carbofuran can be hydrolysed to from 2,3-dihydro-2,2-dimethyl-7-benzofuranol (BF). BF is coupled with 4-aminoantipyrene (AP) in presence of potassium ferricyanide [$K_3Fe(CN)_6$] to generate red coloured derivative (BFAP) having λ=530 nm. The BF molecule has one free phenolic hydroxyl group and no substitute in the *para* position pare to the hydroxyl group. CPE methodology and using TX-100 as extractant was applied as a preconcentration step prior to HPLC, the surfactant-rich phase containing BFAP was then analysed by HPLC in visible region. The coloured analytes are detected in visible region, in which the high back ground absorption of surfactant may not interfere with the determination of the analytes. On the other hand, when the determination is carried out by HPLC–UV system, the response of the

coloured derivate is higher than that of original compound. This method can be used to determine other pesticides which could be hydrolysed into the phenolic compounds. Figure 3. shows Carbofuran hydrolysis and derivatization reaction.

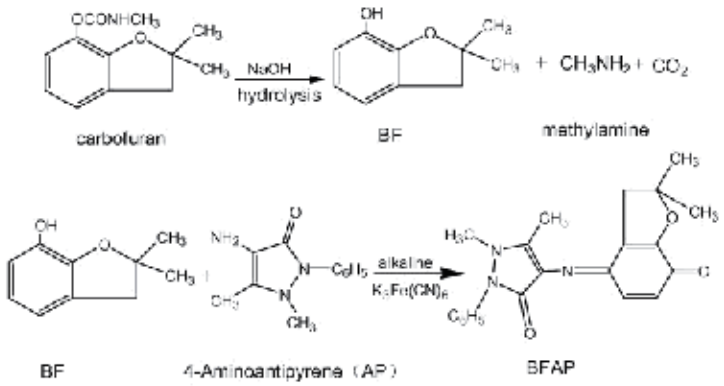


Fig. 3. Reaction mechanism of carbofuran with derivatising reagents (Chen et al., 2008).

Sulfonylurea herbicides are widely used for controlling weeds or grasses in farmland. Three sulfonylurea herbicides (metsulfuron-methyl (MSM), chlorsulfuron (CS), and bensulfuron-methyl (BSM)) in water, soil, and rice grain have been analyzed simultaneously by Wu et al. (2010). TX-114 and PEG-6000 were used for CPE separation of sulfonylurea herbicides in the samples. Impurities in the extracts of soil and rice grains did not interfere with the quantitative determination of MSM, CS, and BSM because the peaks were shown to be located at different places. Optimal extraction recovery for the three herbicides was observed at 12% sodium sulfate, with 92.3%, 93.6%, and 94.5% recoveries were obtained for MSM, CS, and BSM, respectively.

Polychlorinated dibenzofurans (PCDF) are organic compounds with very toxic effects for humans and the environment. Fernández and his coworkers have applied CPE to analyze six polychlorinated dibenzofurans (PCDF). In this work, the methodology of cloud-point extraction, using two non-ionic surfactants oligoethylene glycol monoalkyl ether (Genapol X-080) and polyoxyethylene-10-cetyl ether (Brij 56), is applied to the extraction and preconcentration of PCDF in sea water samples prior to their determination by HPLC with fluorescence detection. The surfactant-rich phase was analysed on a 4 μm Nova-Pak C18 column (15 cm × 3.9 mm i.d.), with aqueous 85% methanol as the mobile phase and the recoveries are 68-105%.

Micelle-mediated extraction with octyl-b-D-thioglucoside (OTG) has been raported by Saitoh et al. (2000). Many hydrophobic compounds are efficiently incorporated into the surfactant-rich phase separated from the aqueous surfactant solution with elimination of hydrophilic matrix components to the bulk aqueous phase. Because of the extremely small volume fraction of the surfactant-rich phase, the analytes can be highly concentrated, thus allowing great enhancement in the sensitivity of chromatographic analysis. However, the appearance of a large number of peaks because of the ultra-violet (UV) absorption of TX-114 or PONPE-7.5, which are mostly used for CPE, limits the subsequent detection method. The use of alkylglucoside surfactants instead of polyoxyethylene-type surfactants may solve these problems. An aqueous solution of octyl-b-D-thioglucoside (OTG) can be separated into

bulk aqueous and surfactant-rich phases by adding an appropriate water-soluble polymer such as polyethylene glycol (PEG) or dextran derivatives. Since OTG has little UV absorption around 254 nm, presence of the concentrated surfactant would not hinder UV detection of analytes. In an extended application, Saitoh et al. (2000) evaluated the possibility of isolating a wide range of organic analytes, including PAHs, alkylbenzenes, alkylphenols, chlorobenzenes, chlorophenols, phthalic esters, pesticides and steroid hormones with octyl-b-D-thioglucoside (OTG) followed by LC–UV detection. Micelle-mediated extraction with octyl-b-D-thioglucoside (OTG) is a viable and attractive method for extracting various organic compounds in aqueous solution prior to HPLC analysis. The surfactant-rich phase containing concentrated OTG could be directly introduced into the hygro-organic mobile phase of HPLC with UV detection. The application of this method greatly enhanced the signal intensity in the chromatogram while reducing the interference of matrix components.

Anionic surfactant micelle-mediated extraction (coacervation extraction) has been evaluated for isolation of Etofenprox before HPLC (Jia et al., 2006). The anionic surfactant sodium dodecylsulfonate (SDoS) has been used for extraction of Etofenprox from aqueous environmental samples and from biological samples by means of coacervation extraction. Genarally, nonionic and zwitterionic surfactants can be used for CPE. The cloud point refers to the phase separation of neutral surfactants induced by temperature. Cationic and anionic surfactants can be used for coacervation extraction. The term "coacervation" is reserved for the phase separation of ionic amphiphiles induced by other conditions. Cationic surfactants (alkyltrimethylammonium bromides) are known to undergo coacervation in the presence of saturated NaCl and 1-octanol. Anionic surfactants such as alkyl sulfates, sulfonates, and sulfosuccinates undergo pH-induced coacervation. For extractions with cationic surfactants the main problem arises from the sharp dependence of the volume of the surfactant-rich phase obtained on the volume of the cosurfactant added, which can result in poor reproducibility (Jia et al., 2006). The recoveries obtained from five real samples ranged from 94.3 to 100.1%.

Ding et al. (2009) analyzed the organophosphate pesticides in aqueous solution. A CPE utilizing polyoxyethylene 10 laurylether ($C_{12}E_{10}$) has been developed to enrich the trace organophosphate pesticide in aqueous solution, including parathion-methyl and phoxime for rapid determination of pesticide residues. As the result reveals, CPE is a good technology with a high enrichment times on parathion-methyl and phoxime, which reaches 95 and 97 at most, respectively, and the CP-extraction yield can exceed 90%, when using 5 g L^{-1} $C_{12}E_{10}$ and 120 g L^{-1} Na_2SO_4 at 35°C. Combining CPE with HPLC, the method detect limit (MDL) of parathion-methyl and phoxime can reach 1 μg L^{-1}.

Six herbicides in milk samples have been analyzed simultaneously (Wang et al., 2007). The feasibility of employing CPE as extraction and preconcentration method for the recovery of herbicides from milk samples followed by HPLC analysis has been demonstrated. An aqueous surfactant solution containing 60 g L^{-1} Tween 20 or Triton X-100 was heated with an appropriate concentration of $(NH_4)_2SO_4$ or NaCl for the extraction of herbicides. The extract was analyzed by HPLC subsequently. The results showed that the linear dynamic ranges of detection were 20-10000 μg L^{-1} for tralkoxydim, metribuzin and bromoxynil, 30-10000 μg L^{-1} for mefenacet, and 50-10000 μg L^{-1} for bensulfuron-methyl and nicosulfuron. The correlation coefficients were 0.9981-0.9997. The average recoveries of the six herbicides ranged from 85.09% to 96.74% .The relative standard deviations for the six herbicides were in the range of 1.90% -3.98%. The limits of detection for the six pesticides were lower than the maximum residue limits (MRL) of China.

Pesticide	Method/ Surfactant/Matrix	Linear range	LOD	Reference
Carbaryl	Uv-vis/TX114/ water	10–500 µg L^{-1}	7.0 µg L^{-1}	(Melchert & Rocha, 2009)
Napropamide Thiabendazole	FD*/Genapol X 080/Water & Soil	NR	0.2 µg L^{-1} 0.2 µg L^{-1}	(Stangl & Niessner, 1994)
Carbaryl	Uv-vis/TX114/ Water & vegetable	0.1–7.0 mg L^{-1}	50 µg L^{-1}	(Santalad et al. 2008)
Napropamide	FD*/Genapol X-080 &X-150/ultrapure water and natural samples.	NR	0.2 ng L^{-1}	(Stangl & Niessner, 1995)
DDT	HPLC/Igepal-ICO-630/ TX-114/Soil	NR	NR	(Evdokimov & von Wandruszka, 1998)
Folfet Captan Captafol	HPLC-ED/ TX-114/River water	NR	4.0 µg L^{-1} 4.0 µg L^{-1} 6.0 µg L^{-1}	(Carabias - Martínez et al., 1996)
4,4′- DDD Dieldrin 4,4′- DDT 2,4′-DDT 4,4′- DDE Aldrin	HPLC-UV/ Cetyl mixture/ Soil sample & aged soils.	80–800 ng g^{-1}	108.8 ng g^{-1} 793.2 ng g^{-1} 167.2 ng g^{-1} 86.4 ng g^{-1} 135.6 ng g^{-1} 806.4 ng g^{-1}	(Moreno et al., 2006)
4,4′- DDD Dieldrin 4,4′- DDT 2,4′-DDT 4,4′- DDE Aldrin	HPLC-UV/ Stearyl mixture/ Soil sample & aged soils.	80–800 ng g^{-1}	269.6 ng g^{-1} 734.0 ng g^{-1} 171.6 ng g^{-1} 285.2 ng g^{-1} 150.8 ng g^{-1} 593.2 ng g^{-1}	(Moreno et al., 2006)
Metsulfuron Chlorsulfuron Bensulfuron	HPLC-UV TX-114/ Water , soil& rice grains	0.004–2.0 mg L^{-1} 0.004–2.0 mg L^{-1} 0.004–2.0 mg L^{-1}	0.8-4.0 µg kg^{-1} 1.2-6.0 µg kg^{-1} 0.8-4.0 µg kg^{-1}	(Wu et al., 2010)
Arprocarb Carbofuran Isoprocarb Fenobucarb	HPLC-UV/ TX-100/ Corn	8×10^{-4}–0.5 mg L^{-1} 8×10^{-4}–0.5 mg L^{-1} 8×10^{-4}–0.5 mg L^{-1} 2×10^{-3}–0.5 mg L^{-1}	2×10^{-4} mg L^{-1} 2×10^{-4} mg L^{-1} 2×10^{-4} mg L^{-1} 5×10^{-4} mg L^{-1}	(Zhou et al., 2009a)
Prometryne	HPLC-UV/ TX-114/ Water&Soil	0.016–10 µg mL^{-1}	3.5 µg L^{-1} 4.0 µg L^{-1}	(Zhou et al., 2009b)

Tricyclazole, Triadimefon, Tebuconazole Diniconazole	PEG600MO/ Tap& River water	0.05–20 µg L^{-1} 0.05–20 µg L^{-1} 0.05–20 µg L^{-1} 0.05–20 µg L^{-1}	6.8 ng L^{-1} 23.2 ng L^{-1} 34.5 ng L^{-1} 21.6 ng L^{-1}	(Tang et al., 2010)
Chlorpyrifos Diazinon Dimethoate, Ethoprophos Malathion, Methidathion Parathion Paration ethyl	LC-UV/ POLE/ Aqueous samples	50–3000 ng mL^{-1} 50–3000 ng mL^{-1} 50–3000 ng mL^{-1} 300–3000 ng mL^{-1} 500–3000 ng mL^{-1} 50–3000 ng mL^{-1} 50–3000 ng mL^{-1} 100–3000 ng mL^{-1}	1.86 ng mL^{-1} 1.65 ng mL^{-1} 1.86 ng mL^{-1} 28.45 ng mL^{-1} 0.88 ng mL^{-1} 2.03 ng mL^{-1} 2.96 ng mL^{-1} 3.54 ng mL^{-1}	(Sanz et al., 2004)
Chlorpyrifos Diazinon Dimethoate, Ethoprophos, Malathion, Methidathion Parathion Paration ethyl	LC-UV/ Genapol X080/ Aqueous samples	25–2500 ng mL^{-1} 25–2500 ng mL^{-1} 25–2500 ng mL^{-1} 100–2500 ng mL^{-1} 250–2500 ng mL^{-1} 25–2500 ng mL^{-1} 25–2500 ng mL^{-1} 50–2500 ng mL^{-1}	0.6 ng mL^{-1} 0.8 ng mL^{-1} 2.1 ng mL^{-1} 0.7 ng mL^{-1} 1.4 ng mL^{-1} 2.6 ng mL^{-1} 1.0 ng mL^{-1} 2.2 ng mL^{-1}	(Sanz et al., 2004)
Polychlorinated dibenzofurans (PCDF)	HPLC-FD*/ Genepol X080 & Brij 56/Seawater	0.17-27.2 µg mL^{-1}	0.5–27.5 ng L^{-1}	(Fernández et al., 1999)
Benomyl Carbendazim Thiabendazole Fuberidazole	HPLC-FD*/ POLE/ Water	10–200 µg L^{-1} 10–200 µg L^{-1} 1.0–100 µg L^{-1} 0.01–0.5 µg L^{-1}	7.1 µg L^{-1} 9.2 µg L^{-1} 4.3 µg L^{-1} 4.5 µg L^{-1}	(Halko et al., 2004)
Benomyl Carbendazim Thiabendazole Fuberidazole	HPLC-FD*/ Genopol/ Water	10–200 µg L^{-1} 10–200 µg L^{-1} 1-100 µg L^{-1} 0.01-0.5 µg L^{-1}	5.8 µg L^{-1} 6.4 µg L^{-1} 0.13 µg L^{-1} 0.08 µg L^{-1}	(Halko et al., 2004)
Paraoxon Methylparathion Fenitrothion Ethylparathion	HPLC-DED TX-114/ Water	0.99–60 ppb 0.97–58 ppb 0.80–47 ppb 0.96–58 ppb	0.35 ppb 0.21 ppb 0.18 ppb 0.33 ppb	(Pinto et al., 1995)
p, *p*′-DDD *p*, *p*′-DDE	HPLC/OGT/ Water	NR	NR	(Saitoh et al., 2000)
Tralkoxydim, Metribuzin Bromoxynil, Mefenacet Bensulfuron-methylsulfuron Nicosulfuron	HPLC/Tween 20 or TX 100/ Milk	20-10000 µg L^{-1} 20-10000 µg L^{-1} 20-10000 µg L^{-1} 30 -10000 µg L^{-1} 50 -10000 µg L^{-1} 50 -10000 µg L^{-1} 50 -10000 µg L^{-1}	NR NR NR NR NR NR NR	(Wang et al., 2007)
Parathion-methyl Phoxime	10-laurylether /aqueous solution	NR	1.0 µg L^{-1} 1.0 µg L^{-1}	(Ding et al., 2009)

Trichlorfon	HPLC/TX-100/ Cabbage	0.01–0.2 mg L^{-1}	2.0 µg L^{-1}	(Zhu et al., 2007)
Etofenprox	HPLC/ SDoS/ Water, Urine & Beer	0.04–2.0 mg L^{-1}	0.004 mg L^{-1}	(Jia et al., 2006)
Phorate, Diazinon, Parathion Fenthion Quinalphos	GC/TX-114/ Human urine	0.10–20 ng mL^{-1} 0.10–20 ng mL^{-1} 0.10–20 ng mL^{-1} 0.10–20 ng mL^{-1} 0.10–20 ng mL^{-1}	0.07 ng mL^{-1} 0.04 ng mL^{-1} 0.08 ng mL^{-1} 0.07 ng mL^{-1} 0.07 ng mL^{-1}	(Jia et al., 2008)
Disulfoton	GC/ TX-114/ Surface water	3.9-150 µg L^{-1}	1.2 µg L^{-1}	Faria et al. 2007
Fenitrothion Chlorpirifos Parathion Methidathion	GC/TX-114/ Honey	1.0–1000 ng g^{-1} 0.3–1000 ng g^{-1} 1.0–1000 ng g^{-1} 1.0–1000 ng g^{-1}	0.06 ng g^{-1} 0.03 ng g^{-1} 0.09 ng g^{-1} 0.47 ng g^{-1}	(Fontana et al., 2010)
Dichlorvos Methamidophos Acephate Diazinon Dimethoate, Chlorpyrifos Parathion-methyl Malathion Parathion-ethyl	GC/PEG 6000/ Fruit juice	5.0–200 µg kg^{-1} 5.5–200 µg kg^{-1} 8.0–200 µg kg^{-1} 4.0–200 µg kg^{-1} 5.5–200 µg kg^{-1} 4.0–200 µg kg^{-1} 4.0–200 µg kg^{-1} 4.0–200 µg kg^{-1} 5.0–200 µg kg^{-1}	1.5 µg kg^{-1} 2.0 µg kg^{-1} 3.0 µg kg^{-1} 0.5 µg kg^{-1} 2.0 µg kg^{-1} 1.0 µg kg^{-1} 1.0 µg kg^{-1} 1.0 µg kg^{-1} 1.5 µg kg^{-1}	(Zhau et al., 2011)
Cyclopentadiene Aimazine Atrazine Alachlor Metolachlor Butachlor	GC/TX-114/ Water	5–4000 µg L^{-1} 1–4000 µg L^{-1} 0.5–4000 µg L^{-1} 0.5–4000 µg L^{-1} 0.1–4000 µg L^{-1} 0.5–4000 µg L^{-1}	481.5 ng L^{-1} 97.1 ng L^{-1} 19.6 ng L^{-1} 31.2 ng L^{-1} 6.59 ng L^{-1} 33.9 ng L^{-1}	(Takagai & Hinze, 2009)
Organochlorine Pyrethroid	GC/TX100/ Viscum coloratum	5-500 µg L^{-1} 10-1000 µg L^{-1}	1.5-7.5 µg kg^{-1} 1.5-7.5 µg kg^{-1}	(Zhang et al., 2009)
Pericyazine Chlorpromazine Fluphenazine	GC/TX-114/ Human serum	12.3–82.1 nmol 9.0–90.3 nmol 14.9–37.3 nmol	3 nmol 3 nmol 3 nmol	(Ohashi et al., 2004)
Ametryne Terbutryne Prometryne Simazine Atrazine Propazine	CE/TX-114/ Drinking & River water	25–500 µg L^{-1} 26–510 µg L^{-1} 26–520 µg L^{-1} 109–2180 µg L^{-1} 66–1310 µg L^{-1} 51–1020 µg L^{-1}	NR NR NR NR NR NR	(Carabias - Martínez et al., 1999)

*FD: fluorescence detection and UV: ultraviolet detection.

Table 3. Cloud point extraction (CPE) applications for the determination of pesticide analytes.

3.3 Gas chromatography

The application of CPE as a preconcentration step prior to gas chromatography (GC) or GC-mass spectrometry (GC/MS) has found very little application due to the viscous nature of the surfactant, which endangered blocking of the capillary column. The primary reason for this stems from the fact that direct introduction of the surfactant-rich extractant phase into a GC system causes difficulties (Carabias-Martinez et al., 2000; Fröschl et al., 1997; Faria et al., 2007; Zygoura et al., 2005; Giokas et al., 2005; Sikalos & Paleologos, 2005; Paleologos et al., 2006; Jia et al., 2008; Ohashi et al., 2004; Shen & Shao, 2006). Namely, the surfactant can (i) adsorb onto (coat) the stationary phase and alter its polarity which causes changing non-reproducible analyte retention times with subsequent injections and/or (ii) it self elute as a series of peaks over a period of time from the column such that it overlaps with and obscures the analyte peak(s) of interest. In addition, it was thought that the introduction of the surfactant-rich extractant phase could "clog or block" the GC column. Several approaches have been employed to circumvent such difficulties. The first involves the use of mini-column liquid chromatography to separate and recover the target analyte(s) from the surfactant-rich extractant phase. Cation exchangers or silica gel and Florisil stationary phases have been employed in conjunction with aqueous methanolic, methanol-hexane or hexane mobile phases for this purpose (Fröschl et al., 1997; Faria et al., 2007). Sikalos and co-worker (2005) created a breakthrough on this problem. They applied microwaves or sonication to back-extract analytes from the surfactant-rich phase into a water immiscible solvent prior to GC-flame ionization detection (FID) without any supplemental cleanup. Analogous back-extraction preceded GC analysis of UV-filters after CPE recently reported by Giokas et al. (2005) and of diethylhexyladipate and acetyltributylcitrate by Zygoura and coworkers (2005). After the components of a mixture are separated using gas chromatography, they must be detected as they exit the GC column. There exist a number of detectors, which can be used in GC. Different detectors give different types of selectivity. When organic molecules that contain electronegative functional groups, such as halogens, phosphorus, and nitro groups pass by the detector, they capture some of the electrons and reduce the current measured between the electrodes. Fröschl et al. (1997) reported the use of TX-100 in the preconcentration of polychlorinated biphenyls (PCBs) from water and extensive clean-up with two columns (Silica gel and Florisil) prior to GC analysis with electron capture detector (ECD). After the preconcentration of PCBs from water, the surfactant-rich phase passes through a silica gel column and is eluted with n-hexane. Then a small volume of eluate is collected. The rest of TX-100 in the eluate is removed by a second column filled with Florisil column. After the two clean-up procedures, the surfactant is eliminated completely and the final eluate is injected into GC-ECD for further analysis. The recoveries of PCBs obtained by CPE were compared with those obtained by liquid-liquid extraction. Both methods are comparable with recoveries ranging 86–116% for spiked ultra-pure and tap water samples. The micellar extraction for PCBs is superior to the liquid-liquid extraction (LLE) for land fill seepage water.

Cloud point extraction coupled with microwave-assisted backextraction has been combined with GC-FPD successfully. The preconcentration of organophosphorous pesticides (OPPs) (phorate, diazinon, parathion-methyl, fenthion and quinalphos) from human urine samples by CPE coupled with microwave-assisted back-extraction prior to gas chromatography with flame photometry detection (GC-FPD) analysis has been developed by Jia et al. (2008). The preconcentrated analytes were back-extracted from the obtained surfactant-rich phase into isooctane by short-term microwave application. The isooctane solution obtained from back-

extraction was centrifuged for further cleanup and then directly injected into the GC. A preconcentration factor of 50 was obtained for these five OPPs extracted from only 10 mL of a sample. Precision was also good; the relative standard deviations (RSDs) were less than 9%. The method showed to be potential for biological monitoring. Compared with solid phase micro extraction (SPME), the proposed method only need some cheap surfactant and does not require special instrument. The LODs of this method are lower than the others.

Disulfoton [O,O-diethyl-S-[2-(ethylthio)ethyl]-phosphorodithioate] is a systemic insecticide and acaricide, also marketed as Di-Syston. Faria et al. (2007) reported the use of TX-114 in the preconcentration of Disulfoton from surface water and extensive clean-up with two columns (Silica gel and Florisil column) prior to GC analysis with flame ionization detector (FID). A cleanup stage is essential for analysis by GC using the cloud-point methodology. The presence of surfactant molecules can lead to rapid deterioration of the analytical column. After the preconcentration of disulfoton from water, the surfactant-rich phase passes through a silica gel column and is eluted with methanol:hexane (1:1). Then a small volume of eluate is collected. The rest of Triton X-114 in the eluate is removed by a second column filled with Florisil. After the two clean-up procedures, the surfactant is eliminated completely and the final eluate is injected into GC-FID. The recoveries of PCBs obtained by CPE were compared with those obtained by LL extraction. Both methods are comparable with recoveries ranging 86–116% for spiked ultra-pure and tap water samples.

Ohashi et al. (2004) studied three non-ionic surfactants (i.e. Triton X-100, Triton X-114 and PONPE 10) for preconcentration of phenothiazine derivatives before their determination by GC with flame ionization detector (FID). TX-114 provided the most efficient recovery of the phenothiazines tested. It was difficult to determine phenothiazine derivatives in the surfactant-rich phase by GC directly. Therefore, the separation of phenothiazine derivatives from surfactants can be accomplished by passing the surfactant rich phase through a cation exchange column. This surfactant clean-up procedure permits the determination of phenothiazine derivatives extracted in the surfactant-rich phase by GC-FID. The recoveries of pericyazine, chlorpromazine and fluphenazine from spiked serum samples were 95.1%, 87.1% and 84.7%, respectively.

Zhau et al., (2011) described a competitive method of CPE for the rapid and effective extraction and preconcentration of nine OPPs (Dichlorvos, methamidophos, acephate, diazinon, dimethoate, chlorpyrifos, parathion-methyl, malathion, parathion-ethyl) from concentrated fruit juice coupled with ultrasonic-assisted back-extraction prior to GC with flame photometric detection (GC-FPD) analysis. CPE coupled with ultrasonic-assisted back-extraction has been combined with GC-FPD successfully. Under optimum conditions: a solution containing 6% (w/v) polyethylene glycol 6000 (PEG 6000) and 20% (w/v) Na_2SO_4 for the extraction of the OPPs. The coacervation phase obtained was back extracted with ethyl acetate. The upper ethyl acetate solution was centrifugated simply for further cleanup for the sake of automatic injection. A preconcentration factor of 50 was obtained for these 9 pesticides. Using this method, the limits of detection (LOD) and limits of quantification (LOQ) were in the range of 0.5-3.0 and 1.5-9.0 μg kg^{-1} in concentrated fruit juice, respectively; the relative standard deviations (RSD) were less than 9%.

An alternative approach for GC or GC/MS analysis of seven herbisides has been proposed by Takagai & Hinze (2009). In this method, a post-extraction derivatization step is employed in which the surfactant in the surfactant-rich extractant phase is derivatized with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) prior to introduction into the GC. Such derivatization step improved the chromatographic performance yielding a fairly wide

elution time window absent of surfactant peaks, reproducible analyte retention times, and quantitative results. This approach enables the direct use of the surfactant-rich phase following CPE without any subsequent laborious column chromatographic or back-extraction analyte isolation procedures. It should prove to be an attractive alternative approach for the GC or GC/MS analysis of analytes following their preconcentration by CPE in many situations.

In the recent times, OPPs were analyzed by Fontana et al. (2010) using a coacervative microextraction ultrasound-assisted back-extraction technique (CME-UABE) followed by GC–MS. The extraction/preconcentration technique is supported on the micellar organized medium based on non-ionic surfactant. To enable coupling the proposed technique with GC, it was required to back extract the analytes into hexane. CME-UABE use alternative solvents such as surfactants and only require 60 μL of hexane on the overall extraction procedure to achieve a satisfactory performance. The back-extracted analytes were introduced to GC–MS successfully without declining the separation efficiency of the capillary column. The recoveries were ≥90%, indicating satisfactory robustness of the methodology, which could be successfully applied for determination of OPPs in honey samples of different Argentinean regions. Under optimal experimental conditions, the enrichment factor (EF) was 167.

To establish a GC method for simultaneous determination of organochlorine and pyrethroid pesticide residues in Viscum coloratum by CPE has been proposed by Zhang et al. (2009). Pesticides were extracted with the non-ionic surfactant TX-100. The apparatus was gas chromatography with electron capture detector and the separation was performed on an Hp-5 column. The pesticide residues were calculated by external standard method. The average recoveries of organochlorine and pyrethroid were 74.15%-111.6% with corresponding RSD of 4.0%-9.1%.

3.4 Capillary electrophoresis

Capillary electrophoresis (CE), has increasingly gained importance in pesticide analysis (Balinova, 1996) and represents an attractive alternative for their determination. CPE has been applied as a preconcentration step prior to capillary electrophoresis (CE). The use of CPE as sample pretreatment techniques for pesticides prior to CE analysis has not been extensively investigated. The main problem of applying CPE to CE is that the surfactant-rich phase introduced into a bare fused-silica capillary using aqueous buffers would be adsorbed onto the wall of the capillary, leading to a marked loss of efficiency and reproducibility both in migration times and solute peak areas. To solve this problem, Carabias-Martínez et al. (1999b) used non-aqueous media in the separation buffer that can permit the electrophoretic separation of samples with high-surfactant contents, thus avoiding the adsorption of surfactant onto the wall of the capillary. The application of CPE to CE has been described and successfully applied for the determination of triazine herbicides in water samples (Carabias-Martínez et al., 1999; 2003). TX-114 was employed as the extraction solvent. The behaviour of a surfactant-rich micellar phase injected into a capillary electrophoresis system was studied using different separation modes. One problem that appeared on introducing a surfactant-rich phase into a bare fused-silica capillary, using aqueous buffers was that the surfactant was adsorbed onto the wall of the capillary, leading to a marked loss of efficiency and reproducibility. The use of dynamic coatings in the capillary, such as those obtained when the cationic surfactant CTAB is added to the separation buffer, afforded reproducible results,

although half-life of the capillary was short (Xie et al., 2010). The most satisfactory results were obtained when the surfactant-rich samples were suitably diluted and injected, in the electrokinetic mode, into a non-aqueous separation medium of acetonitrile–methanol (50:50).

4. Conclusion

The present review has focused on the recent developments in CPE and its applications in conjunction with different analytical techniques. GC, HPLC, and CP have been used for the determination of different classes of pesticides by means of CPE. CPE was applied for preconcentration of organophosphorus, organochlorine and pyrethroid pesticides (Jia et al. , 2008; García-Pinto et al., 1995; Zhang et al., 2009; Fontana et al., 2010), carbamate pesticides (Zhou et al., 2009), triazole fungicides (Tang et al., 2010), benzimidazole fungicides (Halko et al., 2004), triazine herbicides (Carabias-Martínez et al., 1999), polychlorinated biphenyls (Fröschl et al., 1997). CPE using non-ionic surfactant such as POLE and Genapol X-080 provides good extraction efficiency of the studied fungicides, as compared to the conventional extraction method, such as solid-phase extraction (SPE). GC is very useful for the simultaneous determination of several pesticides at trace levels, and, in general, provides higher sensitivity rather than HPLC. But the use of CPE in combination with GC and also CE has appeared in few publications. The determination of pesticides has been carried out by GC coupled to sensitive and specific detection systems, such as the electron capture detector (ECD), flame photometry detector (FDP), flame ionization detector (FID) and MS detector. The performance of CPE in aqueous samples is excellent; however, it is not yet suitable in complex matrixes such as biological samples. Therefore, it needs further improvement.

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Determination of Pesticides in Complex Samples by One Dimensional (1D-), Two-Dimensional (2D-) and Multidimensional Chromatography

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1. Introduction

An important goal in the field of analytical chemistry is to achieve continous improvement in the analysis of toxic pollutants, e.g., pesticides. Pesticides are widespread throughout the world. The composition of pesticide mixtures occurring in environmental samples depends on geographical area, season of the year, number of farms, and quantity and intensity of use of plant-protection agents. The variety of their mixtures in different matrices, for example, rivers, fruits and vegetables, medicinal plants, is very large. Sometimes, a monodimensional chromatographic separation is not sufficient to resolve all the components of interest of multicomponent mixtures. The problem of peak overlapping may occur, and a pre-separation of the sample is often necessary. This pre-separation aims at reducing the complexity of the original matrix, by isolating simpler fractions from the original mixture. The fractions should contain the same amounts of the analytes as in the whole sample, ready for analysis and free from substances that can interfere during the chromatographic analysis. Multidimensional chromatography brings together separations often based on different selectivity mechanisms.

Multidimensional or comprehensive two-dimensional chromatography (GCxGC) is a relatively new technique that can analyze multicomponent samples on different GC phases in the same analysis. GCxGC has a number of advantages over single column techniques. In this mode, two different chromatographic columns are connected in series through a modulator, which traps the analytes eluting from the primary column and re-injects them in small compressed packets onto the secondary column. Columns coupled in series provide better separation of components of sample through different physical and chemical properties (e.g., boiling point/polarity versus shape selection) in the two steps. The GCxGC provides much better chromatographic resolution and peak capacity than single column system. GCxGC can also be used as screening method for various groups of pesticides.

Multidimensional liquid chromatography also represents a powerful tool and alternative procedure to classical one-dimensional HPLC methods with optimum efficiency and selectivity for the separation of the component of interest, while simultaneously minimizing

the analysis time by decreasing the time spent in separating those components of the sample that are of no analytical interest. Multidimensional LC separation has been defined as a technique which is mainly characterized by two distinct criteria, as follows:

- the first criterion for multidimensional system is that sample components must be displaced by two or more separation techniques involving an orthogonal separation mechanism,
- while the second criterion is that components that are separated by any single separation dimension must not be recombined in any further separation dimension.

Coupled-column liquid chromatography (LC-LC coupling) refers to the conventional two-dimensional mode of chromatography in which fractions from one column are selectivly transferred to a secondary column for further separation. The volume of the eluate transferred from the first column to the second column can correspond to a group of peaks, a single peak or a fraction of a peak, so that different parts of the sample may follow different paths through the LC-LC configuration.

The separation of complex mixtures of pesticides is also possible by combination of different modes of multidimensional planar chromatography. Multidimensional planar chromatography (MDPC) can be performed using different mobile phases in systems with single-layer or bi-layer plates by graft thin-layer chromatography. Graft thin-layer chromatography is a complex system in which chromatographic plates with similar or different stationary phases are used. Compounds from the first chromatographic plate after chromatogram development are transferred to the second plate of different selectivity, without scraping, extraction, or re-spotting the bands by use of a strong mobile phase.

In the second mode of MDPC the separation of complex mixtures of compounds has been realized on a monolayer. In these experiments a solvent system (mobile phase A) was selected for one adsorbent, e.g., silica stationary phase to separate groups of compounds from the investigated complex mixture. Afterwards the mobile phases were optimized (mobile phases B, C, D, E) for the different group of compounds regarding each group as an individual separation problem. By the help of this new procedure numerous compounds from a complex mixture were separated on 10 cm x 10 cm TLC or/and HPTLC plates. In this mode of MDPC the largest differences for separation of complex mixtures can be obtained by combination of normal-phase and reversed-phase systems with the same chromatographic layer, e.g., cyanopropyl one.

In the third mode of MDPC the separations of multicomponent mixtures were realized on multiphase plates. Also in this mode the largest differences were obtained by combination of a normal-phase system of the type silica/nonaqueous eluent in the first step of MDPC and a reversed-phase system of the type octadecyl silica/water + organic modifier (methanol, acetonitrile, tetrahydrofuran) in the next steps of MDPC on multiphase plates, e.g., with a narrow zone of SiO_2 and a wide zone of RP-18 (or vice versa) which are commercially available from Whatman (Multi K SC5 or CS5 plates).

In the fourth mode of MDPC the separations of mixtures were realized on a monolayer of, e.g., silica. Separations of compounds were performed on polar stationary phases with a non-aqueous system (step A) and with partly aqueous eluents (step B) in the next step of MDPC. Application of multidimensional planar chromatography (MDPC) with different systems in steps, e.g., adsorption chromatography (step A) and hydrophilic interaction chromatography (HILIC) or ion exchange (step B) is especially useful for correct identification of components of difficult, complicated mixtures, e.g., pesticides in rivers or plant extracts. MDPC combined with different modes of scanning, e.g., with diode array

detection (MDPC-DAD) or mass spectrometry (MDPC-MS) enables quantitative analysis and identification of compounds. Heart-cut spots of analytes from the stationary phase can also be injected on, e.g., a C18 column and analysed by HPLC-DAD.
The new multidimensional methods with fast scanning detectors should enable us to detect and determine many more compounds in sample extracts and continue to improve modern analytical methods for better research to control the environment for persistent toxic compounds, e.g., pesticides.
The most efficient approach to pesticide analysis involves the use of chromatographic methods. Sometimes, the resolving power attainable with a single chromatographic system is still insufficient for the analysis of complex mixtures. The coupling of chromatographic techniques is clearly attractive for the analysis of multicomponent mixtures of pesticides. Truly comprehensive two-dimensional (2D) hyphenation is generally achieved by frequent sampling from the first column into the second, which permits a very rapid analysis.
This chapter is a continuation of an earlier published study by the same author: *Multidimensional chromatography in pesticides analysis*. In: Pesticides – strategies for pesticides analysis. (Ed.) Margarita Stoytcheva. InTech, Rijeka 2011, pp. 155-196 (Tuzimski, 2011a). In the previous study (Tuzimski, 2011a) were presented different modes of multidimensional chromatographic separation techniques including multidimensional gas chromatography (MDGC), multidimensional liquid chromatography (MDLC) and multidimensional planar chromatography (MDPC) applied to analysis of pesticides. In this study are presented sequential aspects of one dimensional (1D-), two-dimensional (2D-) and multidimensional chromatographic separation techniques including also MDGC, MDLC and MDPC applied to analysis of pesticides (without repetition of information included in the previous study (Tuzimski, 2011a)).

2. Chromatographic techniques applied to analysis of pesticides

2.1 Planar chromatography

2.1.1 Advantages of planar chromatography

Planar chromatography is most effective for the low-cost analysis of samples requiring minimal sample clean-up. Planar chromatography is also selected for pesticides screening analysis, because (Tuzimski, 2011b):

- single use of stationary phase minimizes sample preparation requirements;
- parallel separation of numerous samples enhances high throughput;
- ease of post-chromatographic derivatization enables improved method selectivity and specifity;
- detection and/or quantitation steps can easily be repeated under different conditions;
- all chromatographic information is stored on the plate and can be (re-) evaluated if required;
- several screening protocols for different analytes can be carried out simultaneously;
- selective derivatizing reagents can be used for individual or group identification of the analytes;
- detection of the separated spots with specific and sensitive color reagents;
- visual detection of UV-absorbing compounds is possible in field analyses by use of a UV lamp;
- detection by contact with X-ray film, digital bio- and autoradiography, and even quantitative assay by use of enzymes is possible;

- TLC plates can be documented by videoscans or photographs;
- planar chromatography combined with modern videoscanning and densitometry enables quantitative analysis;
- planar chromatography coupled with densitometry enables detection of the spots or zones through scanning of the chromatograms with UV-Vis light in the transmission, reflectance, or fluorescence mode;
- with multi-wavelength scanning of the chromatograms, spectral data of the analytes can be directly acquired from the TLC plates and can further be compared with the spectra of the analytes from software library;
- additional information for structural elucidation can be obtained by planar chromatography combined with MS (fast atom bombardment (FAB) and liquid secondary mass spectrometry (SIMS));
- the whole procedure of chromatographic development can be followed visually, so any distortion of the solvent front, etc., can be observed directly;
- the chromatogram can be developed simply by dipping the plate into a mobile phase;
- the possibility of two-dimensional development with a single adsorbent;
- the possibility of two-dimensional development on e.g., silica – octadecyl silica coupled layers (Multi-K SC5 and CS5 dual phase);
- planar chromatography is also the easiest technique which performs multidimensional separation (e.g., by graft chromatography or multidimensional chromatography).

Summing up, planar chromatography is one of principal separation techniques, which plays an important role also in environmental analysis.

2.1.2 From one dimensional (1D-) planar chromatography by two-dimensional (2D-) planar chromatography to multidimensional planar chromatography

Planar chromatography analysis of pesticides is especially suitable at sites where the concentration of pesticides might be high, e.g., sites of dumping grounds of toxic substances; after car or train accident during conveyance of pesticides and contamination of the environment. The analysis of complex mixtures of pesticides can be simplified by preliminary fractionation of the mixture into simpler mixtures by micropreparative chromatography (Tuzimski and Soczewiński, 2004). The main purpose of preparative layer chromatography is isolation of pure compounds from a mixture with maximal yield. Sample application is one of the most important steps of a successful preparative separation. The zonal application of the sample from the edge of the layer is preferable (Soczewiński and Wawrzynowicz, 2000; Soczewiński 2001). Nyiredy (Nyiredy 2001; Nyiredy 2003) described sample application and other characteristics of classical preparative layer chromatography, overpressured layer chromatography, other special techniques, and trends in preparative layer chromatography. Waksmundzka-Hajnos and colleagues (Waksmudzka-Hajnos and Wawrzynowicz, 2002; Waksmudzka-Hajnos et al., 2002) described the strategy of preparative separation in TLC. Dzido et al. (Dzido et al., 2002) also described the effect of temperature on the separation of test solutes in preparative TLC. Guiochon and colleagues (Guiochon, 2002; Felinger and Guiochon, 1998; Jandera et al., 1997; Gritti and Guiochon, 2003) described theory, instruments, and practical issues of preparative chromatography. The separation of a certain target component from a multicomponent mixture using isocratic preparative elution chromatography was also studied theoretically (Shan and Seidel-Morgenstern, 2003).

A good perspective of separation of compounds is obtained by use, in the second stage, systems of different selectivities compared with the first stage, e.g., NP system on silica in the first stage followed by a reversed-phase (RP) system on octadecyl silica adsorbent in the second stage [TLC or high performance liquid chromatography (HPLC)] or vice versa especially for samples with matrix rich in water.

Also possible is the use of NP or RP systems with different retention mechanisms on hydrophilic modified stationary phases, e.g., cyanopropyl, aminopropyl, and diol. These phases have many other advantages—an extended range of selectivity, and graduated surface polarity, and show less influence of the vapour phase on retention behaviour and, therefore, have better reproducibility (Rabel, 2003). NP HPLC has also several advantages (Jandera, 2000): pressure drop across the column is lower in non-aqueous RP system than in aqueous RP system (because of lower viscosity of non-aqueous eluents); columns are usually more stable in non-aqueous solvents than in aqueous solvents; some samples are more soluble, or less prone, to decomposition in organic mobile phases. However, RP chromatography generally offers better selectivity for the separation of molecules with different sizes of their hydrocarbon part.

Figure 1 illustrates possibilities for the separation of the 10-component mixture of pesticides (nos. 1–10) into fractions (nos. I–VIII) in the NP system, silica–ethyl acetate–diisopropyl ether (10 : 90, v/v) (Tuzimski, 2005a). A solution of the mixture of pesticides (1–3% of pesticides, 0.4 mL) was applied to the plate from the edge of the silica layer (0.5mm) through the glass distributor. The plate was developed with ethyl acetate–diisopropyl ether (10 : 90, v/v). Bands were visualised in UV light at λ = 254 nm (**Figure 2**). Next, the zones were scraped from the plate, and the adsorbed fractions were isolated by elution with methanol. Each of the pesticide fractions (I–VIII) was applied on a silica gel 60 F_{254} HPTLC plate. In addition, standard substances were applied to the plates (1–10) and the plates developed to a distance of 9 cm with ethyl acetate–diisopropyl ether (10 : 90, v/v) as mobile phase. The real picture of the silica gel plate obtained by videoscanning (**Figure 3**) showed the separation of eight fractions of pesticides (Tuzimski, 2005a).

The fractions were applied 0.5 cm from the edge on a RP-18W plate and developed with a RP aqueous eluent, acetonitrile–water (60 : 40, v/v) [**Figure 4(a) and (b)**]. It can be seen that the fractions are separated into single components. In the next series of experiments, the selectivity was investigated for cyanopropyl silica adsorbent. The cyanopropyl-bonded layer was developed with the RP system, acetonitrile–water (55 : 45, v/v). [**Figure 5(a) and (b)**] and with the NP system, dioxane–n-heptane (30 : 70, v/v) [**Figure 6(a) and (b)**]. It follows from **Figure 6** that the selectivity of the CN/NP system is satisfactory for HPLC analysis on a CN column (Tuzimski, 2005a).

The fractions (I–VIII) were also injected on a cyanopropyl column (LC–CN) and developed by NP eluents composed of dioxane and n–heptane (2 : 98, 3 : 97, and 5 : 95, v/v) (Tuzimski, 2005a). The fractions were also separated on a octadecyl silica column (LC-18) and analysed by the RP system with acetonitrile–water (70 : 30, v/v). The data of **Table 1** (Tuzimski, 2005a) showed larger R_S values for the majority of a set of pesticides of the I–VIII fractions in acetonitrile–water system on an LC-18 column, than nonaqueous system on an LC–CN column. The more sensitive HPLC analysis shows that some peaks are accompanied by small peaks of neighbouring compounds, e.g., in the fraction II: peak of metazachlor (no. 2) is accompanied by a small peak of neighbouring triadimefon (no. 3). The values of R_S of the pair were 0.78 and 2.78, respectively, on Supelcosil™ LC–CN and Supelcosil™ LC-18 columns. These values indicate successful resolution of the compounds in the acetonitrile–

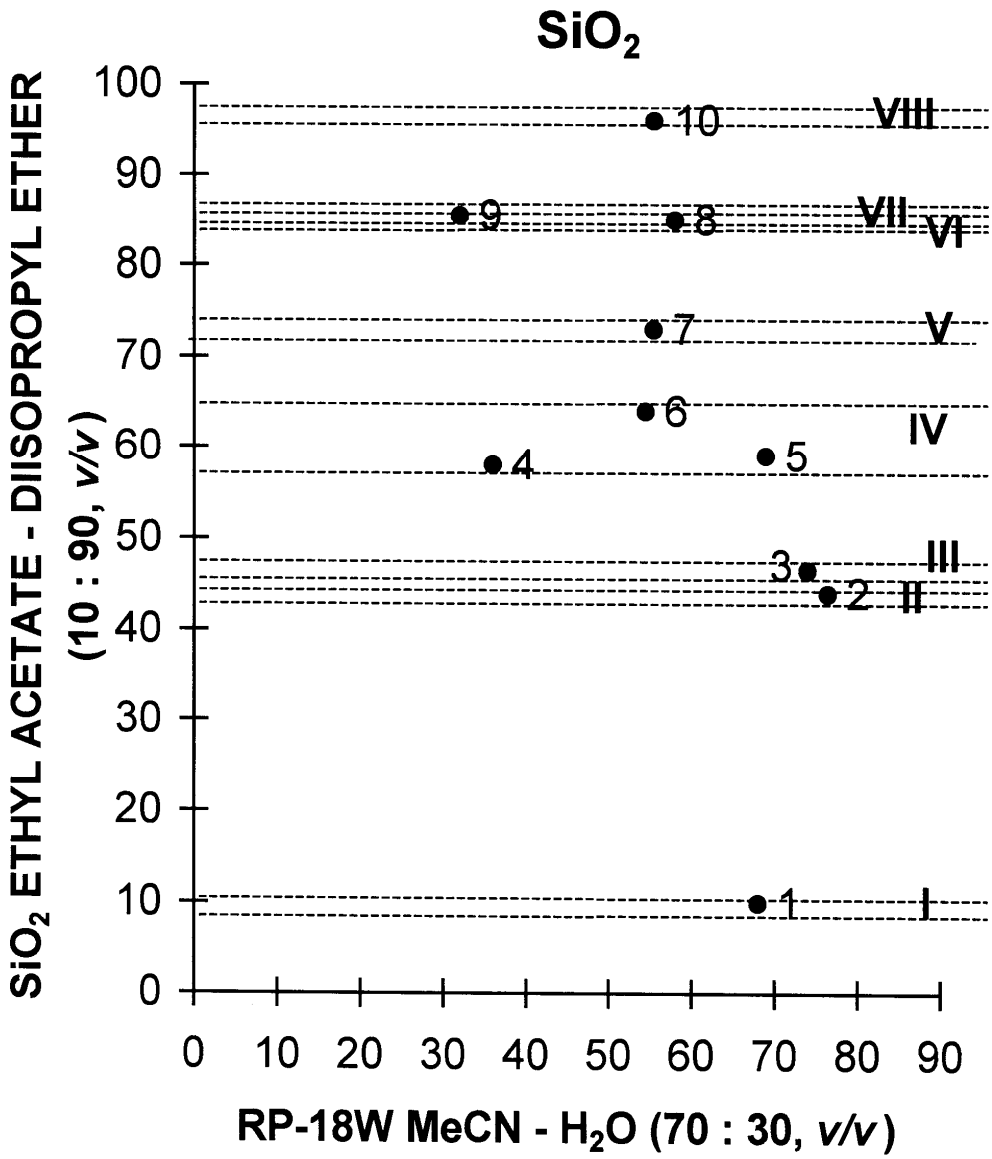


Fig. 1. Correlation hR_F vs. hR_F system—RP: acetonitrile–water (70 : 30, v/v) on octadecyl silica wettable with water (RP-18W) and NP: ethyl acetate–diisopropyl ether (10 : 90, v/v) on silica gel. Illustrates possibilities for the separation of the 10-component mixture of pesticides (nos. 1–10) into eight fractions (nos. I–VIII) in the NP system on silica (From Tuzimski, 2005a. With permission.).

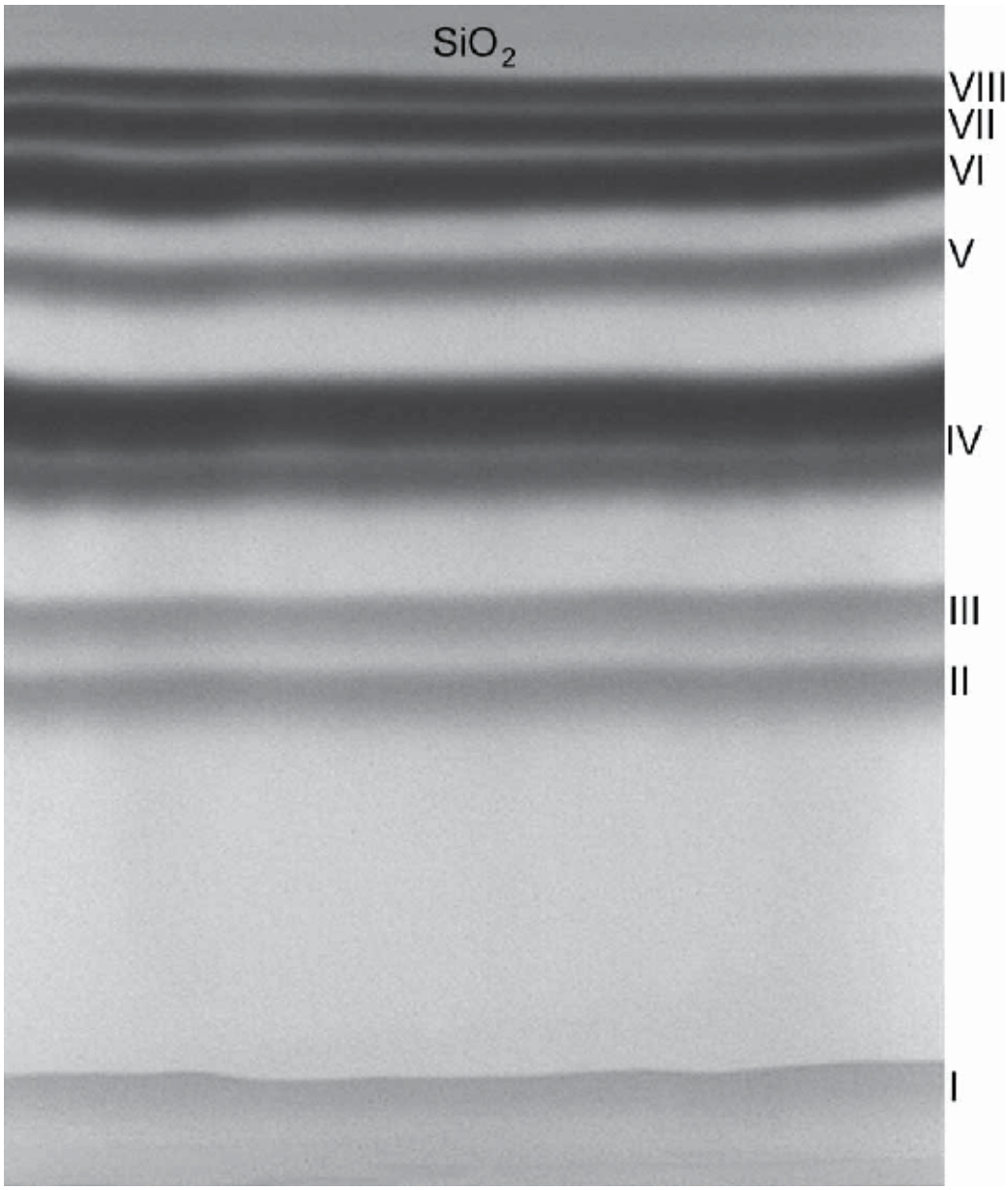


Fig. 2. Chromatographic separation of pesticides on silica (0.5mm layer) with ethyl acetate–diisopropyl ether (10 : 90, v/v) as mobile phase: zone diagrams of eight fractions of mixture of 10 pesticides applied as a solution (1–3%, 0.4 mL) to the plate from the edge of the silica layer through the glass distributor. (From Tuzimski, 2005a. With permission.).

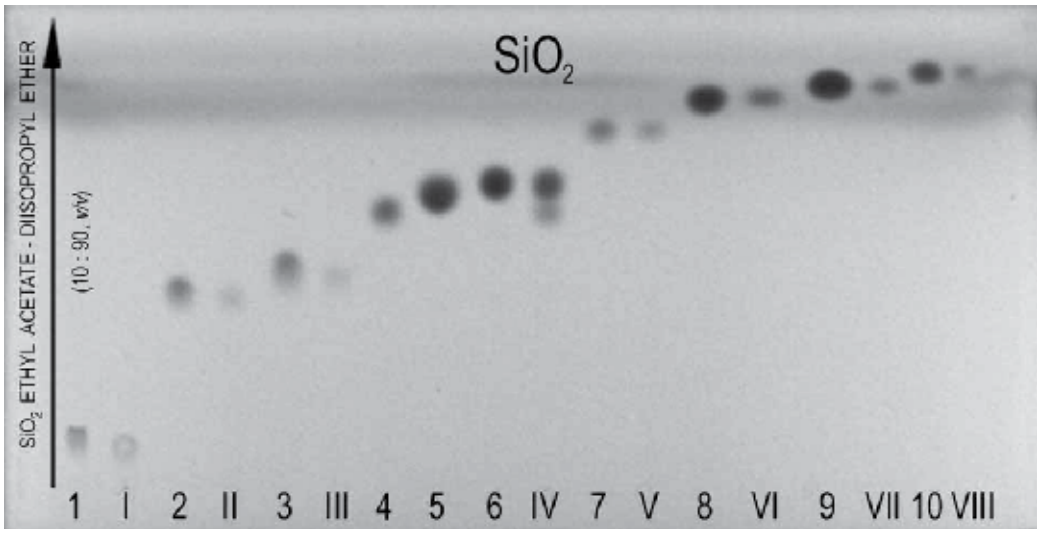


Fig. 3. Videoscan showing a real picture of the silica plate and fractionation of the mixture of 10 pesticides (fractions I–VIII) by use of 10% ethyl acetate in diisopropyl ether as mobile phase (From Tuzimski, 2005a. With permission.).

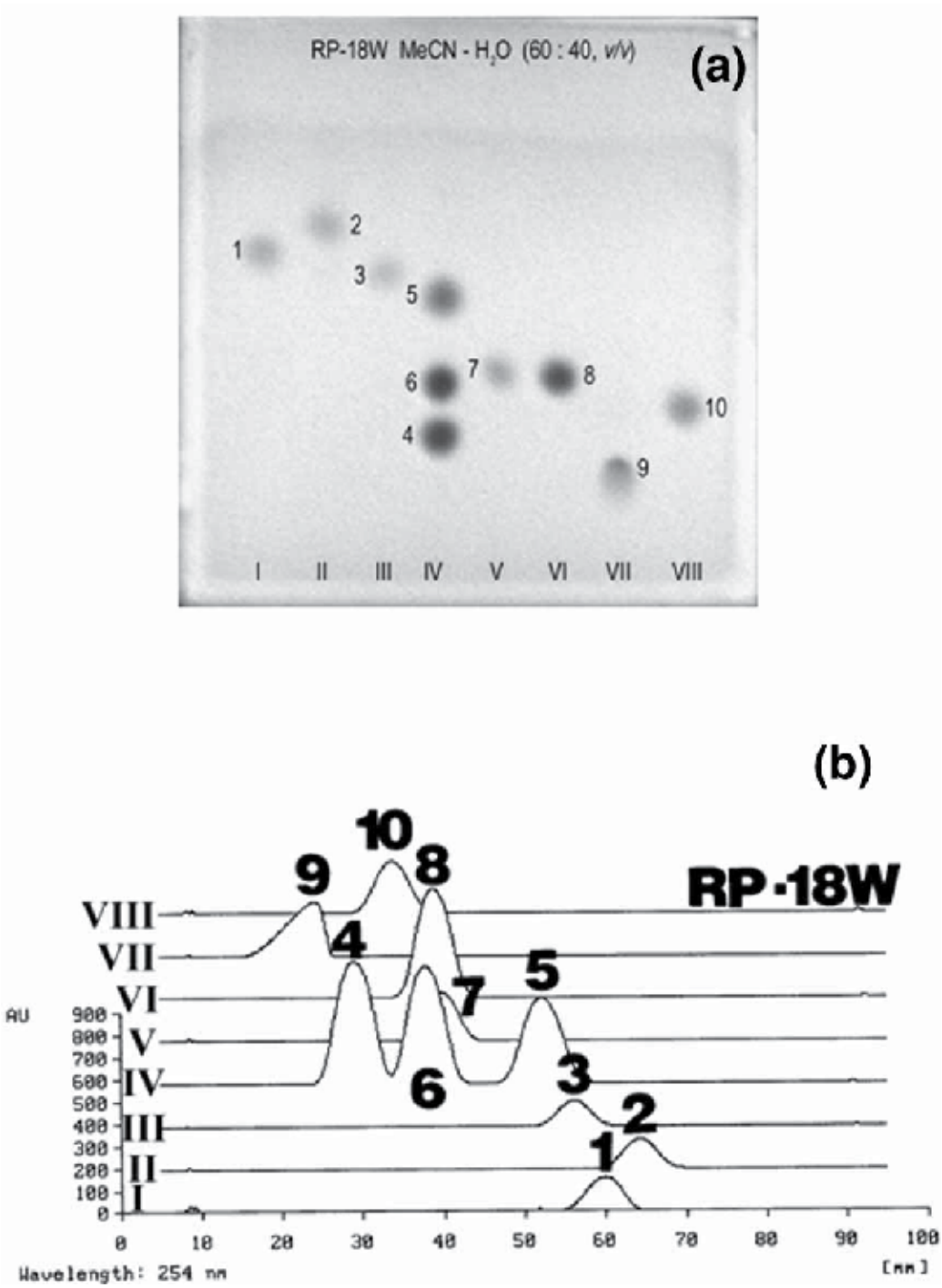


Fig. 4. Videoscan (a) and densitogram (b) of the RP-18W plate, which shows separation of eight fractions of mixture of 10 pesticides (5 μL of each fraction) by RP system: acetonitrile–water (60 : 40, v/v) (From Tuzimski, 2005a. With permission.).

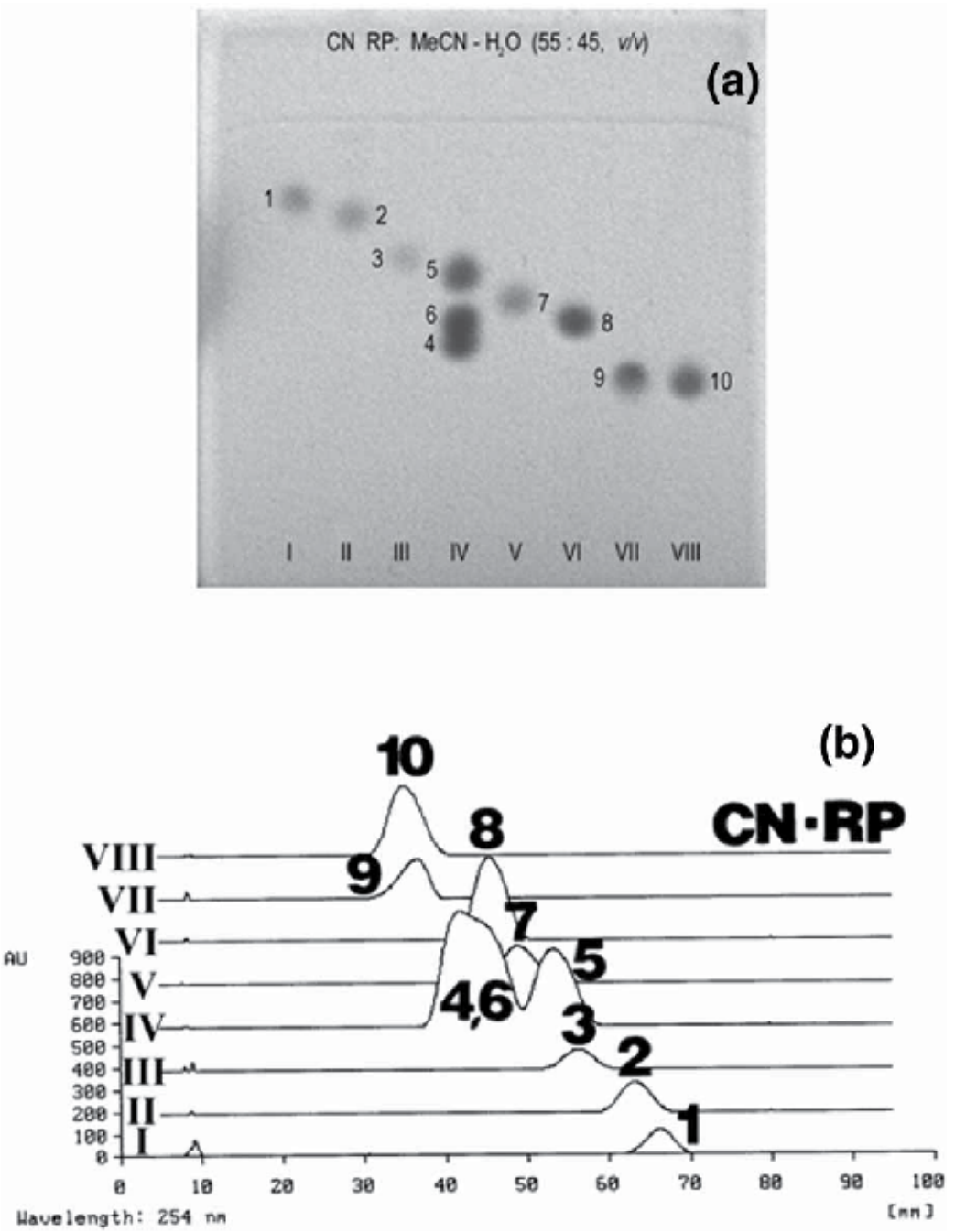


Fig. 5. Videoscan (a) and densitogram (b) of the cyanopropyl plate, which shows separation of eight fractions of mixture of 10 pesticides (5 µL of IV–VII fractions; 10µL of remaining fractions) by RP system: acetonitrile–water (55 : 45, v/v) (From Tuzimski, 2005a. With permission.).

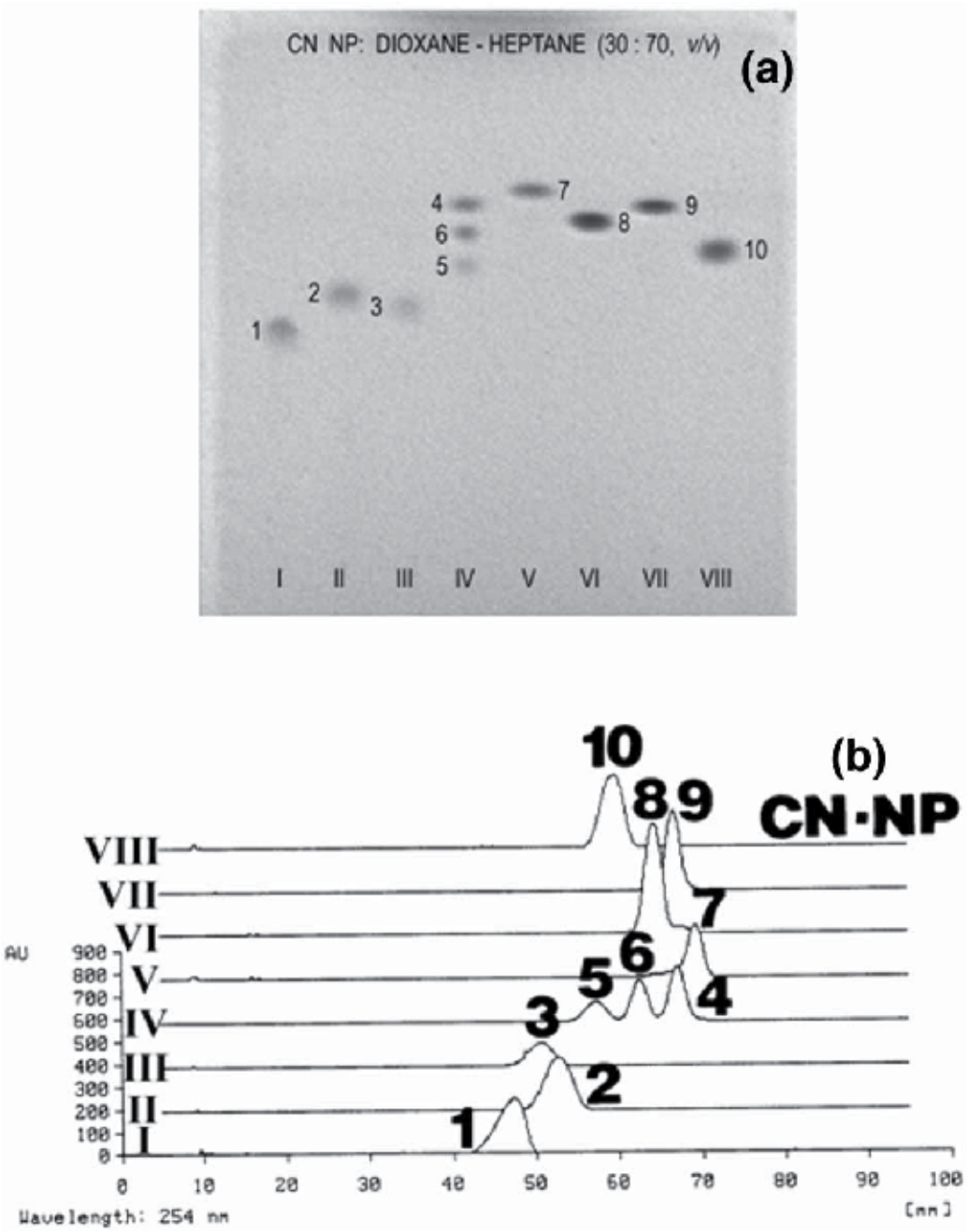


Fig. 6. Videoscan (a) and densitogram (b) of the cyanopropyl plate, which shows separation of eight fractions of mixture of 10 pesticides (5 µL of IV, VI, VII fractions; 10µL of remaining fractions) by NP system: dioxane–n-heptane (30 : 70, v/v) (From Tuzimski, 2005a. With permission.).

water system on the octadecyl silica column. For the majority of the compounds, larger values of *N* were for the RP system on the LC-18 column, besides compounds (nos. 2, 3, 5). The solutions of some pesticides, during prolonged storage, development of chromatograms, elution and visualisation of bands in UV light, may decompose and can cause the appearance of additional peaks on their chromatograms. The correct identification and separation of the decomposition of the labile pesticides is possible.

		Supelcosil™ LC-CN column dioxane-n-heptane (2:98, v/v)		
Fraction no.	Pesticide No.	t_r	N/m	R_s
I	1	7.967	1,890	-
II	2	7.075	241,560	-
	3	7.317	25,980	2.78
III	2	7.092	180,860	-
	3	7.358	189,590	1.59
IV	4	3.245	22,880	-
	6	5.408	31,830	11.64
	5	7.075	165,880	3.91
V	7	3.800	35,310	-
	8	4.183	26,940	2.97
VI	8	4.175	26,920	-
VII	9	4.275	41,060	-
VIII	10	6.308	46,080	-
		Supelcosil™ LC-18 column Acetonitrile-water (70:30, v/v)		
Fraction no.	Pesticide No.	t_r	N/m	R_s
I	1	2.650	12,940	-
II	2	2.558	31,370	-
	3	3.083	26,980	2.78
III	3	3.067	24,070	-
	5	3.400	26,830	1.59
IV	5	3.392	28,140	-
	6	6.433	44,860	11.64
	4	7.875	36,850	3.91
V	8	6.258	48,310	-
	7	7.200	47,540	2.97
VI	8	6.250	46,480	-
VII	9	10.100	53,510	-
VIII	10	7.442	54,310	-

(From Tuzimski, 2005a. With permission).

Table 1. Data of HPLC analysis of the I-VIII fractions of 10-component mixture of pesticides on cyanopropyl LC-CN column and on octadecyl silica LC-18 column.

A good perspective of separation of all components of fractions was obtained by using systems of different selectivity in both stages, e.g., NP system on silica in first stage and RP

system on octadecyl silica adsorbent in the second stage (TLC or HPLC). The procedure gives successful separation of the fractions by using the two methods with the possibility of full quantitative TLC and HPLC analysis (Tuzimski, 2005a). Another example of preparative separation of a complex mixture of pesticides by TLC on silica (non-aqueous mobile phase, normal-phase (NP) chromatography) combined with TLC and HPLC (aqueous mobile phase, reversed-phase (RP) chromatography) is presented in **Figures 7-11** (Tuzimski, 2005b). A ten-component mixture of pesticides was applied to the edge of the layer in 'frontal + elution' mode for preliminary fractionation by zonal micropreparative TLC. **Figure 7** shows the plate with four separate zones, visualized in UV light at λ = 254 nm. The located zones were scraped from the plate, in the conventional manner, and the fractions were extracted

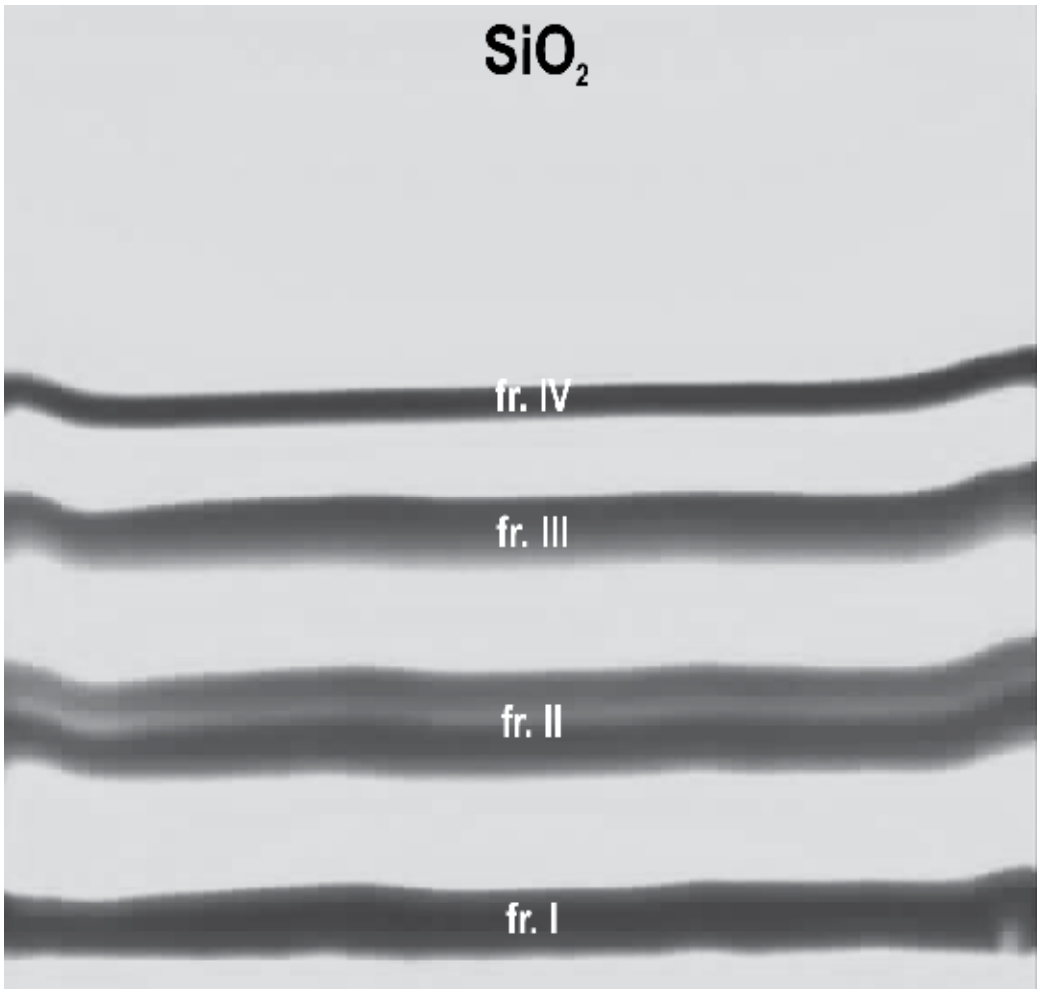


Fig. 7. Chromatographic separation of the pesticides on silica gel (0.5 mm layer) with tetrahydrofuran–*n*-heptane, 20 + 80 (*v/v*), as mobile phase. Zonal chromatogram of four fractions (I–IV) of the mixture of ten pesticides (1–10) applied as a solution (1% of each, 0.7 μL) to the edge of the silica layer through the glass distributor (videoscan at λ = 254 nm) (From Tuzimski, 2005b. With permission.).

with 50:50 (v/v) ethyl acetate–methanol. **Figure 8** shows the silica gel plate with separation both of pesticide fractions I–IV and the standard substances (1–10) applied to the plate, to verify the purity of separated fractions I–IV. The separated, simpler, fractions were applied to an octadecyl silica layer wettable with water (TLC, RP-18W) and re-chromatographed. The plate was scanned and videoscanned, furnishing a real picture of the plate and showing complete separation of the fractions. **Figure 9** shows the videoscan and densitogram obtained from the RP-18W plate with the separated components of the fractions. Separation of fraction II was better with acetonitrile–water, 60 + 40 (v/v), as mobile phase, whereas the components of fractions I, III, and IV were better separated with methanol–water, 60+40 (v/v), although two components of fraction I (isoproturon and diuron) were only partly separated. The mixture of ten pesticides and fractions I–IV were also injected on to an octadecyl silica column (RP-18) and chromatographed by use of a water–methanol mobile phase gradient. The ten pesticides cannot be completely separated by gradient elution (**Figure 10**).

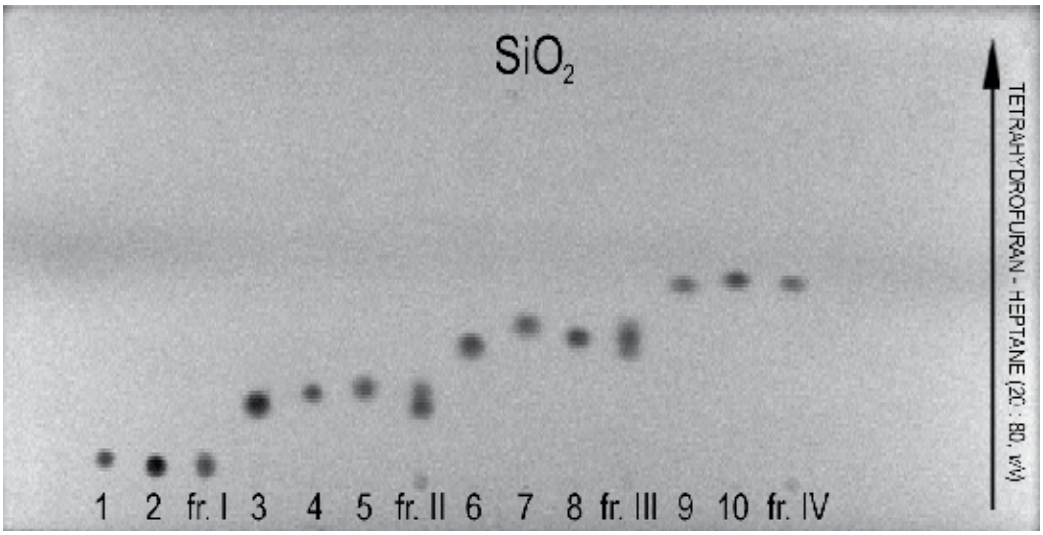


Fig. 8. Videoscan showing a picture of the silica plate and fractionation of the mixture of the ten pesticides (fractions I–IV) by use of tetrahydrofuran–*n*-heptane, 20 + 80 (*v/v*), as mobile phase. Standard substances (1–10) were also applied to the plate (From Tuzimski, 2005b. With permission.).

The simpler fractions were also analyzed by HPLC on an octadecyl silica (LC-18) column (**Figure 11**). **Figures 11a–11d** show separations of the four pesticide fractions (I–IV) by use of this gradient. The resolution (R_S) obtained for separation of most of the pesticide peaks in each of the fractions was >1.5. The more sensitive HPLC analysis enables almost complete separation of the components of fraction I. R_S for this pair was 1.1. Comparison of **Figures 10** and **11** shows that although direct separation of the mixture of the pesticides is impossible, identification and quantitative analysis of the components is possible after preliminary fractionation by preparative TLC. Results obtained by the author shows possibility of full separation of the simpler fractions in the second stage by use of the two independent methods, owing to the different selectivity of NP and RP systems (TLC and HPLC) (Tuzimski, 2005b).

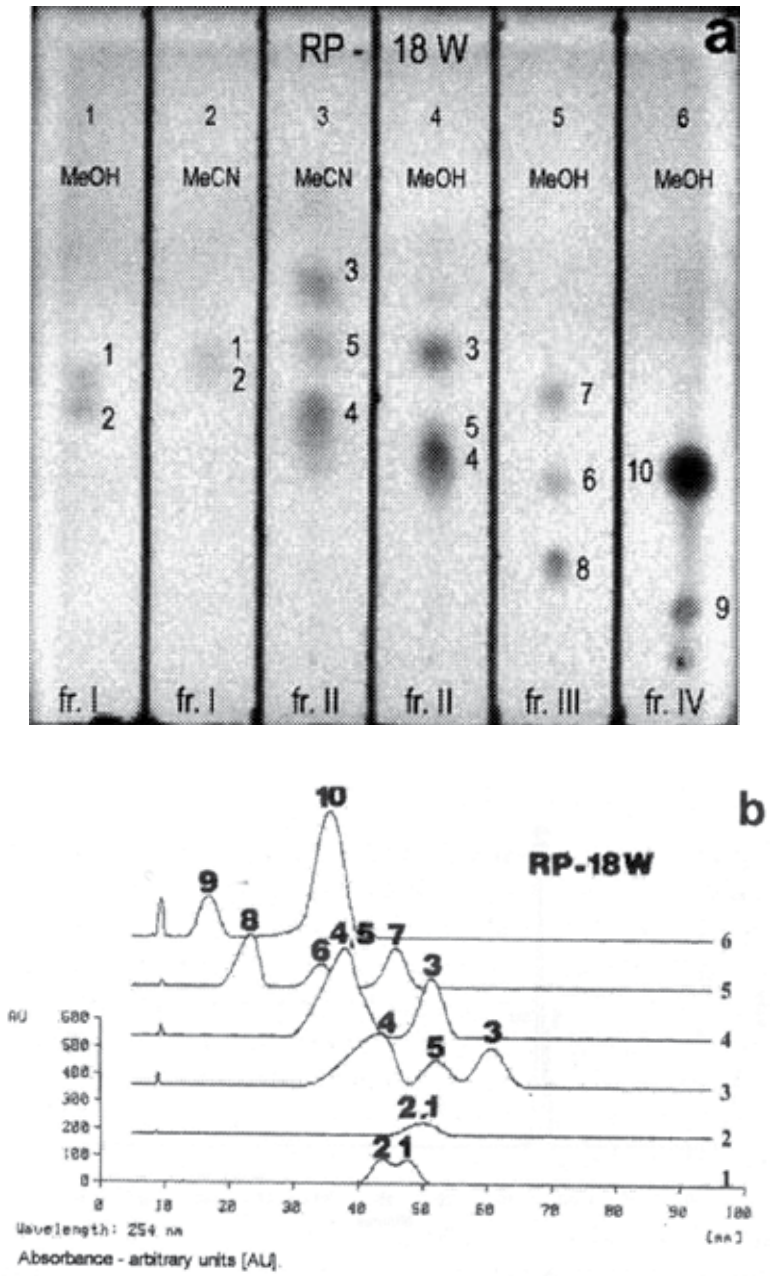


Fig. 9. Videoscan (a) and densitogram (b) showing preliminary fractionation on an RP-18W plate developed with the reversed-phase mobile phases methanol–water, 60 + 40 (*v/v*), (chromatograms 1, 4–6) and acetonitrile–water, 60 + 40 (*v/v*), (chromatograms 2 and 3) in a DS-M type of chamber (From Tuzimski, 2005b. With permission.).

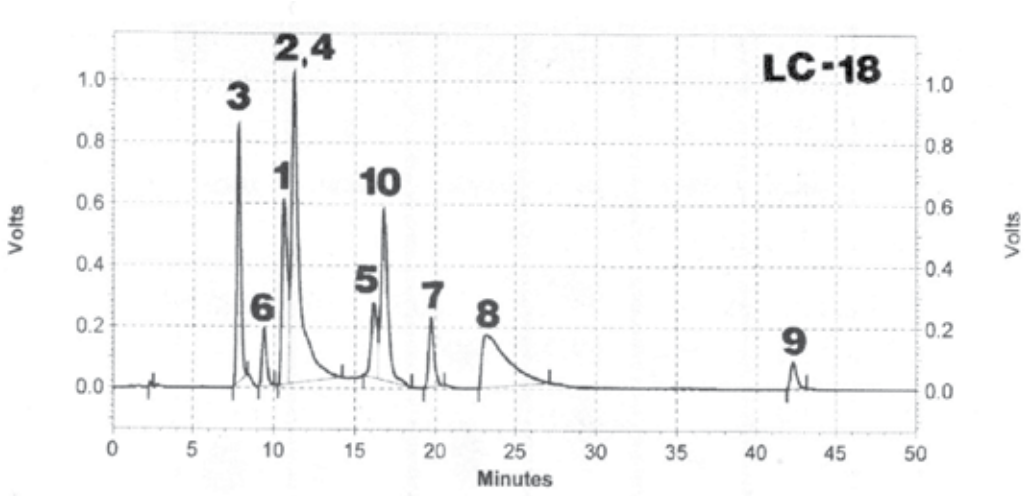
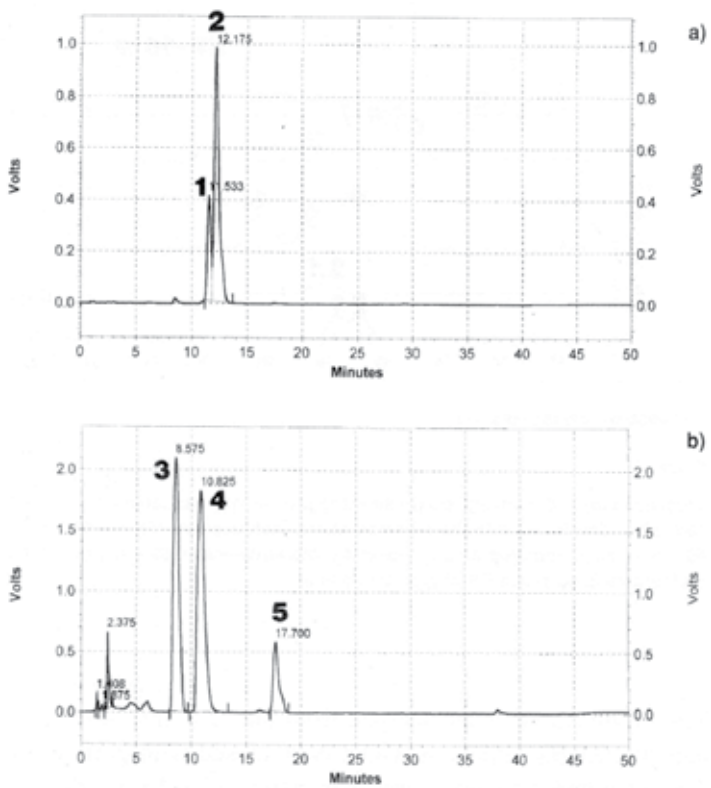


Fig. 10. HPLC separation of the ten-component pesticide mixture on a 150 mm × 4.6 mm, *d*p = 5 µm, Supelcosil LC-18 column (Supelco) by use of a methanol–water gradient (50 to 85% methanol from 0–50 min). The flow rate was 1.0 mL min^{-1}, detection was at 254 nm, and the temperature was 22 ± 1°C. (From Tuzimski, 2005b. With permission.).



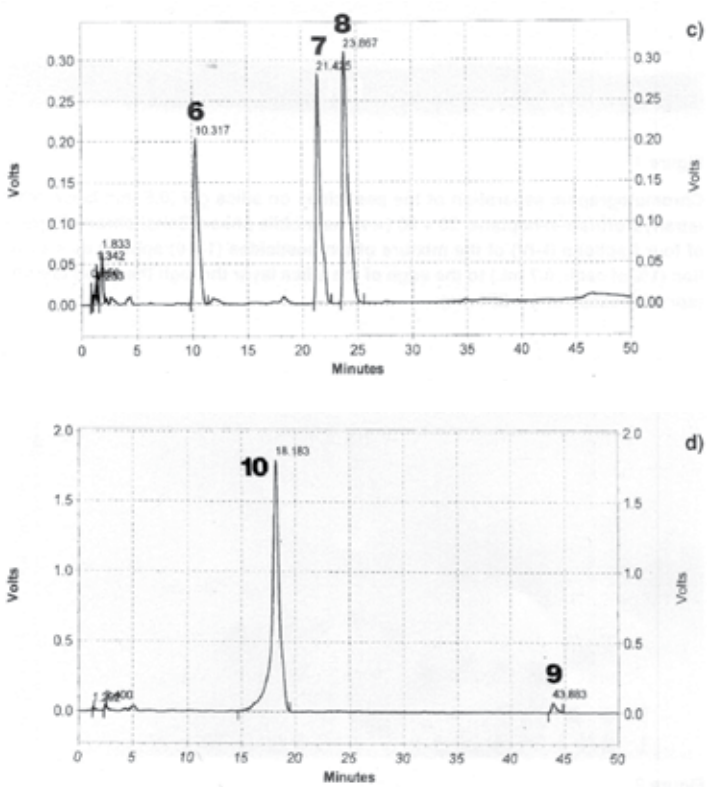


Fig. 11. HPLC separation of the four pesticide fractions (I–IV) on a 150 mm × 4.6 mm, *d*p = 5 µm, Supelcosil LC-18 column (Supelco) by use of a methanol–water gradient (50 to 85% methanol from 0–50 min). The flow rate was 1.0 mL min^{-1}, detection was at 254 nm, and the temperature was 22 ± 1°C (From Tuzimski, 2005b. With permission.).

One of the most attractive features of planar chromatography is the ability to operate in the two-dimensional (2D) mode. In 2D development the mixtures can be simultaneously spotted at each corner of the chromatographic plate so that the number of separated samples can be higher in comparison to the 'classical 2D development' (Nyiredy, 2001; Szabady and Nyiredy, 1996). An example of this type of 2D development is illustrated in **Figure 12**. **Figure 12** shows a videoscan of the plate which shows separation of three fractions of the mixture of nine pesticides by 2D planar chromatography with NP/RP systems on a chemically bonded-cyanopropyl stationary phase.

Nyiredy (Nyiredy, 2001; Szabady and Nyiredy, 1996) described the technique of joining two different adsorbent layers to form a single plate. The largest differences were obtained by combination of normal-phase systems of the type silica/nonaqueous eluent and reversed-phase systems of the type octadecyl silica/water + organic modifier (methanol, acetonitrile, tetrahydrofuran, dioxane) on multiphase plates with a narrow zone of SiO_2 and a wide zone of RP-18 (or vice versa) which are commercially available from Whatman (Multi K SC5 or CS5 plates) (Tuzimski and Soczewiński, 2002a-d; Tuzimski and Bartosiewicz, 2003). Tuzimski and Soczewiński were the first to use bilayer Multi K plates for the separation of complex mixtures (Tuzimski and Soczewiński, 2002a-d; Tuzimski and Bartosiewicz, 2003).

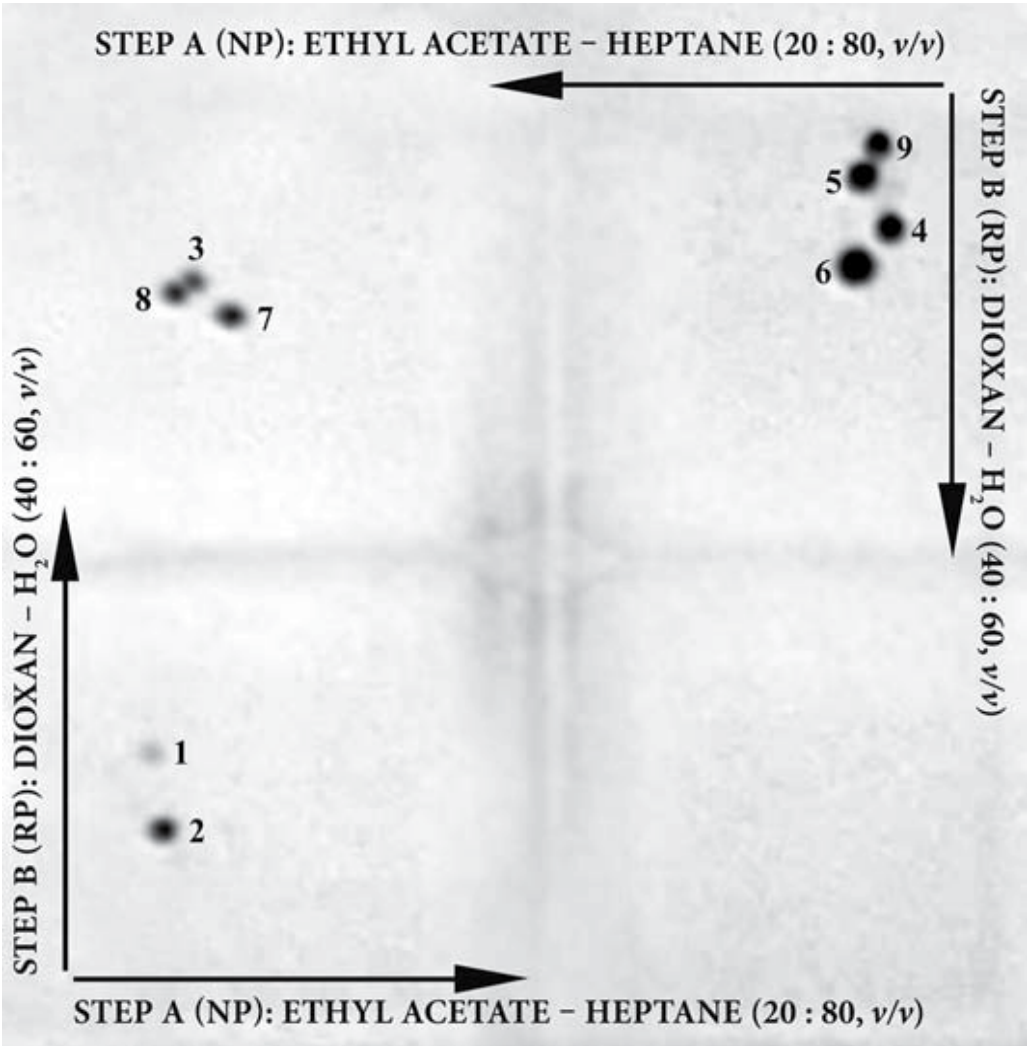


Fig. 12. Videoscan of the plate which shows separation of three fractions of mixture of nine pesticides for 2D-TLC system: NP: ethyl acetate - n-heptane (20 : 80, v/v) (step A) and RP: dioxane – water (40 : 60, v/v) (step B), both on plates with chemically bonded-cyanopropyl stationary phase (From Tuzimski & Soczewiński, 2004a. With permission.).

The multidimensional separation can be performed using different mobile phases in systems with single-layer or bi-layer plates. Graft thin-layer chromatography is a multiple system in which chromatographic plates with similar or different stationary phases are used. Graft-thin layer chromatography, a novel multiplate system with layers of the same or different adsorbents for isolation of the components of natural and synthetic mixtures on preparative scale, was first described by Pandey et al. (Pandey et al., 1979). The procedure of performing reproducible graft-TLC analysis was described in detail by Tuzimski (Tuzimski, 2007a) and in the previously study (Tuzimski, 2011a).

Thin-layer chromatography (TLC) combined with modern scanning densitometry provides the possibility of quantitative analysis (Ahrens et al., 2002; Hiegel and Spangenberg, 2009;

Spangenberg and Klein, 2000; Spangenberg and Klein, 2001; Spangenberg et al., 2003; Spangenberg, 2006; Tuzimski, 2010a; Tuzimski, 2011a). The method offers a simple and economical alternative to other chromatographic techniques, especially column high-performance liquid chromatography (HPLC). Application of modern fiber optic TLC scanner with a diode array detector (DAD) has several advantages (Ahrens et al., 2002; Hiegel and Spangenberg, 2009; Spangenberg and Klein, 2000; Spangenberg and Klein, 2001; Spangenberg et al., 2003; Spangenberg, 2006; Tuzimski, 2010a; Tuzimski, 2011a) e.g., the scanner can measure TLC plates simultaneously at different wavelengths without destroying the plate surface and permits parallel recording of chromatograms and in situ UV spectra in the range 191-1033 nm; therefore, it is possible to obtain doubly credible correct identification of the compounds on a chromatogram. The TLC scanner permits analysis of each compound at its optimum wavelength, thus offering optimum sensitivity for detection of each component. The TLC-DAD scanner permits measurement of a three-dimensional chromatogram, $A = f(\lambda, t)$, with absorbance as a function of wavelength and migration distance. The TLC-DAD scanner can compare parallel UV spectra of an unknown compound and a standard from the library of spectra.
Software is available that allows the user access to all common parameters used in HPLC-DAD: peak purity, resolution, identification via spectral library match, etc. The TLC-DAD scanner is especially useful for correct identification of components of difficult, complicated mixtures, such as in plant extract and toxicological analysis.

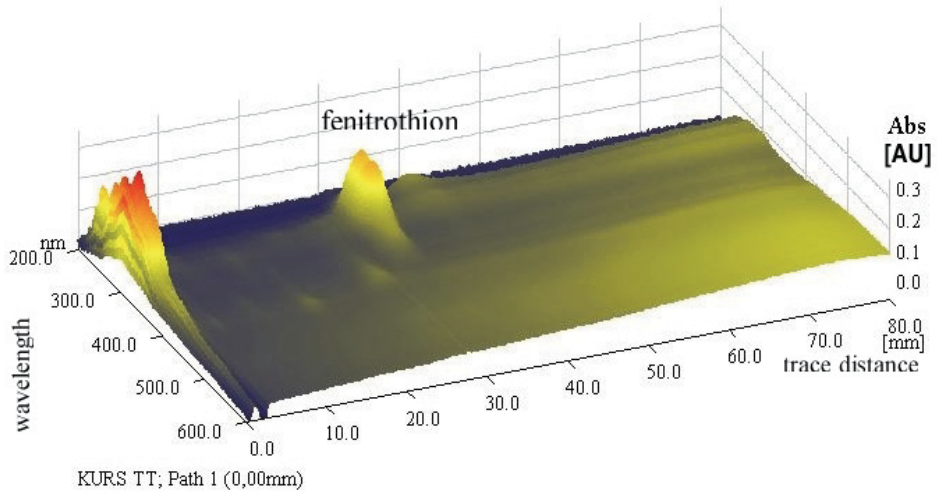


Fig. 13. Three-dimensional plot obtained from an apple extract containing fenitrothion (From Tuzimski, 2005. With permission.).

Figure 13 shows an example of the three-dimensional plot (scanning range x trace distance x absorbancy) obtained from an apple extract (Tuzimski, 2005). Identification was achieved by comparing the UV spectrum obtained from the extract and a fenitrothion standard. **Figure 14** shows UV spectra obtained from fenitrothion standards at eight concentrations (100-1000 μg mL^{-1}) and the UV spectrum obtained from fenitrothion in an extract from freshly squeezed apple juice (Tuzimski, 2005). The instrument detection limit (IDL) for fenitrothion was also determined: was 10 μg mL^{-1}. The concentration of fenitrothion in the extract from

fresh squeeze apple juice forty-five days after spraying apples was below the method detection limit (MDL) for fenitrothion (Tuzimski, 2005).
The peak purity index is a numerical index for the quality of the coincidence between two datasets. It is given by the least-squares-fit coefficient calculated for all intensity pairs in the two datasets under consideration. The following equation is applied:

$$P = \frac{\sum_i (s_i - \bar{s})(r_i - \bar{r})}{\sqrt{\sum_i (s_i - \bar{s})^2 \sum (r_i - \bar{r})^2}} \quad (1)$$

where s_i and r_i are the respective intensities for the same abscissa value, *i* is the number of data points, and $\bar{s}$ and $\bar{r}$ are the average intensities of the first and second dataset. A peak purity index has values in the range from 0 to 1. A peak-purity index of 1 indicates that the compared spectra are identical (Tuzimski, 2005).

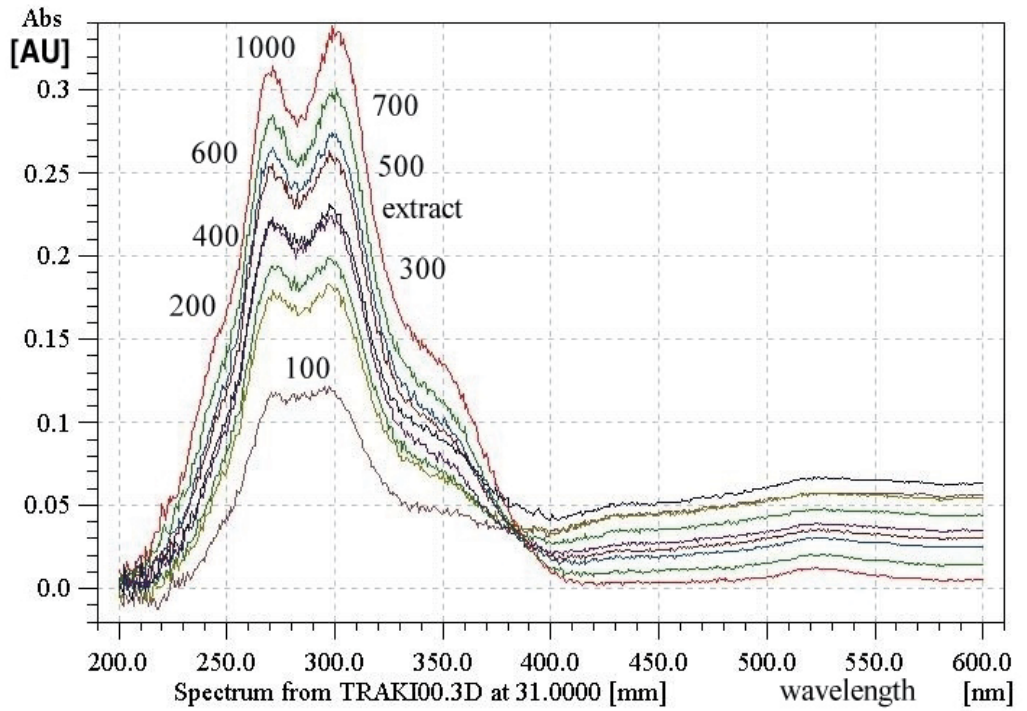


Fig. 14. UV spectra obtained from fenitrothion standards at eight concentrations (100-1000 μg mL^{-1}) and from an extract of freshly squeezed apple juice containing fenitrothion (From Tuzimski, 2005. With permission.).

Very difficult separations of multicomponent mixtures of compounds require the application of multidimensional planar chromatography combining different separation systems. A new procedure for separation of complex mixtures by combination of different modes of multidimensional planar chromatography were described (Tuzimski, 2008a;

Tuzimski, 2007b). MDPC combined with different modes of scanning, e.g., with diode array detection (MDPC-DAD) or mass spectrometry (MDPC-MS) enables quantitative analysis. The best combination for multidimensional planar chromatography is the parallel combination of stationary and mobile phases. In the next mode of multidimensional planar chromatography the separations of multicomponent mixtures were realized on multiphase plates. Also the largest differences were obtained by combination of normal-phase systems of the type silica/nonaqueous eluent in the first step of MDPC and reversed-phase systems of the type octadecyl silica/water + organic modifier (methanol, acetonitrile, dioxane, tetrahydrofuran) in the next steps of MDPC on multiphase plates, e.g., with a narrow zone of SiO_2 and a wide zone of RP-18 (or vice versa) which are commercially available from Whatman (Multi K SC5 or CS5 plates) (Tuzimski, 2010a, Tuzimski, 2011a).
In another mode of MDPC the separations of mixtures were realized on a monolayer of, e.g., silica (Tuzimski, 2010b, Tuzimski, 2011a). Separations of compounds were performed on polar stationary phases with a non-aqueous eluent (step A or in both A and B steps) and with partly aqueous eluents (step B) in the next step of MDPC. Application of multidimensional planar chromatography (MDPC) with different systems in steps, e.g., adsorption chromatography (step A) and hydrophilic interaction chromatography (HILIC) or ion exchange (step A) adsorption (step B) is especially useful for correct identification of components of difficult, complicated mixtures, e.g., pesticides in plant extracts. The procedure described for the separation of complex mixtures of compounds is inexpensive and can be applied to routine analysis of analytes in samples of natural origin, e.g., in water or plant extracts, after preliminary clean-up and concentration, e.g., by solid-phase extraction (SPE). Application of multidimensional planar chromatography and modern fiber optical TLC densitometric scanners with DAD are especially useful for correct identification of components of difficult, complicated mixtures, e.g., clofentezine in Herba Thymi (Tuzimski, 2010a, Tuzimski, 2011a). The identification of analytes was confirmed by the comparison of the UV spectra of the components of plant extracts and standards of analytes by DAD densitometer (Tuzimski, 2010a,b; Tuzimski, 2011a).
The LOD and LOQ for clofentezine were 0.23 and 0.70 μg per spot (TLC-DAD), and 0.35 and 1.06 μg/mL, (HPLC-DAD), respectively. The method recovery was studied by analyzing five replicates of samples spiked with clofentezine at four concentrations levels (4.5, 6, 9 and 12 μg/g in plant material). Average recoveries from the spiked samples, and the SD, were 55.8%±4.5 and 44.5%±6.5 (SPE: C18/SDB-1, THF eluates) after step B determined by MDPC-DAD and HPLC-DAD, respectively. The methanol eluates contained traces of clofentezine (<0.09%). The determined quantity of the clofentezine in the extract of Herba Thymi (*T. vulgaris* L., Lamiaceae) ranged from 0.78 to 0.86 μg/g in plant material (n=7) in samples from the year 2009. The proposed procedure is efficient and uncomplicated. It allows to analyze the quantity of clofentezine in medical herbs without the necessity of applying additional purifying and absorbents (silica or Florisil) of the matrix in SPE. Moreover, it does not necessitate the use of additional columns in HPLC experiments to purify the matrix from the ballast substances (Tuzimski, 2010b; Tuzimski, 2011a).
MDPC has many advantages, for example wide possibilities of optimization of the chromatographic system, special development modes, diverse detection methods, and low-cost analysis of samples, requiring minimal sample cleanup. The purpose of next study was to demonstrate an application of 2-D high-performance planar chromatography-diode array detector (DAD) and HPLC-DAD after solid-phase extraction (SPE) for identification and quantitative analysis of some pesticides (isoproturon, aziprotryne, hexazinone,

flufenoxuron, methabenzthiazuron, procymidone, and a-cypermethrin) in *Melissa officinalis* L. (Labiatae) samples (Tuzimski, 2011c). In the preliminary part of described experiments (Tuzimski, 2011c), the eluates from fortified samples were injected on C18 column and analyzed by HPLC-DAD. The chromatogram obtained from a sample of *M. officinalis* L. (Labiatae) fortified by seven pesticides is shown in **Figure 15**. The analytes were identified on the basis of their retention times and by comparison between the UV spectrum of the reference compound in the library and the UV spectrum of the detected peak in the sample (**Figure 16**, left column). A match equal to or higher than 990 was fixed to confirm identification between both spectra for all of the pesticides determined. Aziprotryne, flufenoxuron, and a-cypermethrin were obtained as pure peaks (**Figure 16**, right column), but for other analytes, the peaks were impure. If the peaks of analyte are pure, then the surface area under the compared spectra of standard and analyte is green (on black-and-white print it is light-grey). If the peak of the analyte is contaminated, the surface area would be red (dark-grey on black-and-white print). The right column of **Figure 16** (and **Figure 20**) represents the purity of peaks of three pesticides: aziprotryne, flufenoxuron, and cypermethrin. Since these peaks are pure, the calculated surface areas of the compared peaks are light-grey (green in original).

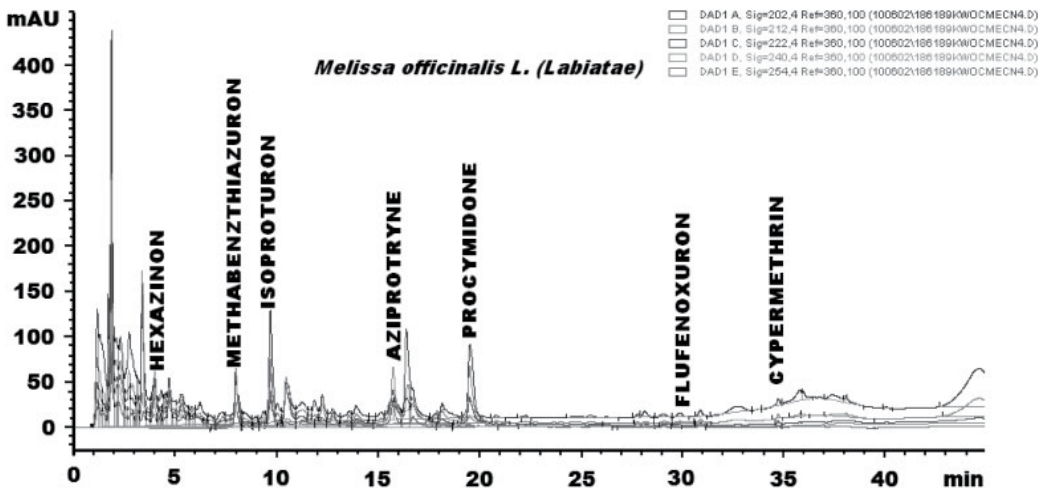


Fig. 15. Chromatogram obtained by HPLC-DAD after SPE from *M. officinalis* L. (Labiatae) (From Tuzimski, 2011c. With permission.).

Applications of NP and RP systems during a single experiment enable separation not only of analytes in a multicomponent sample, but also separation of analytes from impurities and other components of the matrix. In the next experiments, the eluates were applied on bilayer Multi K CS5 plates. On the basis of correlations of R_F values in NP and RP systems (**Figure 17**), it was possible to separate seven analytes from the components of the matrix. The Multi-K CS5 plates were scanned in the wavelength range of 200–400 nm. Identification of analytes was confirmed by comparison of UV spectra of the components of plants extract with those of standards of analytes. The purities of the peaks were also determined. Example of the least-squares fit values (obtained by cross-correlation) of spectra from a fortified sample of M. officinalis L. and spectra from pesticide standards were also calculated and the purity index (Pearson's r) for compared spectra was always between 0.9911 and 0.9997.

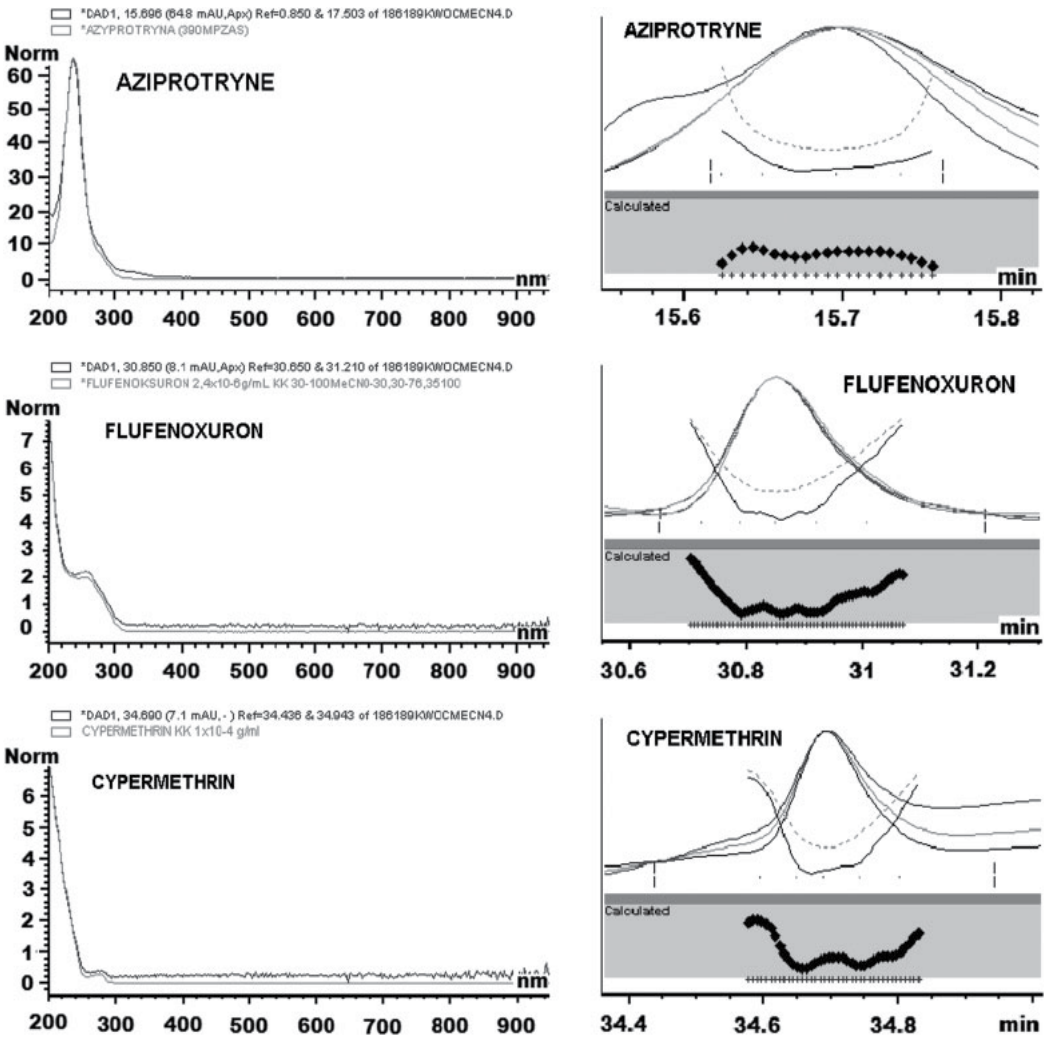


Fig. 16. Left column: Comparisons of the UV spectra of standards (library) and spectra found in *M. officinalis* L. (Labiatae); right column: purities of peaks found in *M. officinalis* L. (Labiatae) by HPLC experiments before 2-D-HPTLC (From Tuzimski, 2011c. With permission.).

Heart-cut bands of analytes from the stationary phase (after 2-D-TLC experiments) were also analyzed by HPLC-DAD on C18 column. The HPLC chromatograms obtained from extract of *M. officinalis* L. (Labiatae) after 2-D-TLC show the purities of peaks of analytes separation from components of matrix (**Figure 18**). Identification of the analytes was accomplished on the basis of retention times of the analytes (**Figure 18**) and by comparing the UV spectrum of the reference compound in the library with the UV spectrum of the peak detected in the sample (**Figure 19**). The purities of the peaks were also determined (**Figure 20**).

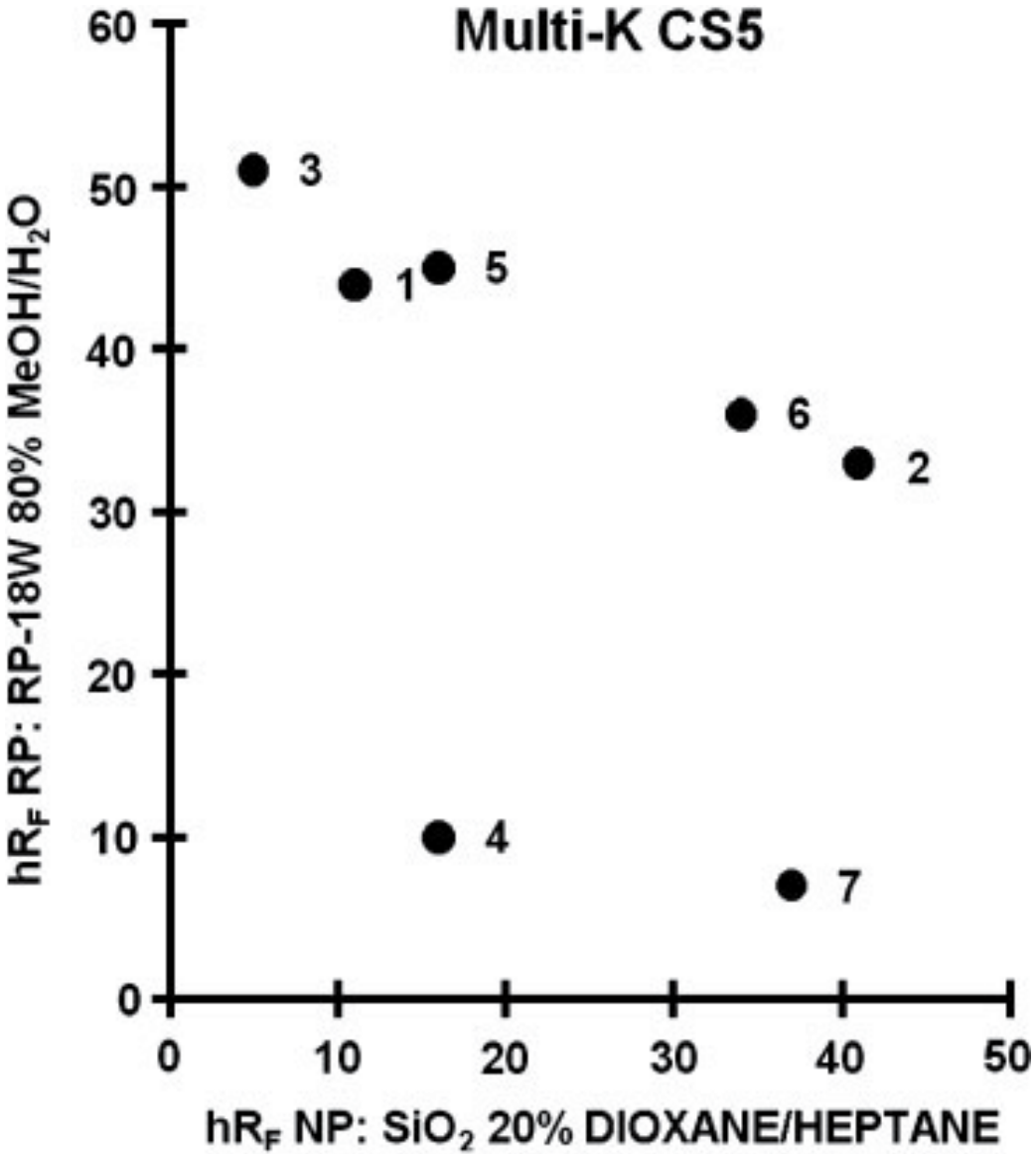


Fig. 17. Correlation hR_F (100xR_F) *versus* hR_F (100xR_F) for 2-D HPTLC system: RP, methanol–water (80:20, v/v) on octadecyl silica adsorbent wettable with water (RP-18W) and NP, dioxane–*n*-heptane (20:80, v/v) on silica gel. 1, isoproturon; 2, aziprotryne; 3, hexazinon; 4, flufenoxuron; 5, methabenzthiazuron; 6, procymidone; 7, a-cypermethrin (From Tuzimski, 2011c. With permission.).

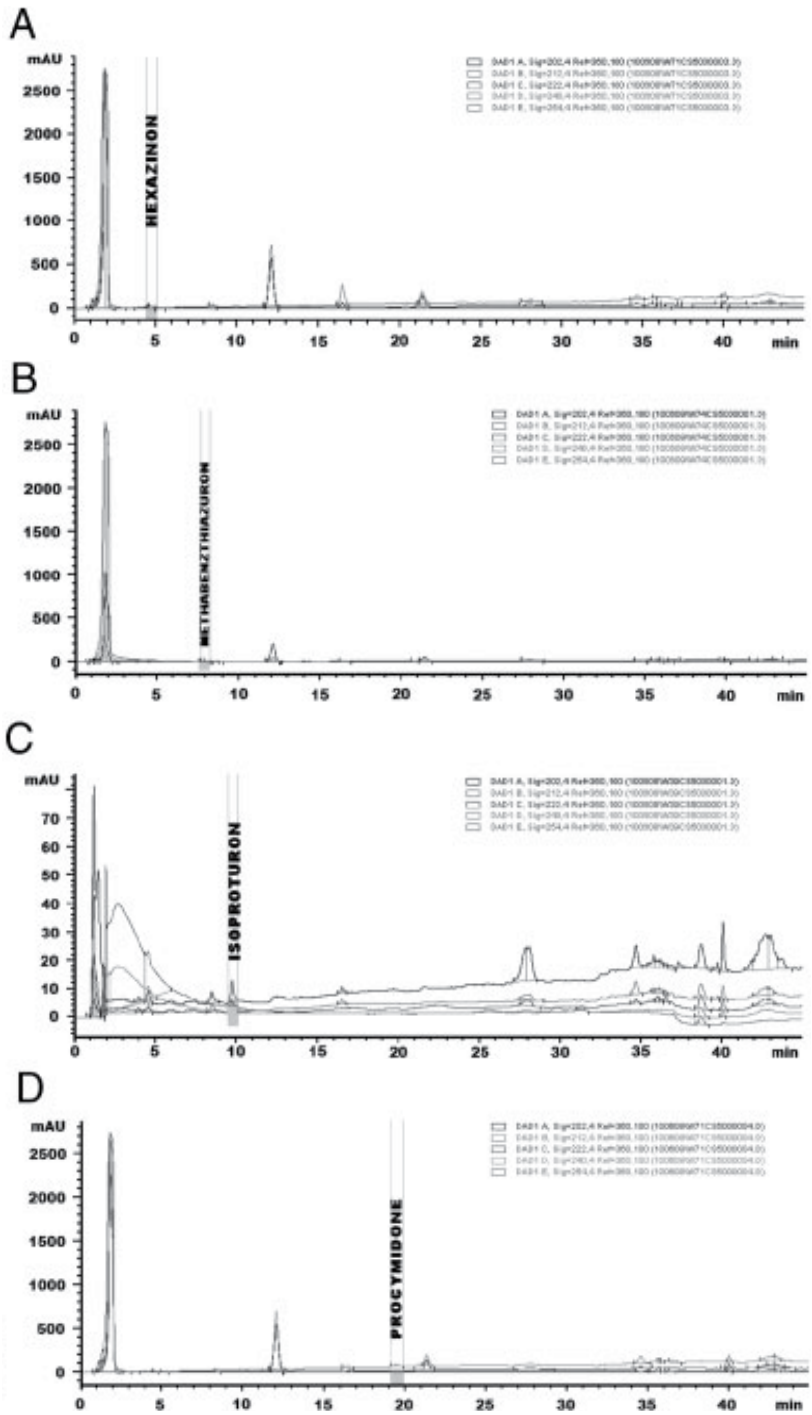


Fig. 18. The heart-cut bands of the analytes from the stationary phase of Multi-K CS5 were analyzed by HPLC-DAD: chromatograms obtained by HPLC-DAD after SPE and 2-D-HPTLC from *M. officinalis* L. (Labiatae) (From Tuzimski, 2011c. With permission.).

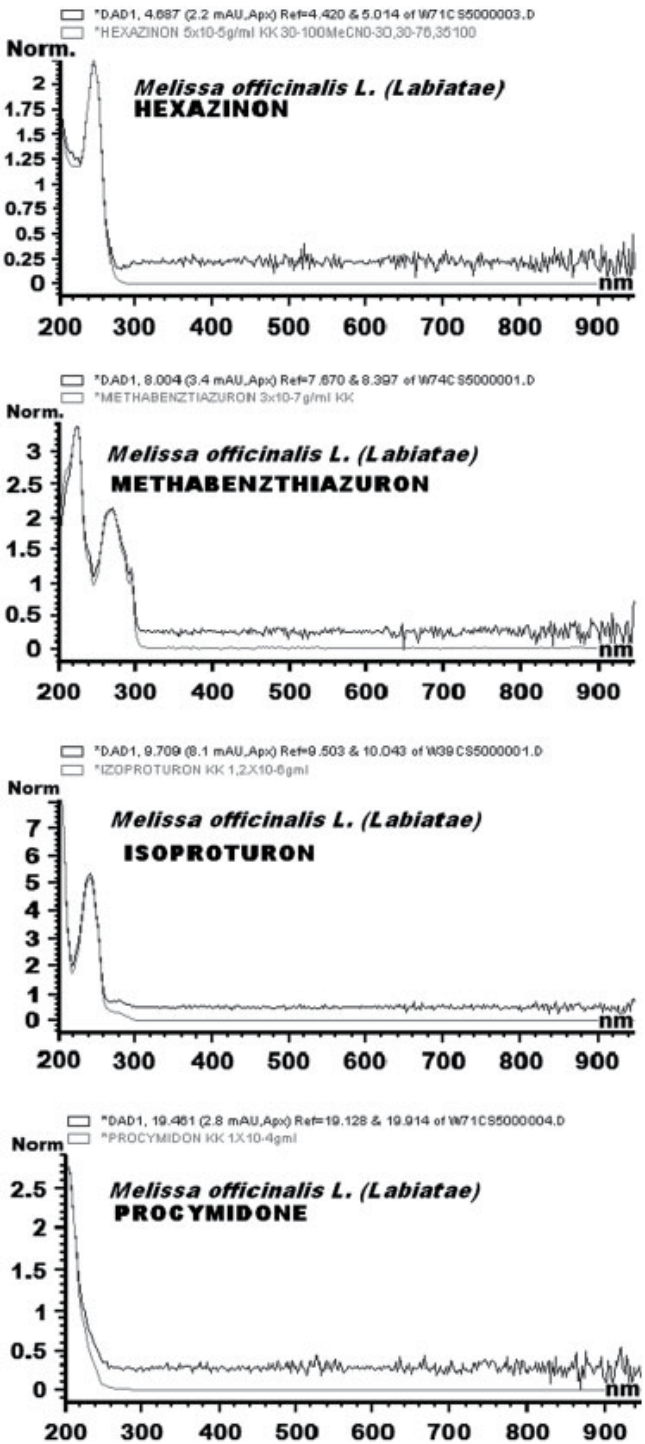


Fig. 19. Comparisons of the UV spectra of standards (library) and spectra found in *M. officinalis* L. (Labiatae) (From Tuzimski, 2011c. With permission.).

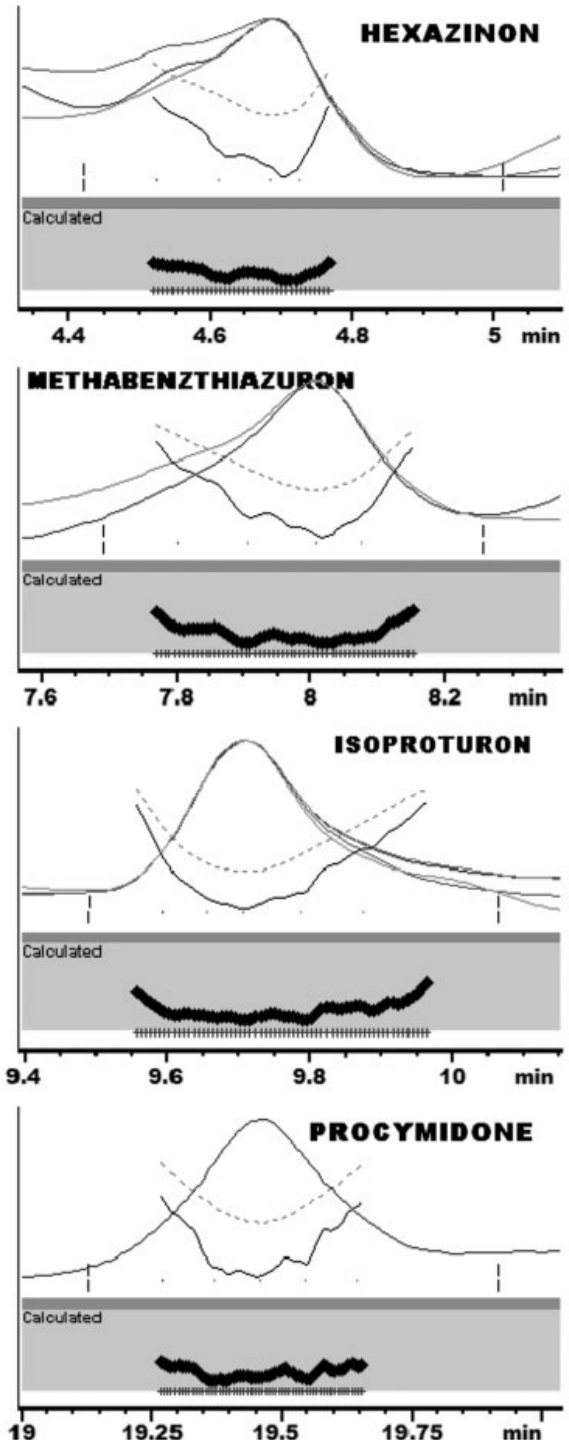


Fig. 20. Purities of peaks found in *M. officinalis* L. (Labiatae). (From Tuzimski, 2011c. With permission.).

The proposed procedure was proved correct for spiked samples with seven pesticides at concentration levels equal to 10 μg/g in plant material after 1, 5, and 11 days. The method characterized good reproducibility. Application of both chromatographic techniques with different systems, e.g. NP and RP (in both steps of 2-D-TLC-DAD on the bilayer Multi-K CS5 plates) and RP (HPLC-DAD) can be useful for correct identification of pesticides in complicated mixtures and separation of analytes from the components of the matrix. Thanks to 2-D-TLC experiments, it is possible to obtain pure peaks of almost all pesticides investigated and acceptable values of recoveries of analytes (except for very nonpolar pesticides, e.g. pyrethroids, benzoylphenylurea chitin synthesis inhibitors) (Tuzimski, 2011c).

2.2 Liquid chromatography

In the last past decade high performance liquid chromatography has emerged as a technique for the separation of complex environmental samples because of its outstanding chromatographic resolving power, the possibilities to automate the analysis and its compatibility with mass-spectrometric detection using electrospray interfacing (ES-MS). Mutiresidue analysis of 95 pesticides at low nanogram/liter levels in surface waters by HPLC with mass spectrometry was described (Jasson and Kreuger, 2010). Analysis of pesticide residues in apples based on QuEChERs (quick, easy, cheap, effective, rugged and safe) was described by Stevens et al. (Zhao, 2009). The maximum peak capacity obtained in one-dimensional (1D) liquid chromatography is mainly determined by the column technology such as column length and particle size, and also the duration of the gradient (Eeltink et al., 2009). The maximum allowable column length is determined by the permeability of the chromatographic bed and the maximum pressure drop of the HPLC instrumentation (Poppe, 1997; Desmet et al., 2009). 1D-LC allows the separation of hundreds of analytes. The excellent power of the multidimensional separation approaches have potential to separate thousands of analytes (Stoll et al., 2007). Dependent on the way transfers from the primary column effluent to the second dimension column are established, distinctions should be made for 2D-LC separations between either off- or on-line 2D-LC. Off-line 2D-LC is propably the most applied 2D-LC approach, since the execution is very simple. Fractions of the first dimension effluent are collected (manually or via fraction collector), after which they are concentrated if necessary and re-injected on the secondary column (François et al., 2009). On-line 2D-LC can be divided into heart-cutting and comprehensive liquid chromatography, abbreviated as LC-LC and LC x LC, respectively (Schoenmakers et al., 2003). Off-line 2D-LC (or LC x LC) offers the most flexibility in terms of separation modes and LC conditions, because matching eluents, flow-rates and transfer volumes are less critical than in on-line 2D-LC (Kohne and Welsch, 1999). However, off-line 2D-LC allows re-analysis of samples when partial injection of the fractions is applied.

Schoenmakers et al. (Schoenmakers et al., 2003) defined the criteria of a comprehensive separation based on postulates earlier reported by Giddings (Giddings, 1990): subjection of the entire sample to two different separations, separation and detection of equal percentages of all sample components and the preservation of the separation obtained in the first dimension (François et al., 2009). A typical comprehensive 2D-LC system consists of two pumps, two columns, injector, interface and detector. An excellent review of method development and instrumentation used in comprehensive liquid chromatography was described by Sandra et al. (François et al., 2009). A typical example of an LC x LC set-up is shown in **Figure 21**. The interface is the key component in all LC x LC systems, since it

enables the continuous transfer of the primary column effluent to the second dimension (François et al., 2009). Schoenmakers et al. (Schoenmakers et al., 2006) published a paper in which a protocol was proposed for establishing suitable column dimensions (length and diameters), particle sizes, flow rates, and second dimension injection volumes (i.e. loop sizes) in comprehensive two-dimensional liquid chromatography (LC×LC). The chromatographer should select the maximum allowable first-dimension retention time, which is approximately equal to the overall analysis time. Also, (s)he should define the maximum allowable pressure in both dimensions and the (minimum) diameter of the first-dimension column. The proposed protocol provides design parameters corresponding to the ideal (theoretically optimal) conditions or to realistic practical conditions. The protocol also allowed us to study the implications of contemporary developments in LC, such as the use of high temperatures (implying reduced viscosities and increased diffusion coefficients), monolithic columns (implying smaller flow-resistance factors), and ultra-high-pressure LC (Schoenmakers et al., 2006). Monolithic columns can mainly be advantageous as first dimension columns in LC×LC. Because of their lower resistance to flow, long monolithic columns can be used to obtain high efficiencies (plate counts). Operating at the highest possible pressure is advantageous in either dimension (Schoenmakers et al., 2006).
The use the full peak capacity offered by comprehensive LC x LC the separation mechanism used in the first dimension should be independent of the retention mechanism in the second-dimension separation and none of the separation achieved should be lost due to understanding (i.e., transferring too few fractions to the second-dimension column) (Eeltink et al., 2009). In the case, the maximum 2D-LC peak capacity ($^{2D}n_c$) that can be obtained is:

$$^{2D}n_c = {}^{1}n_c \times {}^{2}n_c \quad (2)$$

where $^{1}n_c$ and $^{2}n_c$ are the peak capacities obtained in the first dimension and second dimension, respectively.
Tanaka et al. (Horie et al., 2007) showed that the sampling time applied for the second dimension (^{2}D separation) should be adjusted to 2-4 times the standard deviation of the first-dimension (^{1}D separation) peak (i.e., one or two 'cuts' per ^{1}D peak). This yields the best compromise between the time available for the ^{2}D separations and maintaining separation obtained in the first dimension (Eeltink et al., 2009). Schoenmakers and co-workers described an optimization strategy to obtain the best possible performance in the shortest analysis time – called the peak production rate – for comprehensive off-line two-dimensional liquid chromatography (Eeltink et al., 2009). The authors give an overview of the effects of column length, particle size and gradient times on the optimal sampling time (**Table 2**).

Column length (mm)	Particle size (μm)	Gradient time (min)	4σ peak width (s)	Sampling time (s)	Sampling volume (μL)	Number of fractions collected
150	5	20	40	20	17	60
150	5	60	60	30	25	120
250	3	20	26	13	11	92
250	3	33	35	17.5	15	113
250	3	60	50	25	21	144

Table 2. Effect of column properties and gradient time on sampling time (2 cuts per peak) and the number of ^{1}D fractions collected (From Eeltink et al., 2009. With permission.).

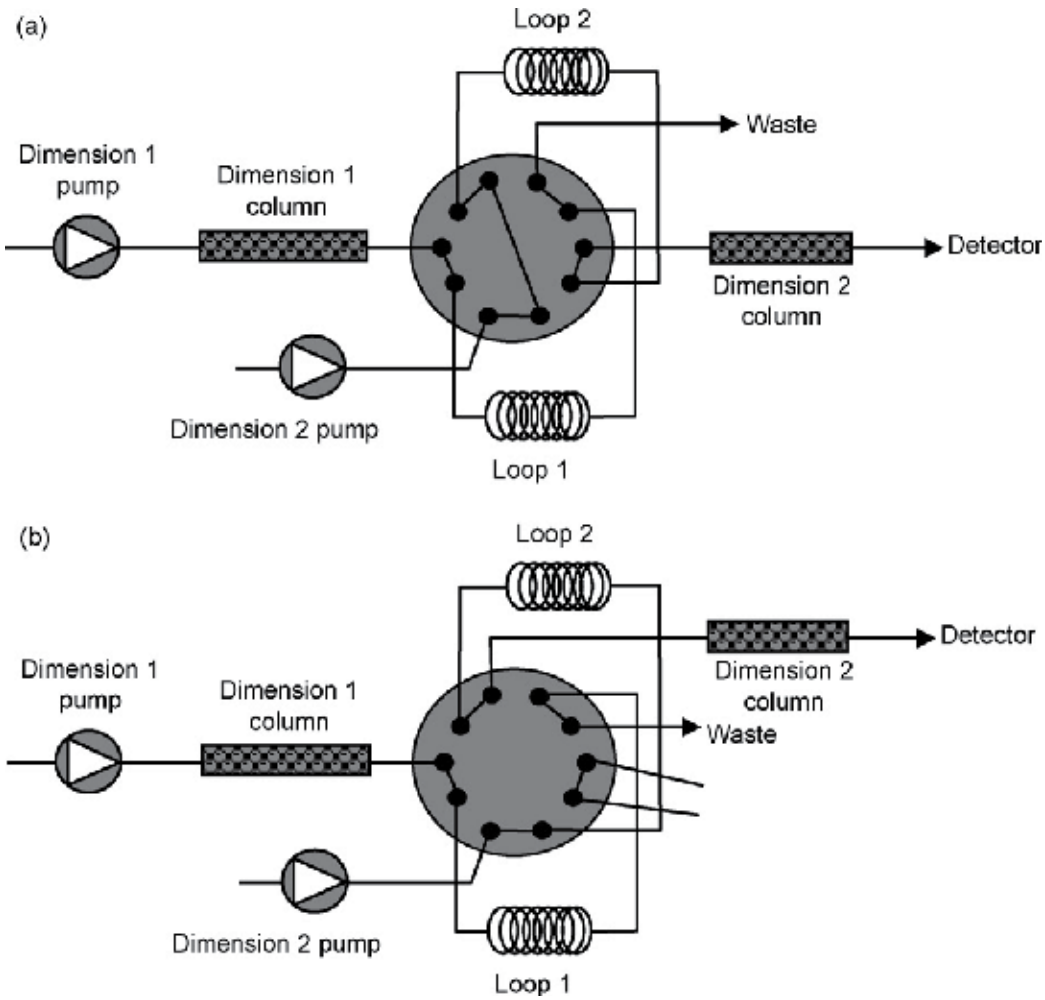


Fig. 21. Common comprehensive set-up with two-position/10-port switching valve; symmetrical (a) and asymmetrical (b) arrangements. (From François et al., 2009. With permission.).

True orthogonality is technically difficult to achieve, as orthogonality not only depends on the separation mechanisms, but also on the properties of the analytes (e.g., physicochemical properties of the sample constituents including size, charge, polarity, hydrophobicity, etc.) and separation conditions (e.g., surface chemistries, support material, carbon load, etc.), whereas the characteristics of the mobile phase can be altered by changing modifier, pH , temperature or adding ion pair agents.

An important issue in comprehensive LC is the compatibility of the mobile phases in the two dimensions. The mobile phase eluting from the primary column is preferably consisting of a weak solvent constituent of the second dimension mobile phase in order to create a focusing effect (Hoffman et al., 1989). Furthermore, if the solvents or solvent mixtures that are used as mobile phases are not completely miscible, serious difficulties arise, resulting in the complication of the combination of various separation modes. As an example, this is the

case when one of the separation dimensions is RP-LC, HILIC or IEX, while the other one is either NP-LC or SEC. The solvent in the former step is usually an aqueous solution, while that in the latter modes is generally an organic solvent that is not necessarily miscible in aqueous solvent mixtures (François et al., 2009).

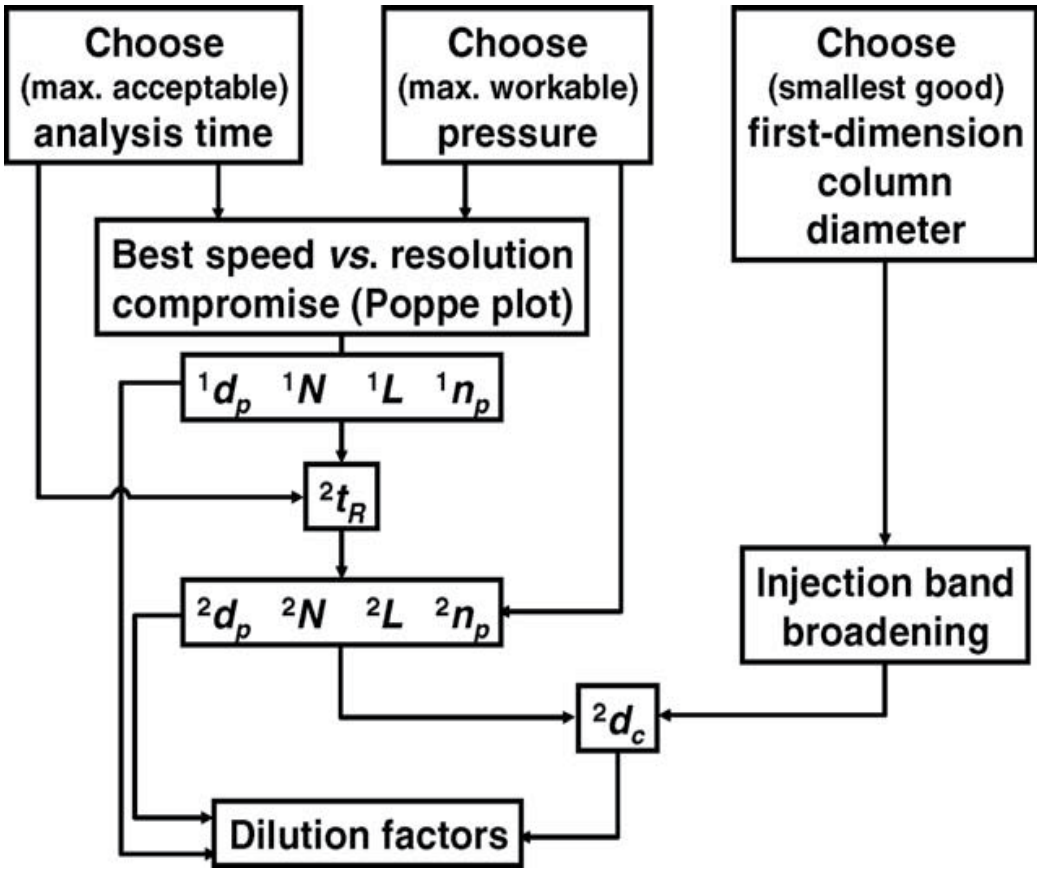


Fig. 22. Illustration of the protocol for designing LC×LC separation systems. (From Schoenmakers et al., 2006. With permission.).

These incompatibility problems were already observed in 1D LC in the past when sample solvents were significantly different than the initial conditions of the mobile phase. When the first-dimension separation is 'undersampled', for example when the resolution obtained in the first-dimension is partially lost by the fraction collecting rate, the first dimension peak capacity (^1n_c) cannot be higher than the number of fractions taken and is defined as (Eeltink et al., 2009):

$$^1n_c = \frac{^1t_G}{s_t} \tag{3}$$

where ^1t_G is the gradient duration in the first-dimension and s_t the sampling time.

The peak capacity (^2n_c) (2D) in gradient-elution chromatography can be approximately defined as (Eeltink et al., 2009):

$$^2n_c = \frac{^2t_G}{W} + 1 = \frac{^2t_G\sqrt{L}}{4t_R\sqrt{H}} + 1 \quad (4)$$

where ^1t_G is the gradient duration of the second-dimension reversed-phase gradient, W the average peak width (which is approximately equal to four times the standard deviation, at σ), L the column length, t_R the retention time and H the plate height.

Details were described by Schoenmakers and co-workers (Eeltink et al., 2009). The conclusions obtained by Schoenmakers and co-workers (Eeltink et al., 2009) indicates that the highest peak production rate in 2D-LC mode was obtained when applying second-dimension RP gradients of 20 min duration on a 150 mm long column packed with 3 μm particles. The optimal ^{2}D gradient time is strongly influenced by the column equilibrium time. In the case when increasing column length, the equilibrium time also increases, decreasing the final peak production rate in 2D-LC (Eeltink et al., 2009).

Gradient elution provides significant improvement of the peak capacity in comparison to isocratic conditions. In the second dimension, gradients are limited to a short-time period available for separation. Various types of second-dimension gradients in comprehensive LC x LC are compared: (i) ''full in fraction'', (ii) ''segment in fraction'' and (iii) ''continuously shifting'' gradients, applied in orthogonal LC x LC separations was described by Jandera et al. (Jandera et al., 2010). The porous shell columns provide narrow bandwidths and fast second-dimension separations at moderate operating pressure that allows important savings of the overall separation time in comprehensive LC x LC separations. The effects of the gradient type on the bandwidths, theoretical peak capacity, separation time and column pressure in the second dimension were investigated by these authors (Jandera et al., 2010).

The coupled-column (LC–LC) configuration consisting of a 3 μm C column (50x4.6 mm I.D.) as the first column and a 5 μm C_{18} semi-permeable-surface (SPS) column (150x4.6 mm I.D.) as the second column appearing to be successful for the screening of acidic pesticides in surface water samples was described by Hogendoorn et al. (Hogendoorn et al., 1999). In comparison to LC–LC employing two C_{18} columns, the combination of C_{18}/SPS-C_{18} significantly decreased the baseline deviation caused by the hump of the co-extracted humic substances when using UV detection (217 nm). The developed LC–LC procedure by Hogendoorn et al. (Hogendoorn et al., 1999) allowed the simultaneous determination of the target analytes bentazone and bromoxynil in uncleaned extracts of surface water samples to a level of 0.05 μg/L in less than 15 min. In combination with a simple solid-phase extraction step (200 mL of water on a 500 mg C_{18} -bonded silica) the analytical procedure provides a high sample throughput. During a period of about five months more than 200 ditch-water samples originating from agricultural locations were analyzed with the developed procedure. Validation of the method was performed by randomly analyzing recoveries of water samples spiked at levels of 0.1 μg/L (n=10), 0.5 μg/L (n=7) and 2.5 μg/L (n=4). Weighted regression of the recovery data showed that the method provides overall recoveries of 95 and 100% for bentazone and bromoxynil, respectively, with corresponding intra-laboratory reproducibilities of 10 and 11%, respectively. The performance of different columns in LC-LC configuration is illustrated in **Figure 23** (Hogendoorn et al., 1999). In

comparison to the use of two C_{18} columns in LC-LC the C_{18}/SPS combination improves significantly the elution profile of humic acid interferences allowing quantification of both herbicides to the required level in these types of samples described by Hogendoorn et al. (Hogendoorn et al., 1999).
The details about LC-LC and LC x LC methods with references were also described in the previous study (Tuzimski, 2011a).

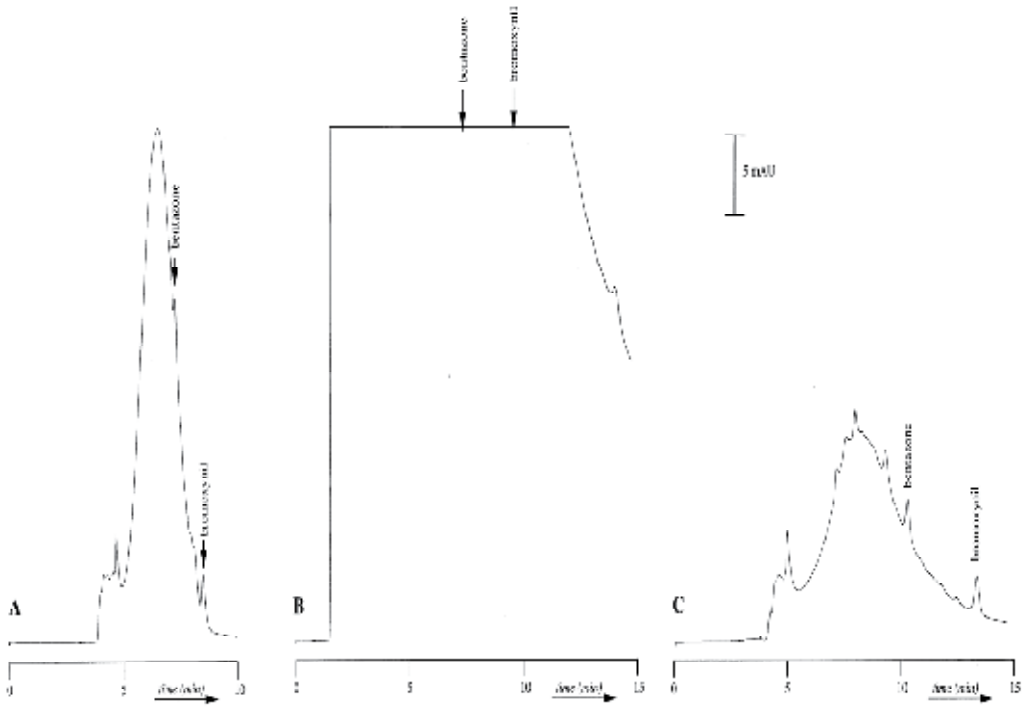


Fig. 23. RPLC–UV (217 nm) of an extract of ditch-water sample spiked with bromoxynil and bentazone at a level of 0.5 µg/ L employing different LC column configurations. (A) LC–LC on two 3 µm C_{18} columns (50x4.6 mm I.D.) /(100x4.6 mm I.D.); M-1, acetonitrile–0.03 *M* phosphate buffer, pH 2.4 (35:65); M-2, acetonitrile–0.03 *M* phosphate buffer, pH 2.4 (40:60); clean-up volume, 2.9 ml; transfer volume, 0.9 ml. (B) LC on a 5 µm SPS C_{18} column (150x4.6 mm), M, acetonitrile–0.03 *M* phosphate buffer, pH 2.4 (40:60). (C) LC–LC on 3 µm C_{18} (50x4.6 mm I.D.) /5 mm SPS (150x4.6 mm I.D) columns; further LC conditions as in (A). (A, B and C): Injection, 200 µl of SPE extract corresponding to 10 ml of water sample; flow-rate, 1 ml /min. (From Hogendoorn et al., 1999. With permission.).

2.3 Gas chromatography

Multidimensional or comprehensive two-dimensional gas chromatography (GCxGC) is a relatively new technique that can analyze multicomponent samples on different GC phases in the same analysis. GCxGC has a number of advantages over single column techniques. In this mode, two different chromatographic columns are connected in series through a modulator, which traps the analytes eluting from the primary column and re-injects them in

small compressed packets onto the secondary column. Columns coupled in series provide better separation of components of sample through different physical and chemical properties (e.g., boiling point/polarity versus shape selection) in the two steps. The GCxGC provides much better chromatographic resolution and peak capacity than single column system. GCxGC can also be used as screening method for various groups of pesticides (Tuzimski, 2011a). The details about GC x GC methods with examples and references were described in the previous study of an earlier published by the same author: *Multidimensional chromatography in pesticides analysis*. In: Pesticides – strategies for pesticides analysis. (Ed.) Margarita Stoytcheva. InTech, Rijeka 2011, pp. 155-196 (Tuzimski, 2011a).

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Recent Techniques Applied for Pesticides Identification and Determination in Natural Products and Its Impact to Human Health Risk

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1. Introduction

Based on the compilation of the British Crop Protection Council, approximately 860 active substances are formulated in pesticide products currently (Tomlin, 2003). These substances belong to more than 100 substance classes. Benzoylureas, carbamates, organophosphorous compounds, pyrethroids, sulfonylureas, or triazines are the most important groups. The chemical and physical properties of these pesticides may differ considerably. There are several acidic pesticides; others are neutral or basic and some compounds contain halogens, others phosphorous, sulfur, or nitrogen. These heteroatoms may have relevance for the detection of pesticides in natural products. Pesticides such as polychlorinated biphenyls PCB'S organochlorines and organophosphates are found in various parts of the environment in quite small concentrations, but they accumulate and thus become a threat to human health and life. Maximum residue levels (or tolerances) have been established for pesticides in foodstuffs and drinking water in most countries to avoid any adverse impact on public health, and to insist on good agricultural practice. For these reasons a large number of researchers are involved in the surveillance of maximum residue levels or in the identification and quantification of pesticide residues in environmental matrices. A lot of these pesticides were registered in Egypt or most frequently detected in fruits and vegetables in Egyptian market as well as in Europe and USA. To control local, imported and exported food, multi-residue analytical methods are preferred to reduce the workload. In this study, simple and reliable multi-residue method of analysis for determination of pesticide residues in different agricultural products was developed. In this method different pesticide groups, e.g. organophosphates, moderately polar organochlorines, benzimidazoles, N-methylcarbamates and phenoxy acids could be analyzed in one multiresidue method using Liquid Chromatography tandem mass spectrometry (LC-MS/MS) and fulfill the Codex and EU regulations. Grape, green beans even vegetable samples were extracted by shaking with acetonitrile .Phase separation was induced by shaking with buffer—salt mixture consisting of magnesium sulfate, sodium chloride, disodium hydrogen citrate sesquihydrate and trisodium citrate dihydrate .The sample was centrifuged and an aliquot of the clear solution dried by shaking with magnesium sulphate . The extract was centrifuged and an aliquot of the clear solution evaporated, re-dissolved in methanol/water buffer solution and injected into LC-system (Afify, 2010) .Quantitation and

identity confirmation was attained by using atmospheric pressure electrospray positive ionization LC-MS/MS in multiple reactions monitoring MRM mode .The recoveries of pesticides at three different concentration levels 0.01, 0.05 and 0.1 mg/kg ranged from 70 to 110 % . The repeatability expressed as relative standard deviation RSDr (was l-25) % n = 6 . Matrix matched standards were used to compensate for the matrix effect.
The present chapter will concern extensively with Multiresidue method for determination of 150 pesticides in grapes and green beans by validating and using Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction method followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). In addition GC systems with three different detectors (GC-ECD, GC-NPD and GC-MSD) were used. Compare between GC (ECD, NPD and MSD) and LC-MS/MS for its efficiency and sensitivity were carried out . The mass spectrometric parameters were optimized to give the best sensitivity, two MRM's were chosen for quantification and conformation of pesticides. The selected MRM's based on the optimized declustring potential and collision energy were used which help pesticides selectivity and justification. Protein binding of Serum Transferrin and Albumin with pesticides during transportation in the living cells will be studied including three pesticides (Trichlorphenol, Fenvalerate and α-Endosulphan). Impact of pesticides contamination to human risk through studying pesticides contamination in milk as well as potatoes tuber were investigated.

2. Materials and methods

2.1 Materials

Polyethylene or PFTE 15 ml and 50 ml with screw cap tubes .Centrifuge Heraeus up to 4000 rcf .(LC–MS/MS was performed with an Agilent 1200 Series HPLC instrument coupled to an API 4000 Q-trap MS/MS from Applied Biosystems with electrospray ionization ESI interface.

2.1.1 Pesticides

Reference standards for 150 pesticides were obtained from Dr. Ehrensdorfer (Augsburg, Germany), Purity was >95%. The common names, KOW log, field of use and chemical class of the tested pesticides are shown in Table (1) (British crop protection council 2002).

2.1.2 Samples

Different types of agricultural products (e.g. green beans and grapes) were purchased from local markets in Egypt. Samples were grinded with high speed grinder 2 litter capacities jar with lid and stored at -20 ±2 ^{0}C till sample analysis according to Codex Alimentarius (2003).

2.2 Methods

2.2.1 Reagents

Acetonitrile from Lab-scan HPLC, assay >99(%, Methanol, 99.9 %HPLC grade Merck. Formic Acid, 98-100) % Riedel–de Haen , Ammonia solution, 33 % Riedel–de Haen , Sodium chloride, 99 % Riedel–de Haen , Disodium hydrogencitrate sesquihydrate , Fluka, Trisodium citrate dihydrate ,Fluka, Sodium chloride and anhydrous magnesium sulphate Merck, De-ionized water was produced by Milli-Q unit Millipore.
Buffer-salt-mixture for second extraction and partitioning was prepared by weighing 4±0.2 g of anhydrous magnesium sulphate, 1±0.05 g of sodium chloride, 1±0.05 g of trisodium

citrate dehydrate, 3.16 and 0.5±0.03 g of disodium hydrogencitrate, sesquihydrate into 25 ml glass tube. LC mobile phase was 10 mM ammonium format solution in methanol-water (1:9), pH 4±0.1 . Sample dilution buffer was 10 mM ammonium format solution in methanol/water (1:1),pH 4±0.1 .Pesticide reference standards purity >95 %were from Dr . Ehrensdorfer ,Augsburg, Germany.

2.3 Standard preparations

2.3.1 Stock solution

Reference standard solutions (1000 µg/ml) of all the analyzed pesticides were prepared in methanol. Stock solution was kept at -20 ± 2 °C. (Banerjee et al., 2007).

2.3.2 Intermediate mixture solution

Mixture 10 µg/ml from all compounds was prepared as intermediate stock solution in methanol and used as spiking mixture and kept at -20 ± 2 °C (Banerjee et al., 2007).

2.3.3 Calibration mixture solution

Calibration mixtures of concentration levels 0.005, 0.01, 0.05, 0.1 and 0.5 µg/ml were prepared in methanol: ammonium format buffer 10 mM pH 4 (1:1) kept at -20 ± 2 °C.

2.4 Pesticide stability mixtures

The calibration mixture was prepared in 5 different pH (3,4,5,6 and 7) in the same solution (methanol : ammonium format buffer 10 mM, 1:1) and concentration (0.5 ppm) to check pesticides stability for 2 weeks then injected 4 different calibration mixture solution after storage at -20 ± 2 °C for two weeks and calibration mixture 0.5 ppm in methanol as reference standard solution fresh prepared.

2.5 Extraction procedure

Extraction procedures used in our studied for analysis of 150 pesticides in grapes as well as in green beans were described as follows:

2.5 Extraction procedures

2.5.1 QuEChERS method as described by Anastassiades, et al., (2008)

Green beans sample (10g) was add in Polyethylene (PFTE) 50 ml tube then 10 ml acetonitrile was add and shacked vigorously for one minute, then buffer-salt-mixture (4g±0.2g of magnesium sulfate anhydrous, 1g±0.05 g of sodium chloride, 1g±0.05 g of trisodium citrate dehydrate and 0.5g±0.03g of disodium hydrogencitrate sesquihydrate) added and shakes immediately for one minute. Centrifugation carried out at 4000 rpm for 5 minutes. Supernatant (4 ml) of the clear solution was transferred to 50 ml round-bottomed flask and evaporated with rotary evaporator at 40 °C. Residues were redissolved in 4 ml (Methanol: ammonium format buffer 10 mM pH 4 (1:1). Injection of 25 µl of the sample into LC-MS/MS system was carried out.

2.5.2 Luke et al., (1975) method

Green beans sample (50g) was add with 100 ml acetone and blended for 2 min at medium speed, homogenized sample is filtered through Buchner funnel containing filter paper

(Whatman no.1) fitted on Buchner flask, the blender jar is rinsed with 50 ml acetone and filtered again on the same funnel, the extracted volume is recovered.
A 40 ml sample extract is transferred to 500 ml separator funnel, 50 ml petroleum ether and 50 ml dichloromethane are added and shake vigorously for 2 min, transfer the lower aqueous layer to graduated cylinder, the upper organic layer is transferred by passing through anhydrous sodium sulphate supported on washed cotton in funnel on receiving flask, about 2g sodium chloride is added to the aqueous phase and shake vigorously for 1 min until most sodium chloride dissolved, transfer it to the same separator funnel, 50ml dichloromethane is added and shake for 1 min, lower dichloromethane layer is filtered through sodium sulphate, the water layer is taken and the last dichloromethane partitioning step is repeated, sodium sulphate is rinsed with 25ml dichloromethane, the received solution is evaporated using rotary evaporator to about 2ml at 35-40 °C, continued evaporation by air just to dryness, the residue was re-dissolved in 10 ml [Methanol: ammonium format buffer 10 mM pH 4 (1:1) and filtered through 0.45 µm syringe filter, the clear filtrate was injected directly into LC/MS/MS system.

2.5.3 Ethyl acetate method by Banerjee et al., (2007)

Green beans sample (50g) was add with 10 ml ethyl acetate in 50 ml PTFE centrifuge tube and blended for 1 min. An aliquot of 4 ml was evaporated using rotary evaporator at 40 ^{o}C just to dryness. The residue was re-dissolved in 4 ml [Methanol: ammonium format buffer 10 mM pH 4 (1:1) and filtered through 0.45 µm syringe filter. The clear filtrate was injected directly into LC-MS/MS system.

2.6 Choosing of pesticides

The 150 chosen pesticides used in this investigation were collected and identified with type of pesticides, chemical class, Field of use and KOW logP as shown in the following table:

Pesticides	KOW logP	Field of use	Chemical class
1-Abamectin	4.4	Insecticide, acaricide	Bio Pesticide
2-Acephate	-0.89	Insecticide	organophosphorus
3-Acetamiprid	0.8	Insecticide	neonicotinoid
4-Aldicarb	1.359	Insecticide, nematicide	carbamate
5-Aldicarb Sulfoxide	0.97	Insecticide, nematicide	carbamate
6-Aldicarb Sulphone	1.13	Insecticide, nematicide	carbamate
7-Ametryn	2.63	Herbicide	triazine
8-Aminocarb	1.73	Insecticide	carbamate
9-Anilofos	3.81	Herbicide	organophosphorus
10-Atrazine	2.5	Herbicide	triazine
11-Azinophos-ethyl	3.18	Insecticide	organophosphorus
12-Azinphos-methyl	2.96	Insecticide	organophosphorus

Pesticides	KOW logP	Field of use	Chemical class
13-Azoxystrobin	2.5	Fungicide	methoxyacrylate
14-Benalaxyl	3.54	Fungicide	phenylamide
15-Bendiocarb	1.72	Insecticide	carbamate
16-Bensulfuron-Me	2.45	Herbicide	sulfonylurea
17-Bromuconazole	3.24	Fungicide	triazole
18-Bupirimate	3.9	Fungicide	pyrimidinol
19-Buprofezin	4.3	Insecticide, acaricide	thiadiazines
20-Butachlor	4.5	Herbicide	chloroacetamide
21-Butralin	4.93	Herbicide	dinitroaniline
22-Carbaryl	1.85	Insecticide	carbamate
23-Carbendazim	1.38	Fungicide	benzimidazole
24-Carbofuran	1.52	Insecticide, nematicide	carbamate
25-Carbofuran-3OH	1.1	Insecticide, nematicide	carbamate
26-Carboxin	2.2	Fungicide	oxathiin
27-Chlorfluazuron	5.8	Insecticide	benzoylurea
28-Chlorpyrifos	4.7	Insecticide	organophosphorus
29-Chlorpyrifos-methyl	4.24	Insecticide	organophosphorus
30-Clodinafop-propargyl	3.9	Herbicide	aryloxyphenoxypropionate
31-Clothianidin	5	Insecticide	neonicotinoid
32-Cyanophos	2.65	Insecticide	organophosphorus
33-Cyhalothrin-L	6.9	Insecticide	pyrethroid
34-Cymoxanil	0.59	Fungicide	Unclassified
35-Cyprodinil	3.9	Fungicide	anilinopyrimidine
36-Deltamethrin	4.6	Insecticide	pyrethroid
37-Demeton-S-methylsulphon	-0.47	Insecticide	organophosphorus
38-Diafenthiuron	5.76	Insecticide, acaricide	thiourea
39-Diazinon	3.3	Insecticide, acaricide	organophosphorus
40-Dichlofuanid	3.7	Fungicide	sulphamide
41-Diclorvos	1.16	Insecticide	organophosphorus
42-Difenoconazole	4.2	Fungicide	triazole

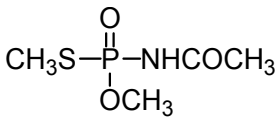
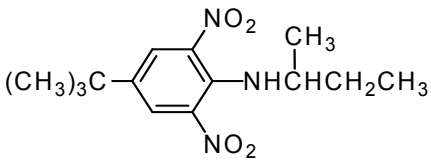
Pesticides	KOW logP	Field of use	Chemical class
43-Diflufenican	4.9	Herbicide	pyridinecarboxamide
44-Dimethoate	0.704	Insecticide, acaricide	organophosphorus
45-Dimethomorph	2.63	Fungicide	cinnamic acid
46-Diniconazole	4.3	Fungicide	triazole
47-Diuron	2.85	Herbicide	urea
48-Edifenphos	3.83	Fungicide	phosphorothiolate
49-Ethion	4.28	Acaricide, insecticide	organophosphorus
50-Ethoprophos	3.59	Nematicide, insecticide	organophosphorus
51-Famoxadone	4.65	Fungicide	oxazolidinedione
52-Fenamiphos	3.3	Nematicide	organophosphorus
53-Fenarimol	3.69	Fungicide	pyrimidine
54-Fenhexamid	3.51	Fungicide	hydroxyanilide
55-Fenoxaprop-P-ethyl	1.83	Herbicide	Aryloxyphenoxy-propionate
56-Fenpropathrin	6	Acaricide, insecticide	pyrethroid
57-Fenpyroximate	5.01	Acaricide	pyrazole
58-Fenthion	4.84	Insecticide	organophosphorus
59-Fipronil	4	Insecticide	phenylpyrazole
60-Flamprop	3.09	Herbicide	Arylalanine
61-Flufenoxuron	4	Insecticide, acaricide	benzoylurea
62-Flumetsulam	0.68	Herbicide	triazolopyrimidine
63-Fluroxypyr	-1.24	Herbicide	pyridinecarboxylic acid
64-Flusilazole	3.74	Fungicide	triazole
65-Flutolanil	3.7	Fungicide	oxathiin
66-Hexaconazole	3.9	Fungicide	triazole
67-Hexythiazox	2.53	Acaricide	thiazolidinone
68-Imazalil	3.82	Fungicide	imidazole
69-Imazamethabenz-methyl	1.54	Herbicide	imidazolinone
70-Imidacloprid	0.57	Insecticide	neonicotinoid
71-Indoxacarb	4.65	Insecticide	oxadiazine
72-Isoprothiolane	3.3	Fungicide	phosphorothiolate

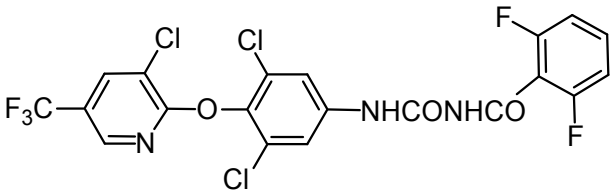
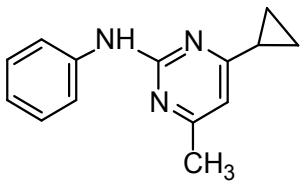
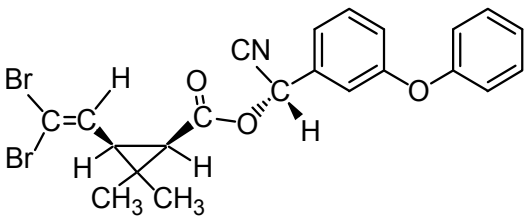
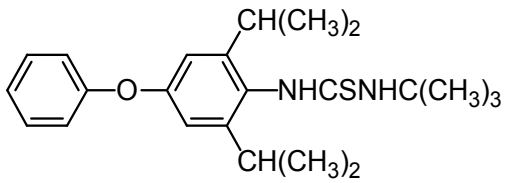
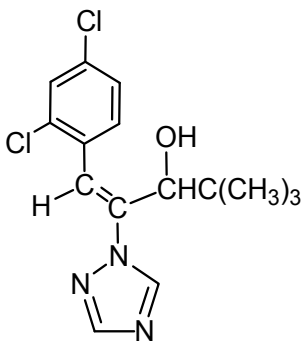
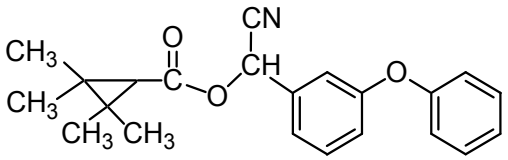
Pesticides	KOW logP	Field of use	Chemical class
73-Isoproturon	2.5	Herbicide	phenyl-urea
74-Linuron	3	Herbicide	phenyl-urea
75-Lufenuron	5.12	Insecticide, acaricide	benzoylurea
76-Malaoxon	2.89	Insecticide, acaricide	organophosphorus
77-Malathion	2.75	Insecticide, acaricide	organophosphorus
78-Metamitron	0.83	Herbicide	triazinone
79-Methamidophos	-0.8	Insecticide, acaricide	organophosphorus
80-Methiocarb	3.08	Molluscicide, insecticide	carbamate
81-Methiocarb Sulfoxid	2.87	Molluscicide, insecticide	carbamate
82-Methiocarb Sulphon	2.95	Molluscicide, insecticide	carbamate
83-Methomyl	0.093	Insecticide, acaricide	carbamate
84-Methoxyfenozide	3.7	Insecticide	diacylhydrazine
85-Metosulam	0.9778	Herbicide	triazolopyrimidine
86-Metribuzin	1.6	Herbicide	triazinone
87-Metsulfuron-methyl	-1.74	Herbicide	sulfonylurea
88-Monocrotophos	-0.22	Insecticide, acaricide	organophosphorus
89-Myclobutanil	2.94	Fungicide	triazole
90-Nuarimol	3.18	Fungicide	pyrimidine
91-Omethoate	-0.74	nsecticide, acaricide	organophosphorus
92-Oxadiargyl	3.95	Herbicide	oxadiazole
93-Oxadiazon	4.91	Herbicide	oxadiazole
94-Oxamyl	-0.44	Insecticide, acaricide	carbamate
95-Oxycarboxin	0.772	Fungicide	oxathiin
96-Oxydemeton-methyl	-0.74	Insecticide	organophosphorus
97-Paraoxon-ethyl	1.98	Insecticide, acaricide	organophosphorus
98-Parathion-ethyl	3.83	Insecticide, acaricide	organophosphorus
99-Penconazole	3.72	Fungicide	triazole
100-Pencycuron	4.68	Fungicide	phenylurea
101-Pendimethalin	5.18	Herbicide	dinitroaniline

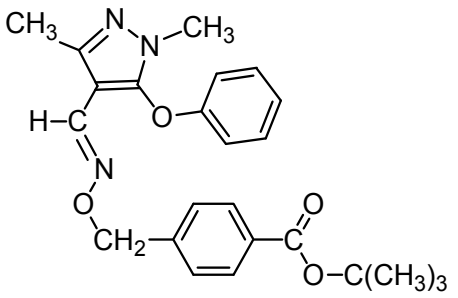
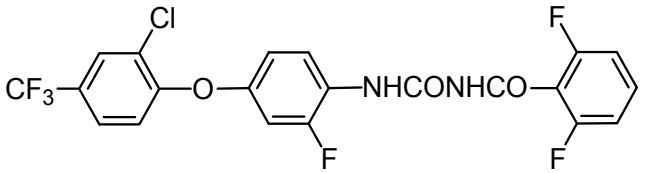
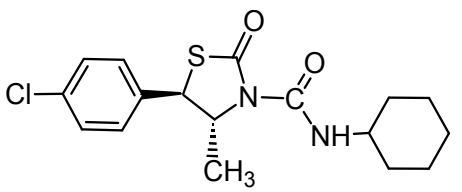
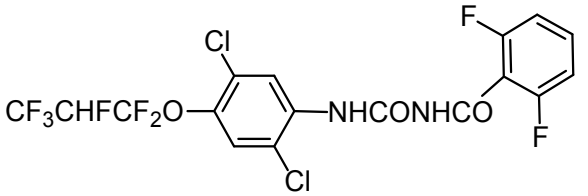
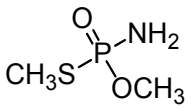
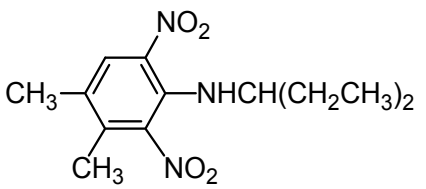
Pesticides	KOW logP	Field of use	Chemical class
102-Phenmedipham	3.59	Herbicide	carbamate
103-Phenthoate	3.69	Insecticide, acaricide	organophosphorus
104-Phosalone	4.01	Insecticide, acaricide	organophosphorus
105-Phosphamidon	0.79	Insecticide, acaricide	organophosphorus
106-Piperonyl butoxide	4.75	Insecticide	hydrocarbone
107-Pirimicarb	1.7	Insecticide	carbamate
108-Pirimiphos-ethyl	5	Insecticide	organophosphorus
109-Pirimiphos-methyl	4.2	Insecticide, acaricide	organophosphorus
110-Prochloraz	4.12	Fungicide	imidazole
111-Profenofos	4.44	Insecticide, acaricide	organophosphorus
112-Promecarb	3.189	Insecticide	carbamate
113-Prometryn	3.1	Herbicide	triazine
114-Propamocarb-HCl	-2.6	Fungicide	carbamate
115-Propargite	3.73	Acaricide	organosulfite
116-Propiconazole	3.72	Fungicide	triazole
117-Propoxur	1.56	Insecticide	carbamate
118-Pymetrozine	-0.18	Insecticide	pyridine
119-Pyrazophos	3.8	Fungicide	phosphorothiolate
120-Pyrazosulfuron-ethyl	1.3	Herbicide	sulfonylurea
121-Pyrethrins	5.9	Insecticide, acaricide	pyrethrin
122-Pyrifenox	3.4	Fungicide	pyridine
123-Pyrimethanil	2.84	Fungicide	anilinopyrimidine
124-Pyriproxyfen	5.37	Insecticide	Unclassified
125-Quizalofop-Et	4.28	Herbicide	Aryloxyphenoxy propionic acid
126-Spinosad-A	2.8	Insecticide	Spinosyns
127-Spinosad-D	3.2	Insecticide	Spinosyns
128-Tebuconazole	3.7	Fungicide	triazole
129-Tebufenozide	4.25	Insecticide	diacylhydrazine
130-Terbuthylazine	3.21	Herbicide	triazine
131-Tetraconazole	3.56	Fungicide	triazole

Pesticides	KOW logP	Field of use	Chemical class
132-Thiabendazole	2.39	Fungicide	benzimidazole
133-Thiacloprid	1.26	Insecticide	neonicotinoid
134-Thiamethoxam	-0.13	Insecticide	neonicotinoid
135-Thifensulfuron-methyl	0.2	Herbicide	sulfonylurea
136-Thiobencarb	3.42	Herbicide	thiocarbamate
137-Thiocyclam-OH	-0.07	Insecticide	Nereistoxin analogues
138-Thiodicarb	1.62	Insecticide, molluscicide	oxime carbamate
139-Thiometon	3.15	Insecticide, acaricide	organophosphorus
140-Thiophanate-methyl	1.5	Fungicide	benzimidazole
141-Tolclofos-methyl	4.56	Fungicide	aromatic hydrocarbon
142-Tolylfluanid	3.9	Fungicide	sulphamide
143-Triadimefon	3.11	Fungicide	triazole
144-Triadimenol	3.08	Fungicide	triazole
145-Triazophos	3.34	Insecticide, acaricide	organophosphorus
146-Triclopyr-butotyl	0.42	Herbicide	pyridinecarboxylic acid
147-Trifloxystrobin	4.5	Fungicide	oximinoacetate
148-Triflumizole	5.06	Fungicide	imidazole
149-Triforine	2.2	Fungicide	piperazine
150-Triticonazole	3.29	Fungicide	triazole

Table 1. Tested pesticides with their KOW logP, field of use and chemical class (British crop protection council 2002).

Pesticides	Structure
1-Acephate KOW logP = -0.89	
2-Butralin less soluble in water	

Pesticides	**Structure**
3-Chlorfluazuron	
4-Cyprodinil	
5-Deltamethrin less soluble in water	
6-Diafenthiuron less soluble in water	
7-Diniconazole less soluble in water	
8-Fenpropathrin KOW logP = 6 less soluble in water	

Pesticides	**Structure**
9-Fenpyroximate	
10-Flufenoxuron Solubility In water 7×10-11 g/l	
11-Hexythiazox	
12-Lufenuron	
13-Methamidophos Solubility In water >200 g/l KOW logP = -0.8	
14-Pendimethalin	

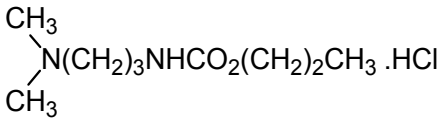
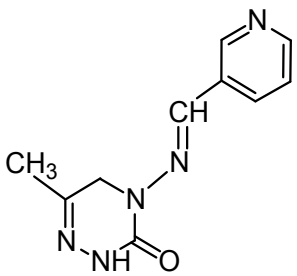
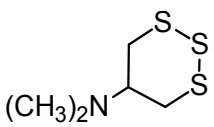
Pesticides	Structure
15-Propamocarb-HCl KOW logP = -2.6	
16- Pymetrozine	
17-Thiocyclam-HO KOW logP = -0.07	

Table 2. Chemical structure of seventeen selected pesticides:

2.8 Risk/safety assessment

The insecticide residues concentrations found in the analyzed potatoes were compared with the tolerance limits established by Codex Alimentarius Commission and the Egyptian Organization for Standardization and Quality Control (EOS), respectively. The dietary intake of insecticides was estimated and compared with the WHO-ADIs, (Tomlin, 2004) as cited by Mansour et al., (2009) as follow:

Estimated dose (mg/kg) = Residues (mg/kg) Food item x daily potato consumption (kg) / Body weight (kg)

3. Analysis methods of pesticides residues

3.1 LC-MS/MS

3.1.1 LC-MS/MS analysis

Separation was performed on a C18 column ZORBAX Eclipse XDB-C18 4.6 mm x 150 mm, 5 µm particle size .The injection volume was 25 µl .A gradient elution program was at 0.3 ml/min flow, in which one reservoir contained 10 mM ammonium format solution in methanol-water 1:9 and the other contained methanol .The ESI source was used in the positive mode, and N2 nebulizer, curtain, and other gas settings were optimized according to recommendations made by the manufacturer; source temperature was 300°C, ion spray potential 5500 V, decluster potential and collision energy were optimized using A Harvard Apparatus syringe pump by introducing individual pesticide solutions into the MS instrument to allow optimization of the MS/MS conditions. The Multiple Reaction monitoring mode MRM was used in which one MRM was used for quantitation and other was used for confirmation .

3.2 GC - measurements with different detectors

3.2.1 GC-NPD parameter

GC-NPD analyses were run on HP 6890 series gas chromatograph equipped with nitrogen phosphorous detector (NPD). Data acquisition, processing, and instrumental control were performed by the Agilent ChemStation software. A split/split less (S/Sl) inlet was used with 1.8 mm id liner. Analytes were separated in an Agilent HP-Pass 5 capillary column, 25 m length ,0.32 mm id, 0.52 µm film thickness. The inlet operating temperature is 225 °C, injection volume 1 µL. The nitrogen carrier gas flow was maintained at a constant flow of 1.3 ml/minute. N_2 make up gas flow rate 8 ml/minute for the NPD and H_2 with flow rate of 4.5 ml/minute. The oven temperature program was 90 °C for 2 minute, programmed to 150 °C at 20 °C/minute, and then to 270 °C at 6 °C/minute, it was kept at this temperature for 15 minute. Detector temperature was maintained at 280 °C with H_2 flow of 3.5 ± 0.1 ml/minute and air flow of 100-120 ml/minute.

3.2.2 GC-ECD parameter

GC-ECD analyses were run on HP 6890 series gas chromatograph equipped with electron capture detector (ECD). Data acquisition, processing, and instrumental control were performed by the Agilent ChemStation software. A split/split-less (S/Sl) inlet was used with 1.8 mm id liner. Analyte samples were separated in an Agilent HP-Pass 5 capillary column, 25 m length, 0.32 mm id, and 0.52 µm film thicknesses. The inlet operating temperature is 225 °C, injection volume 1 µl. The nitrogen carrier gas flow was maintained at a constant flow of 1.3 ml/minute. The oven temperature program was 90 °C for 2 minute, programmed to 150 °C at 20 °C/minute, and then to 270 °C at 6 °C/minute, it was kept at this temperature for 15 minute. Detector temperature was maintained at 300 °C.

3.2.3 GC-MSD parameter

GC-MSD analyses were run on an Agilent 7890 series gas chromatograph (Agilent Technologies, Santa Clara, CA) interfaced to an Agilent 5975 mass selective detector (MSD). Data acquisition, processing, and instrumental control were performed by the Agilent MSD ChemStation software (E.0200.493 version). A split/split less (S/Sl) inlet was used with 1.8 mm id liner. Analyte samples were separated in an Agilent HP-5MS capillary column (5% biphenyl/95% dimethylsiloxane), 30 m, 0.25 mm id, 0.25 µm film thickness. The inlet operating conditions were injection volume, 1 µl, flow rate 1.3 ml/minute; the temperature program was set at 79 °C for 0.25 minute, programmed to 300 °C at 10 °C/minute, and kept at this temperature for 2 minute. The helium carrier gas flow was maintained at a constant pressure of 17.296 psi. The oven temperature program was 70 °C for 1 minute, programmed to 150 °C at 50 °C/minute, then to 200 °C at 6 °C/min, and finally to 280 °C at 16 °C/minute; it was kept at this temperature for 5 minute. Electron impact mass spectra in the full-scan mode were obtained at 70 eV; the monitoring was from m/z 50 to 400. The ion source and quadrupole analyzer temperatures were fixed at 230 and 150 °C, respectively.

4. Results and discussion

4.1 Recovery tests on grapes

The method recoveries for 150 pesticides were tested by performing 6 replicates of spike grapes at different concentration levels; 0.01, 0.05 and 0.1 mg/kg. The average recoveries and relative standard deviation on each level were calculated (Table 3). The precursor ion,

product ion (1) and product ion (2) and retention time will be included in the tables. The injection of 25 µl of acetonitrile into LC system leads to non-symmetrical peak shapes, so that acetonitrile was evaporated and re-dissolved in methanol-water solution. This step improved the pesticide peak shapes and lowered the matrix effect due to precipitation of some insoluble substances. The recovery of most pesticides (143 pesticides) is in the range 70%-110%. The recoveries of 7 pesticides (Chlorfluazuron, L- Cyhalothrin, Deltamethrin, Diafenthiuron, Flufenoxuron, Lufenuron and Pymetrozine) are lower than 70% due to the evaporation of acetonitrile and re- dissolving in methanol-water solution as reported by Afify et al., (2010). The conclusions stated that the proposed method using acetonitrile extraction followed by LC-MS/MS determination is simple, rapid and reliable satisfactory recoveries and repeatability observed .The described method requires little amount of solvents and sample and could be used in controlling levels of pesticides from different classes in natural products samples.

						0.01 mg/kg		0.05 mg/kg		0.1mg/kg	
No.	**Pesticide**	**RT**	**Precursor ion**	**Product ion (1)**	**Product ion (2)**	**Mean Rec.%**	**CV%**	**Mean Rec.%**	**CV%**	**Mean Rec.%**	**CV%**
1	Abamectin	27.5	890.5	305.3	143.0	94%	13%	76%	25%	85%	17%
2	Acephate	10.8	184.0	143.0	126.0	85%	4%	91%	12%	70%	10%
3	Acetamiprid	16.5	223.2	126.0	86.2	97%	5%	95%	7%	83%	3%
4	Aldicarb	12.3	208.2	116.0	186.0	86%	7%	90%	5%	68%	11%
5	Aldicarb Sulfoxide	12.8	207.3	132.0	163.1	88%	6%	97%	10%	81%	5%
6	Aldicarb Sulphone	18.6	223.1	86.2	116.0	66%	27%	77%	18%	85%	14%
7	Ametryn	22.2	228.0	186.0	152.1	93%	4%	99%	8%	84%	3%
8	Aminocarb	16.3	209.0	152.1	198.9	87%	2%	85%	8%	74%	3%
9	Anilofos	23.3	367.9	198.9	174.0	84%	1%	98%	6%	86%	4%
10	Atrazine	21.2	216.1	174.0	132.0	88%	4%	98%	13%	87%	3%
11	Azinophos Ethyl	22.8	346.3	132.0	132.0	85%	6%	94%	18%	70%	8%
12	Azinphos Methyl	21.5	318.0	132.0	372.0	78%	9%	92%	17%	85%	12%
13	Azoxystrobin	21.2	404.0	372.0	148.1	100%	6%	98%	9%	89%	4%
14	Benalaxyl	23.7	326.3	148.1	167.0	98%	3%	107%	9%	79%	4%
15	Bendiocarb	19.5	224.0	167.0	149.0	96%	3%	96%	9%	92%	4%
16	Bensulfuron Methyl	21.2	411.0	149.0	159.0	89%	2%	95%	14%	90%	7%
17	Bromuconazole	22.7	378.0	159.0	166.2	91%	2%	101%	9%	87%	4%
18	Bupirimate	23.2	317.0	166.2	201.0	93%	5%	93%	8%	87%	3%
19	Buprofezine	25.1	306.2	201.0	238.0	95%	7%	76%	13%	82%	6%
20	Butachlor	25.5	312.3	238.0	240.1	73%	8%	89%	8%	69%	7%
21	Butralin	26.7	296.0	240.1	145.1	72%	3%	71%	5%	74%	6%
22	Carbaryl	20.0	202.1	145.1	160.1	99%	3%	99%	10%	91%	5%
23	Carbendazim	17.1	192.1	160.1	165.0	87%	2%	85%	8%	74%	3%
24	Carbofuran	19.6	222.1	165.0	142.9	96%	2%	94%	11%	88%	4%
25	Carbofuran-3OH	16.7	238.3	163.1	209.2	90%	3%	98%	4%	86%	6%
26	Carboxin	20.1	236.0	142.9	158.0	102%	5%	92%	11%	88%	8%
27	Chlorfluazuron	25.7	540.0	158.0	197.9	52%	10%	34%	11%	58%	11%
28	Chlorpyrifos	26.0	349.9	197.9	124.9	86%	3%	82%	5%	79%	5%
29	Chlorpyrifos Methyl	24.6	322.0	124.9	105.0	99%	6%	84%	5%	74%	6%

						0.01 mg/kg		0.05 mg/kg		0.1mg/kg	
No.	Pesticide	RT	Precursor ion	Product ion (1)	Product ion (2)	Mean Rec.%	CV%	Mean Rec.%	CV%	Mean Rec.%	CV%
30	Clodinafop propargyl	23.1	350.0	266.0	169.0	89%	2%	92%	8%	88%	3%
31	Clothianidin	15.9	250.0	169.0	124.9	95%	5%	92%	8%	90%	5%
32	Cyanophos	21.5	261.2	124.9	128.0	91%	7%	106%	11%	87%	7%
33	Cyhalothrin-L	25.5	467.2	225.0	141.0	68%	11%	36%	23%	65%	10%
34	Cymoxanil	17.5	199.2	128.0	93.0	88%	2%	94%	10%	82%	4%
35	Cyprodinil	24.0	226.0	93.0	169.1	88%	3%	88%	2%	80%	2%
36	Deltamethrin	26.3	523.2	281.1	181.1	46%	13%	29%	22%	65%	8%
37	Demeton-S-ethylsulphon	14.1	263.0	169.1	329.1	89%	3%	90%	10%	86%	5%
38	Diafenthiuron	26.0	385.0	329.1	169.1	26%	21%	17%	63%	38%	25%
39	Diazinon	23.8	305.1	169.1	109.0	86%	4%	108%	7%	81%	4%
40	Dichlofuanid	22.6	350.1	224.0	123.0	83%	6%	90%	9%	86%	4%
41	Diclorovs	19.5	221.0	109.0	251.0	90%	8%	96%	13%	84%	5%
42	Difenoconazole	24.0	406.0	251.0	266.0	90%	5%	98%	7%	76%	6%
43	Diflufenican	24.0	395.1	266.0	199.0	86%	3%	76%	6%	83%	2%
44	Dimethoate	16.8	230.0	199.0	301.0	96%	2%	94%	9%	86%	3%
45	Dimethomorph	22.1	388.0	301.0	70.0	90%	5%	95%	10%	95%	5%
46	Diniconazole	24.2	326.1	70.0	72.0	88%	3%	92%	7%	80%	3%
47	Diuron	21.3	233.2	72.0	283.0	92%	2%	95%	9%	88%	5%
48	Edifenophos	23.5	311.0	283.0	171.0	88%	2%	99%	8%	85%	5%
49	Ethion	25.2	385.0	171.0	131.0	86%	4%	89%	6%	80%	5%
50	Ethoprophos	23.1	243.1	131.0	141.0	94%	4%	99%	8%	85%	5%
51	Famoxadone	23.2	392.0	331.0	217.0	99%	10%	91%	7%	84%	1%
52	Fenamiphos	23.0	304.1	217.0	268.1	92%	5%	95%	10%	88%	4%
53	Fenarimol	22.8	331.1	268.1	97.0	94%	3%	93%	10%	87%	5%
54	Fenhexamid	22.6	302.0	97.0	288.1	93%	2%	98%	11%	86%	8%
55	Fenoxap-p-ethyl	24.7	362.0	288.1	366.0	96%	12%	86%	2%	81%	3%
56	Fenpropathrin	25.9	350.2	125.1	97.0	76%	6%	67%	5%	76%	6%
57	Fenpyroximate	26.6	422.0	366.0	368.0	78%	7%	70%	7%	70%	11%
58	Fenthion	23.7	279.2	247.1	169.0	91%	7%	100%	6%	81%	7%
59	Fipronil	22.6	454.0	368.0	105.1	94%	9%	102%	12%	85%	5%
60	Flamprop	21.0	321.9	105.1	129.0	87%	6%	90%	14%	83%	6%
61	Flufenoxuron	24.9	489.1	158.0	129.0	64%	8%	40%	14%	57%	9%
62	Flumetesulam	15.8	326.2	129.0	208.9	95%	4%	100%	8%	90%	6%
63	Fluroxypyr	17.4	255.2	208.9	165.1	92%	8%	106%	19%	84%	8%
64	Flusilazole	22.8	316.1	165.1	262.0	89%	4%	97%	9%	87%	4%
65	Flutolanil	21.9	324.0	262.0	70.0	89%	2%	98%	9%	80%	4%
66	Hexaconazole	23.8	314.0	70.0	228.0	91%	4%	90%	9%	79%	5%
67	Hexythiazox	25.9	353.1	228.0	144.2	88%	3%	85%	3%	72%	6%
68	Imazalil	22.1	297.0	159.0	201.0	85%	4%	92%	6%	83%	5%
69	Imazamethabenz Methyl	19.7	289.0	144.2	99.0	92%	2%	94%	8%	85%	5%
70	Imidacloprid	15.8	256.2	209.2	203.1	97%	3%	96%	5%	80%	6%

						0.01 mg/kg		0.05 mg/kg		0.1mg/kg	
No.	Pesticide	RT	Precursor ion	Product ion (1)	Product ion (2)	Mean Rec.%	CV%	Mean Rec.%	CV%	Mean Rec.%	CV%
71	Indoxacarb	23.7	528.0	203.1	189.0	90%	4%	92%	13%	95%	9%
72	Isoprothiolane	22.5	291.0	189.0	72.0	95%	2%	96%	6%	86%	2%
73	Isoproturon	21.3	207.3	72.0	182.1	94%	6%	97%	7%	86%	3%
74	Linuron	22.2	249.1	182.1	158.0	94%	3%	95%	7%	80%	2%
75	Lufenuron	24.3	511.0	158.0	99.0	83%	11%	45%	22%	61%	10%
76	Malaoxon	19.6	315.1	99.0	220.2	95%	3%	100%	5%	87%	3%
77	Malathion	22.2	331.0	99.0	185.0	92%	4%	105%	9%	91%	4%
78	Metamitron	17.1	203.1	175.1	94.0	90%	10%	101%	7%	78%	4%
79	Methamidophos	9.8	142.2	94.0	122.0	96%	18%	83%	3%	66%	5%
80	Methiocarb	15.9	243.0	169.1	88.0	91%	2%	93%	10%	88%	4%
81	Methiocarb Sulfoxid	16.8	242.1	185.0	122.0	91%	3%	99%	3%	85%	3%
82	Methiocarb Sulphon	22.2	275.1	122.0	169.1	91%	4%	91%	4%	70%	8%
83	Methomyl	14.2	163.2	88.0	149.0	90%	5%	90%	2%	82%	4%
84	Methoxyfenozide	22.2	369.0	149.0	175.0	92%	5%	97%	9%	80%	3%
85	Metosulam	19.4	418.0	175.0	187.1	96%	7%	92%	5%	89%	5%
86	Metribuzin	20.1	215.2	187.1	167.2	94%	4%	96%	5%	86%	3%
87	Metsulfuron Methyl	18.8	382.3	167.2	127.0	96%	4%	100%	7%	90%	7%
88	Monocrotophos	14.7	224.0	127.0	70.0	89%	2%	97%	2%	84%	3%
89	Myclobutanil	22.4	289.0	70.0	252.0	94%	2%	95%	7%	88%	3%
90	Nuarimol	22.0	315.0	252.0	183.0	93%	4%	102%	7%	80%	4%
91	Omethoate	11.8	214.0	183.0	223.1	85%	3%	94%	5%	76%	4%
92	Oxadiargyl	23.7	340.8	223.1	303.0	91%	3%	90%	8%	91%	6%
93	Oxadiazon	25.4	345.3	303.0	175.0	86%	7%	94%	15%	82%	7%
94	Oxamyl	13.0	237.0	72.0	88.1	96%	3%	95%	11%	85%	8%
95	Oxycarboxin	17.5	268.0	175.0	169.0	94%	1%	99%	5%	87%	4%
96	Oxydemeton Methyl	13.8	247.0	169.0	219.9	85%	2%	96%	6%	79%	5%
97	Paraoxon Ethyl	20.7	276.2	219.9	235.9	96%	2%	100%	6%	84%	3%
98	Parathion Ethyl	23.4	292.2	235.9	159.0	109%	14%	82%	19%	90%	16%
99	Penconazole	23.7	284.0	159.0	125.0	91%	5%	92%	8%	89%	5%
100	Pencycuron	24.1	329.1	125.0	168.1	91%	3%	90%	7%	90%	5%
101	Pendimethalin	26.5	282.1	212.0	194.0	83%	4%	76%	17%	82%	5%
102	Phenmedipham	21.2	301.3	168.1	247.2	95%	5%	90%	8%	90%	3%
103	Phenthoate	23.4	321.0	247.2	182.0	90%	4%	95%	10%	86%	2%
104	Phosalone	23.9	368.0	182.0	174.0	93%	2%	95%	10%	90%	5%
105	Phosphamidon	18.9	300.1	174.0	177.0	96%	3%	98%	7%	82%	2%
106	Piperonyl butoxid	25.9	356.0	177.0	182.3	92%	2%	95%	11%	78%	17%
107	Pirimicarb	21.0	239.2	182.3	198.0	94%	2%	98%	3%	81%	4%
108	Pirimiphos Ethyl	25.6	334.1	198.0	164.0	94%	5%	96%	13%	80%	12%
109	Pirimiphos Methyl	24.4	306.2	164.0	308.0	94%	4%	109%	12%	80%	11%
110	Prochloraz	24.1	376.0	308.0	302.9	92%	5%	92%	11%	88%	8%

						0.01 mg/kg		0.05 mg/kg		0.1mg/kg	
No.	Pesticide	RT	Precursor ion	Product ion (1)	Product ion (2)	Mean Rec.%	CV%	Mean Rec.%	CV%	Mean Rec.%	CV%
111	Profenofos	25.1	373.0	302.9	109.1	103%	15%	98%	13%	76%	21%
112	Promecarb	22.4	208.0	109.1	158.0	98%	3%	97%	5%	82%	3%
113	Prometryn	23.2	242.0	158.0	102.0	93%	2%	94%	8%	85%	3%
114	Propamocarb	12.2	189.0	102.0	175.1	82%	3%	88%	4%	71%	7%
115	Propargite	26.0	368.1	175.1	159.0	85%	3%	76%	17%	82%	13%
116	Propiconazole	23.8	342.1	159.0	111.1	95%	3%	92%	9%	83%	6%
117	Propoxur	19.7	210.1	111.1	105.1	90%	2%	103%	6%	84%	3%
118	Pymetrozine	13.6	218.2	105.1	222.0	40%	2%	56%	4%	30%	12%
119	Pyrazophos	24.2	374.0	222.0	182.1	99%	3%	86%	10%	89%	5%
120	Pyrazosulfroun Ethyl	22.5	415.3	182.1	161.1	91%	2%	79%	22%	88%	14%
121	Pyrethrins	26.4	329.1	161.1	93.0	84%	2%	84%	20%	82%	6%
122	Pyrifenox	23.4	295.0	93.0	107.2	90%	3%	92%	6%	78%	6%
123	Pyrimethanil	22.7	200.1	107.2	96.0	89%	3%	95%	7%	86%	3%
124	Pyriproxyfen	25.9	322.2	96.0	299.0	84%	4%	79%	15%	81%	16%
125	Quizalofop Ethyl	25.2	373.0	299.0	178.4	88%	9%	111%	14%	78%	19%
126	Spinosad-A	23.4	732.0	142.0	142.0	96%	2%	88%	11%	83%	4%
127	Spinosad-D	24.1	746.0	142.0	70.1	87%	10%	97%	10%	78%	5%
128	Tebuconazole	23.6	308.0	70.1	133.0	90%	2%	91%	11%	91%	6%
129	Tebufenozide	23.1	353.0	133.0	174.0	92%	5%	95%	13%	90%	7%
130	Terbuthialzine	22.5	230.0	174.0	159.0	92%	2%	96%	8%	87%	3%
131	Tetraconazole	22.6	372.0	159.0	175.0	97%	4%	96%	10%	83%	2%
132	Thiabendazole	18.5	202.1	175.0	126.0	79%	4%	101%	1%	75%	5%
133	Thiacloprid	17.4	253.2	126.0	211.0	93%	6%	96%	6%	84%	4%
134	Thiamethoxam	14.4	292.0	211.0	167.0	94%	4%	98%	4%	78%	5%
135	Thifensulfuron Methyl	18.6	388.2	167.0	125.0	95%	1%	105%	10%	87%	4%
136	Thiobencarb	24.5	258.3	125.0	137.1	87%	3%	101%	10%	83%	8%
137	Thiocyclam	11.1	182.0	137.1	151.0	73%	7%	73%	5%	50%	5%
138	Thiodicarb	19.8	355.1	88.1	108.0	79%	24%	96%	12%	91%	6%
139	Thiometon	23.3	247.1	88.9	169.0	85%	25%	103%	22%	88%	14%
140	Thiophanate Methyl	19.4	343.0	151.0	175.0	80%	13%	99%	8%	82%	5%
141	Tolclophos Methyl	24.3	301.1	175.0	238.0	89%	7%	91%	13%	88%	10%
142	Tolylfluanid	23.4	364.0	238.0	197.0	88%	4%	95%	13%	90%	6%
143	Triadimifon	22.6	294.1	197.0	162.0	98%	3%	94%	8%	85%	1%
144	Triadiminol	22.6	296.1	70.0	277.0	87%	3%	98%	13%	88%	7%
145	Triazophos	22.6	314.2	162.0	155.0	93%	3%	94%	7%	88%	2%
146	Triclopyr Butatyl	25.5	356.2	237.7	186.0	89%	3%	84%	15%	83%	9%
147	Trifloxystrobin	24.1	409.0	186.0	278.0	93%	2%	94%	8%	89%	5%
148	Triflumizole	24.4	346.3	278.0	387.8	90%	5%	95%	12%	76%	17%
149	Triforine	21.3	432.4	387.8	70.0	103%	8%	92%	8%	87%	6%
150	Triticonazole	23.0	318.3	70.0	567.4	89%	3%	92%	7%	83%	5%

Table 3. Recovery tests at different concentration levels on grapes sample

4.2 Recovery tests on green beans

The optimized LC-MS/MS parameters and the best extraction procedures (QuEChERS) were used to study the method performance by carrying out recovery tests of pesticides at different levels on green beans samples. Six replicates of recovery tests were done at concentration levels 0.01 mg/kg, 0.05 mg/kg and 0.1 mg/kg on grapes and green beans, Table(4).

Pesticides	Green beans			Pesticides	Green beans		
	0.01 mg/kg (%)	0.05 mg/kg (%)	0.1 mg/kg (%)		0.01 mg/kg (%)	0.05 mg/kg (%)	0.1 mg/kg (%)
Abamectin	74 ±20	104 ±11	68 ±13	Malaoxon	89 ±15	93 ±5	87 ±4
Acephate	94 ±19	75 ±6	78 ±5	Malathion	95 ±18	97 ±3	81 ±6
Acetamiprid	91 ±12	92 ±4	78 ±3	Metamitron	85 ±14	92 ±6	84 ±3
Aldicarb	90 ±14	91 ±4	77 ±6	Methamidophos	73 ±18	78 ±3	77 ±2
Aldicarb Sulfoxide	81 ±31	84 ±6	83 ±3	Methiocarb	80 ±15	98 ±9	91 ±7
Aldicarb Sulphone	98 ±14	90 ±4	83 ±3	Methiocarb Sulfoxid	93 ±12	90 ±3	77 ±3
Ametryn	96 ±13	96 ±2	78 ±4	Methiocarb Sulphon	89 ±16	91 ±5	86 ±3
Aminocarb	86 ±9	85 ±4	73 ±4	Methomyl	95 ±22	90 ±5	89 ±3
Anilofos	84 ±12	92 ±1	77 ±4	Methoxyfenozide	82 ±21	102 ±1	83 ±4
Atrazine	91 ±15	92 ±4	80 ±3	Metosulam	87 ±12	93 ±4	86 ±4
Azinophos-ethyl	85 ±16	97 ±10	82 ±6	Metribuzin	98 ±25	86 ±6	81 ±4
Azinphos-methyl	97 ±10	106 ±9	79 ±8	Metsulfuron-methyl	85 ±20	104 ±3	87 ±4
Azoxystrobin	98 ±14	96 ±4	78 ±2	Monocrotophos	89 ±22	87 ±5	87 ±4
Benalaxyl	95 ±14	93 ±3	76 ±4	Myclobutanil	92 ±17	95 ±5	85 ±4
Bendiocarb	93 ±11	91 ±3	78 ±2	Nuarimol	78 ±20	89 ±4	81 ±2
Bensulfuron-Me	105 ±14	92 ±4	79 ±3	Omethoate	82 ±24	81 ±5	84 ±2
Bromuconazole	87 ±12	90 ±6	81 ±4	Oxadiargyl	75 ±16	84 ±4	77 ±7
Bupirimate	85 ±11	93 ±4	75 ±4	Oxadiazon	71 ±17	85 ±5	73 ±10
Buprofezin	98 ±18	94 ±4	74 ±6	Oxamyl	99 ±16	89 ±5	80 ±4
Butachlor	81 ±17	110 ±5	66 ±13	Oxycarboxin	87 ±17	92 ±5	87 ±2
Butralin	63 ±19	68 ±7	79 ±6	Oxydemeton-methyl	91 ±24	83 ±3	86 ±4
Carbaryl	93 ±13	93 ±2	79 ±2	Paraoxon-ethyl	89 ±15	91 ±3	86 ±4
Carbendazim	89 ±11	90 ±3	74 ±3	Parathion-ethyl	88 ±36	118 ±12	80 ±20
Carbofuran	99 ±16	93 ±2	80 ±2	Penconazole	78 ±16	89 ±3	85 ±5
Carbofuran-3OH	90 ±15	87 ±5	87 ±4	Pencycuron	71 ±16	84 ±2	81 ±7
Carboxin	76 ±15	67 ±18	73 ±5	Pendimethalin	61 ±19	74 ±1	62 ±11
Chlorfluazuron	24 ±17	30 ±5	24 ±16	Phenmedipham	88 ±18	88 ±2	86 ±3
Chlorpyrifos	65 ±15	76 ±6	68 ±4	Phenthoate	87 ±19	91 ±4	86 ±5
Chlorpyrifos-methyl	80 ±16	82 ±10	77 ±3	Phosalone	77 ±19	82 ±3	80 ±9

Pesticides	Green beans			Pesticides	Green beans		
	0.01 mg/kg (%)	0.05 mg/kg (%)	0.1 mg/kg (%)		0.01 mg/kg (%)	0.05 mg/kg (%)	0.1 mg/kg (%)
Clodinafop-propargyl	82 ±13	90 ±3	68 ±4	Phosphamidon	90 ±15	94 ±5	93 ±7
Clothianidin	93 ±12	85 ±4	82 ±3	Piperonyl butoxide	77 ±23	90 ±2	85 ±4
Cyanophos	95 ±11	94 ±8	72 ±6	Pirimicarb	91 ±18	97 ±3	89 ±1
Cyhalothrin-L	25 ±27	32 ±12	31 ±17	Pirimiphos-ethyl	84 ±18	99 ±3	87 ±4
Cymoxanil	97 ±12	88 ±4	79 ±4	Pirimiphos-methyl	83 ±19	113 ±8	88 ±6
Cyprodinil	80 ±10	89 ±3	68 ±4	Prochloraz	86 ±18	87 ±2	72 ±11
Deltamethrin	23 ±22	29 ±5	24 ±14	Profenofos	75 ±19	85 ±4	82 ±5
Demeton-S-methylsulphon	95 ±15	94 ±4	83 ±5	Promecarb	88 ±20	95 ±3	87 ±4
Diafenthiuron	11 ±30	6 ±35	42 ±64	Prometryn	83 ±16	91 ±3	83 ±4
Diazinon	96 ±15	118 ±4	82 ±4	Propamocarb-HCl	81 ±23	73 ±5	81 ±3
Dichlofuanid	87 ±16	69 ±12	53 ±4	Propargite	78 ±19	71 ±4	64 ±12
Diclorvos	113 ±19	69 ±16	79 ±6	Propiconazole	78 ±25	89 ±3	86 ±4
Difenoconazole	86 ±17	86 ±4	74 ±4	Propoxur	87 ±15	92 ±4	86 ±3
Diflufenican	64 ±12	79 ±4	68 ±5	Pymetrozine	59 ±28	68 ±8	56 ±5
Dimethoate	96 ±14	91 ±4	77 ±3	Pyrazophos	73 ±20	91 ±3	83 ±5
Dimethomorph	99 ±13	94 ±4	85 ±24	Pyrazosulfuron-ethyl	82 ±32	96 ±2	85 ±14
Diniconazole	92 ±14	90 ±3	80 ±7	Pyrethrins	62 ±20	86 ±4	61 ±15
Diuron	100 ±10	95 ±3	77 ±2	Pyrifenox	96 ±9	95 ±4	78 ±8
Edifenphos	82 ±11	92 ±3	77 ±4	Pyrimethanil	87 ±17	87 ±3	81 ±4
Ethion	64 ±16	79 ±5	68 ±4	Pyriproxyfen	65 ±17	80 ±1	71 ±8
Ethoprophos	86 ±31	91 ±6	80 ±7	Quizalofop-Et	72 ±17	85 ±3	74 ±7
Famoxadone	68 ±14	79 ±6	69 ±6	Spinosad-A	78 ±17	84 ±5	61 ±13
Fenamiphos	81 ±9	86 ±5	72 ±3	Spinosad-D	87 ±6	92 ±8	69 ±11
Fenarimol	78 ±14	90 ±5	77 ±4	Tebuconazole	76 ±21	95 ±13	84 ±9
Fenhexamid	88 ±7	88 ±7	58 ±5	Tebufenozide	88 ±17	89 ±6	90 ±7
Fenoxaprop-P-ethyl	112 ±20	81 ±6	117 ±17	Terbuthylazine	82 ±21	93 ±2	86 ±4
Fenpropathrin	48 ±17	60 ±7	52 ±7	Tetraconazole	92 ±20	93 ±3	87 ±5
Fenpyroximate	66 ±19	65 ±6	60 ±8	Thiabendazole	86 ±16	92 ±6	81 ±3
Fenthion	82 ±16	88 ±9	67 ±4	Thiacloprid	84 ±16	90 ±4	82 ±3
Fipronil	99 ±13	87 ±6	85 ±7	Thiamethoxam	87 ±21	84 ±4	91 ±4
Flamprop	86 ±13	84 ±5	76 ±3	Thifensulfuron-methyl	86 ±20	99 ±3	89 ±2
Flufenoxuron	29 ±17	39 ±7	34 ±15	Thiobencarb	77 ±20	86 ±3	87 ±4
Flumetsulam	105 ±14	91 ±4	78 ±5	Thiocyclam-OH	75 ±23	66 ±4	68 ±4

Pesticides	Green beans			Pesticides	Green beans		
	0.01 mg/kg (%)	0.05 mg/kg (%)	0.1 mg/kg (%)		0.01 mg/kg (%)	0.05 mg/kg (%)	0.1 mg/kg (%)
Fluroxypyr	104 ±20	88 ±9	77 ±5	Thiodicarb	95 ±12	96 ±2	73 ±3
Flusilazole	92 ±14	92 ±6	75 ±4	Thiometon	115 ±35	90 ±19	86 ±12
Flutolanil	94 ±12	99 ±4	76 ±3	Thiophanate-methyl	71 ±35	83 ±19	79 ±30
Hexaconazole	97 ±16	91 ±4	78 ±4	Tolclofos-methyl	84 ±18	82 ±6	76 ±8
Hexythiazox	68 ±16	74 ±5	79 ±5	Tolylfluanid	76 ±21	89 ±6	80 ±9
Imazalil	113 ±20	98 ±4	74 ±3	Triadimefon	84 ±19	89 ±3	87 ±3
Imazamethabenz-methyl	98 ±14	90 ±2	78 ±3	Triadimenol	97 ±23	87 ±8	80 ±4
Imidacloprid	85 ±16	93 ±5	85 ±4	Triazophos	88 ±19	92 ±2	89 ±2
Indoxacarb	70 ±22	90 ±3	74 ±10	Triclopyr-butotyl	68 ±16	84 ±2	77 ±8
Isoprothiolane	86 ±18	94 ±2	90 ±3	Trifloxystrobin	73 ±19	86 ±2	85 ±6
Isoproturon	90 ±17	90 ±3	90 ±2	Triflumizole	93 ±21	85 ±4	76 ±8
Linuron	77 ±16	94 ±3	88 ±4	Triforine	53 ±18	87 ±10	76 ±39
Lufenuron	43 ±18	55 ±18	42 ±18	Triticonazole	85 ±19	89 ±3	84 ±4

Table 4. Recovery tests on green beans samples at 0.01 mg/kg, 0.05 mg/kg and 0.1 mg/kg.

The results in Table (4) showed that the 150 pesticides could be determined at concentration 0.01 mg/kg with accepted recovery and precision. The recovery of most pesticides (143 pesticides) is in the range 60%-120%, as cited for grapes. The recoveries of the same 7 pesticides (Chlorfluazuron, L-Cyhalothrin, Deltamethrin, Diafenthiuron, Flufenoxuron, Lufenuron and Pymetrozine) are lower than 60% due to the evaporation of acetonitrile and re-dissolving in methanol-water solution mixture as approved in the recovery tests of pesticides in grapes(Afify et al ., 2010) .0n the other hand recovery test of some pesticides exceeds 100 at concentration at 0.01 mg/kg (Flumetsulam, Fluroxypyr Imazalil), at concentration of 0.05 mg/kg (Butachlor, Pirimiphos-methyl, Metsulfuron-methyl, Parathion-ethyl, Methoxyfenozide) and at concentration of 0.1 mg/kg (Fenoxaprop-P-ethyl).

5. Optimization of sample extraction

Different types of extraction procedures were tested as described in materials and methods using three method (e.g. Luke, QuEChERS and ethyl acetate according to Luke et al. (1975), Anastassiades et al., (2008): and Banerjee et al., (2007). Extraction was done on green beans sample at spiking level of 0.5 mg/kg .

Blank samples, standard in solvent and standard in matrix were injected in parallel to spike samples and in the same run. Due to suppression effect of these types of matrices (decreasing in signal intensity) standard prepared in matrix were used for recovery calculations, the results of recovery tests on green beans samples using the different three methods were discussed by the compound with recovery less than 60% Table (5).

Pesticides	Luke	Ethyl-acetate	QuEChERS
Acephate	57%	a	a
Butralin	54%	41%	a
Chlorfluazuron	34%	25%	26%
Cyhalothrin-L	31%	23%	29%
Cyprodinil	a	58%	a
Deltamethrin	19%	24%	25%
Diafenthiuron	12%	17%	20%
Diniconazole	54%	a	a
Fenpropathrin	48%	55%	53%
Fenpyroximate	50%	41%	a
Flufenoxuron	37%	30%	34%
Hexythiazox	56%	a	a
Lufenuron	a	41%	47%
Methamidophos	31%	a	a
Pendimethalin	56%	51%	a
Propamocarb-HCl	11%	1%	a
Pymetrozine	57%	58%	a
Thiocyclam-HO	46%	35%	a
Total	16	14	7

a = Accepted recovery of pesticide at ≥ 60%.

Table 5. Recovery tests on green beans samples using different extraction methods for pesticides < 60%.

As shown in Table (5) propamocarb-HCl is an example for high polar pesticides which had a low recovery in the extraction by ethyl acetate (1 %) and not completely recovered in the partitioning step in Luke method (11%) .On the other hand the solubility of the pesticides in the different methods are different depending on its polarity which could seen in the results of the recovery test of the three methods such as Pymetrozine and Fenpropathrin pesticides The same results were observed by D´ıez et al. (2006) that Luke was significantly more effective for the extraction of non-polar and medium-polar compounds, but the best recoveries for polar compounds were achieved by QuEChERS and ethyl acetate methods. QuEChERS was the only method that provided an overall recovery value of 60–70% for none, medium and polar compounds, also Kruve *et al*. (2008) reported in his comparison between Luke method and matrix solid-phase dispersion (MSPD) that the best recoveries were obtained with the QuEChERS method.
Therefore the QuEChERS extraction method was found to be better than Luke method and ethyl acetate method because of higher recovery, less solvent and short time of analysis were observed.

6. Comparison of pesticides chromatograms using GC-NPD, ECD, MSD and LC-MS/MS

The chromatograms of the 150 pesticides injected into GC systems with three different detectors ECD, NPD and MSD (Fig. 4.a,b,c) were used to compare between GC efficiency and LC-MS/MS (Fig. 5.a) in separation and sensitivity.

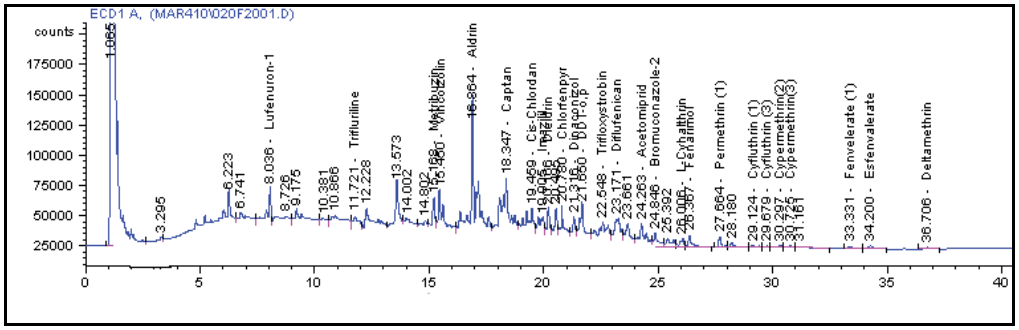


Fig. 4. a. All tested pesticides chromatogram detected by GC-ECD.

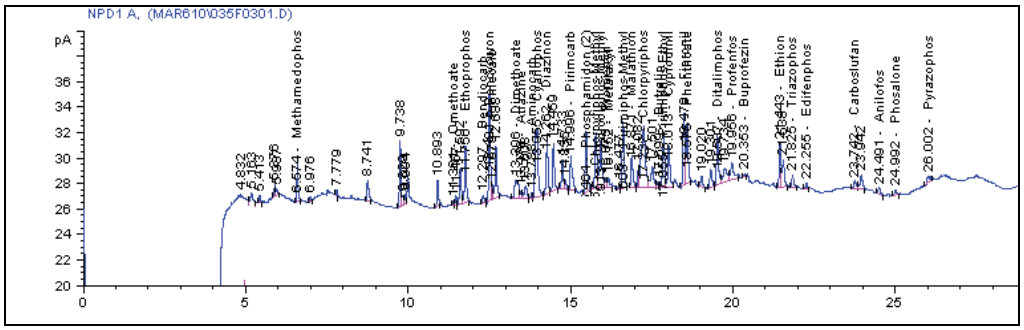


Fig. 4. b. All tested pesticides chromatogram detected by GC-NPD.

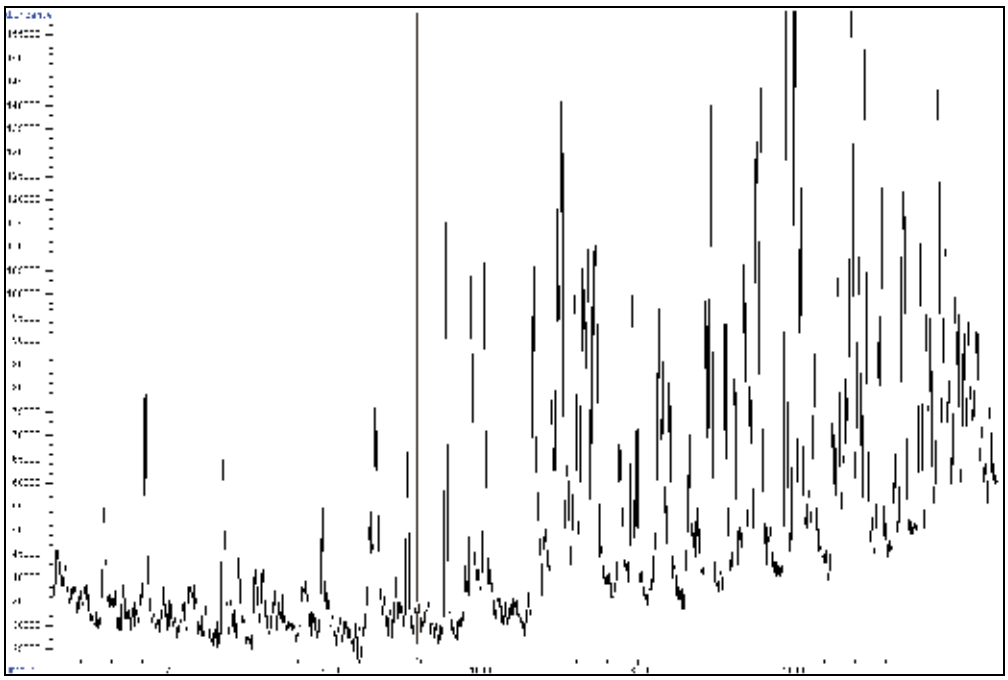


Fig. 4. c. All tested pesticides chromatogram detected by GC-MSD.

The total ion chromatogram for the 150 pesticides injected into LC-MSMS system illustrate in (Fig. 5a). It looks that the pesticide peaks are not resolved but in fact due to the high selectivity of the MS/MS system the peaks can be resolved easily (Fig. 5 b, c,d).

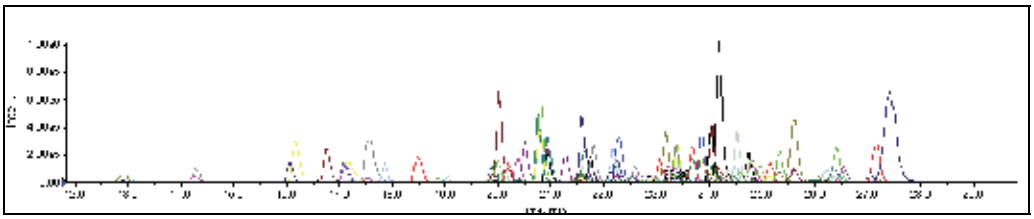


Fig. 5. a. Chromatogram of total 150 pesticides as 300 MRM.

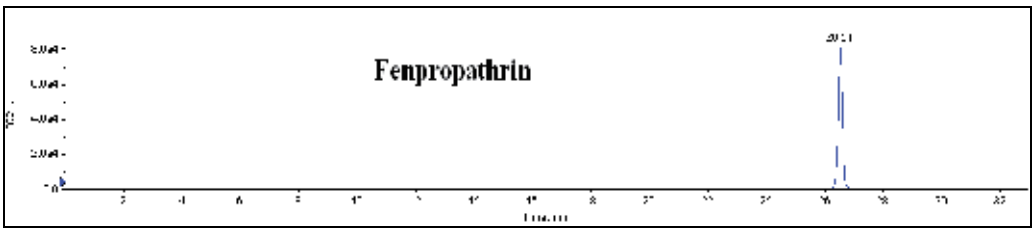


Fig. 5. b. Chromatogram of selected MRM for fenpropathrin.

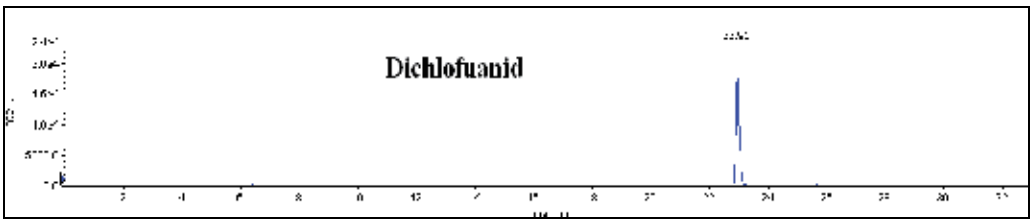


Fig. 5. c. Chromatogram of selected MRM for dichlofuanid.

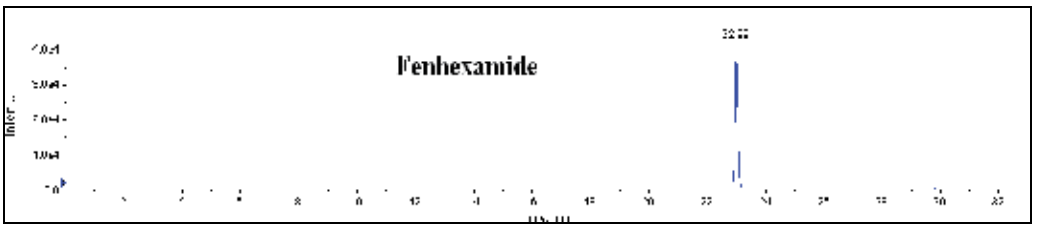


Fig. 5. d. Chromatogram of selected MRM for fenhexamide

It is observed that the pesticide peaks showed in (Fig. 4.a,b,c) by using ECD,NPD and MSD are not resolved and have very low sensitivity while in (Fig. 5a) separation of the 150 pesticides could be analyzed by single chromatographic run of 33 minutes and each MRM could be separated as single peak in a chromatogram by LC-MS/MS system as shown in

(Fig 5, a, b, c and d) for fenpropathrin, dichlofuanid and fenhexamide pesticides as studied by El-Gammal (2010).
It is clear that although dichlofuanid (Fig. 5.c) has the same molecular weight of fenpropathrin (Fig. 5b) (absence of cross talk) and has the same retention time of fenhexamide (Fig. 5d), but it is easily resolved from both compounds. These results were supported by Applied Biosystems (2004) (Application Note: Mass Spectrometry) for fenoxycarb 302/88 and methomyl 163/88 that are measured using the same product ions (but with different precursor ions) they are completely separated, (Publication 114AP30-01).

7. Optimization of mobile phase

A modified multi-residue method for analysis of 150 pesticide residues in green beans using liquid chromatography-tandem mass spectrometry by using three methods as described in material and methods ;QuEChERS as described by Pya, et al., (2008), Luke et al., (1975) method and Ethyl acetate method by Banerjee et al., (2007). The extracts solution of three methods were re-dissolved in methanol ,buffer solution (1:1) 10 mM in pH 4 as modification to increased injection volume to 25 µl without losing our good peak shape. Stabilities of tested pesticides in five different calibration mixture pH for two weeks were studied. Quantitation and identity confirmation was attained by using atmospheric pressure electrospray positive ionization LC-MS/MS in multiple reactions monitoring (MRM) mode. The signal intensity in LC-MS/MS can be influenced by the mobile phase composition. In order to optimize the signal intensity, standard mixtures in methanol were injected into the LC-MS/MS, using different mobile phase compositions. Four different buffer constituents were tested: ammonium format (0.1, 1, 5, and 10 mM) at three pH (3, 3.5 and 4). Evaluation was done by recording the MS/MS signal for each pesticide with a calculation based on 5 mM, pH 4. The mobile phase during this test was composed of 50% buffer constituent in water and 50% methanol.
Generally results showed that there is no variation in signal more than 4% between all of tested mobile phase except the 10 mM in pH 4 which had increasing in 26 compounds more than 15% as shown in the following table(6) .

Pesticides	SE	Pesticides	SE	Pesticides	SE
Pyrazophos	15%	Cyanophos	20%	Carboxin	24%
Thifensulfuron_Me	15%	Flamprop	20%	Thiocyclam HO	27%
Pyrimethanil	15%	Chlorpyrifos-Me	20%	Bensulfuron-Me	28%
Methoxyfenozide	16%	Carbofuran-3OH	21%	Aldicarb	29%
Thiobencarb	17%	Diafenthiuron	21%	Myclobutanil	30%
Triadimifon	17%	Tolylfluanid	21%	Nuarimol	32%
Butralin	19%	Isoproturon	21%	Diuron	33%
Pyrifenox	19%	Linuron	22%	Tetraconazole	38%
Prochloraz	19%	Phenthoate	23%	-	-

Table 6. Comparison between pesticides sensitivity using 10 mM buffer compared to 5 mM buffer.

SE: Signal enhancement in 10 mM buffer compare by 5 mM buffer.
Pesticides which had high matrix effect suppress its standard signals in the compounds with intensity increased up to 38 % (Tetraconazole) . However, when analyzing different samples, which themselves can influence the signal by altering the mobile phase composition, it is important to use a buffer with a sufficient buffering capacity to stabilize the system. Therefore, higher ionic strength contributes to a more stable system, both for retention and signal. By using ammonium format buffer 10 mM with pH 4, the results of 26 pesticides out of 150 pesticides compounds has increased in its sensitivity. These results approved by Jansson et al. (2004) reported that the best signal response was obtained with pH ranging from 4.0 to 4.2 and that the buffer strength of 10 mM was chosen as a compromise on 57 pesticides. Finally the use of ammonium format mobile phase 10 mM in pH 4 represented the most suitable condition for the separation and sensitivity of tested pesticides, which should be considered during determination of pesticides residues.

7.1 Effect of pH on tested pesticides stability

Standard solution of the 150 pesticides was prepared at concentration 0.5 µg/ml and kept in freezer for 15 days at -20± 2 °C and compared to fresh prepared standard solution, the stability of these pesticides at different pH showed by storage recovery in (Fig. 6) and Table (7) also the degradation of pesticides with decreasing more than 10% in different pH were measured Table (8).

Pesticides Recovery	pH 3	pH 4	pH 5	pH 6	pH 7
90-110%	133	150	148	145	142
80-90%	10	0	1	4	4
70-80%	4	0	0	0	2
<70%	3	0	1	1	2

Table 7. Effect of pH on stability of 150 pesticides standard solution.

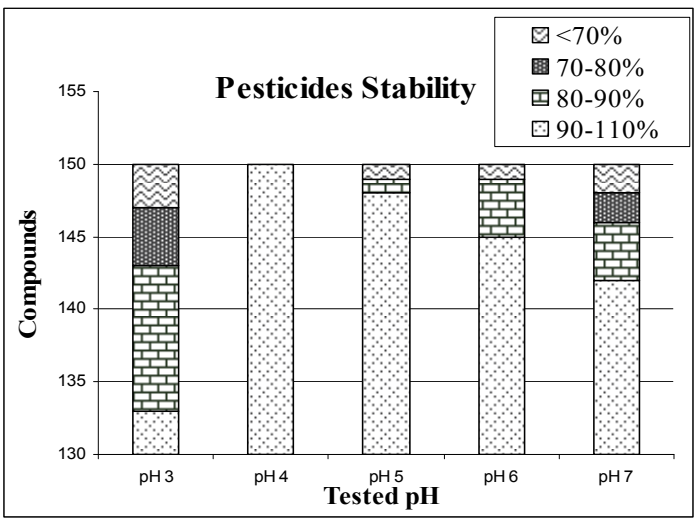


Fig. 6. Pesticides stability in pH 3, 4, 5, 6 and 7.

Pesticides	pH 3	pH 5	pH 6	pH 7
Triflumizole	56%	a	a	a
Fenoxaprop-ethyl	50%	a	a	a
Aldicarb Sulfoxide	30%	a	a	a
Thiophanate-methyl	22%	a	a	a
Metribuzin	20%	a	a	a
Propamocarb-HCl	20%	a	a	a
Thiocyclam-HO	18%	a	a	a
Acephate	16%	a	a	a
Diazinon	14%	a	a	a
Metosulam	14%	a	a	a
Diclorovs	13%	a	a	a
Omethoate	12%	a	a	a
Edifenophos	12%	a	a	a
Butachlor	12%	a	a	a
Pymetrozine	11%	a	a	a
Diafenthiuron	25%	43%	45%	37%
Diniconazole	12%	12%	11%	18%
Parathion-ethyl	a	a	24%	28%
Flamprop	a	a	17%	17%
Piperonyl-butoxide	a	a	13%	14%
Dimethomorph	a	a	a	35%
Thiobencarb	a	a	a	12%
Diflufenican	a	a	a	24%

a = Accepted stability of pesticide at tested pH (>90%).

Table 8. Degradation of pesticides at different pH 3,5,6 and 7.

The pesticides which had lost more than 10% of their concentration were showed by degradable percentage in Table (8), for example triflumizole which showed a degradation of 56% followed by 50 % for Fenoxaprop-ethyl at pH 3 . The result was in agreement with the US- EPA (EPA Pesticide Fact Sheet 10/91) studies on triflumizole which showed that hydrolysis studies of phenyl-labeled Carbon 14 triflumizole (radiochemical purity greater than 99%), at 5 ppm, degraded in sterile aqueous 0.01 M buffered solutions with half-lives of 7 to 15 days at pH 5, greater than 30 days at pH 7 and pH 3 to 17 days at pH 9 when incubated in the dark at 25± 2 ^{o}C. Fenoxaprop-ethyl showed degradation of 50% which is in agreement with the study done by Zablotowicz et al. (2000) stated that stability was pH sensitive in acidic buffered solutions; that is, below pH 4.6, rapid nonenzymatic hydrolysis of the benzoxazolyl-oxy-phenoxy ether linkage occurred, forming 6-chloro-2,3-dihydro-benzoxazol-2-one (CDHB) and ethyl 4-hydroxyphenoxypropanoate or 4-hydroxyphenoxypropanoate. Due to high sensitivity, high duty cycle and simple cleaning of the interface of the API 4000 QT, method development and recovery tests were done using methanol/buffer in pH 4 as calibration mixture solution, using this instrument.

7.2 Optimization of MS/MS

7.2.1 Optimization for precursor ion (parent) and product ion (daughter)

Pesticide standard solutions were prepared in methanol/ ammonium format buffer (1/1) at concentration level of 0.1-0.5 µg/ml and injected individually to optimize for parent ion (MS1 scanning & MS2 static) by scanning at different declustering potential (DP). The optimum DP, which gave the highest sensitivity, was used and changing the collision energy (CE) to optimize for the daughter ion (MS1 static & MS2 scanning). The standard solutions were injected directly into LC/MS/MS system without analytical column, the protonated ions were chosen in ESI+ (MW+1) mode. The compounds which gave accepted intensity with the optimized DP and CE were divided into 3 mixtures and injected into LC/MS/MS system in presence of analytical column using Multiple Reaction Monitoring mode (MRM, MS1 scanning, MS2 scanning) at the optimum DP and CE were used.

Optimization of six pesticides will be discussed as an example. In this chapter we will discuss the optimization of Acetamiprid pesticide and the detailed results of the five remaining pesticides (Lambada-Cyhalothrin, Malathion, Methomyl Propargite and Tetraconazole) were described by El-Gammal (2010).

7.2.1.1 Acetamiprid optimization

7.2.1.2 Calculation of isotopic distribution

The analyst software is used to calculate the isotope distribution, the expected nominal molecular weight of 222.1 for the parent compound also isotopic mass of 224.1 of 33% abundance due to the presence of one chlorine atom (37Cl) (Fig. 7).

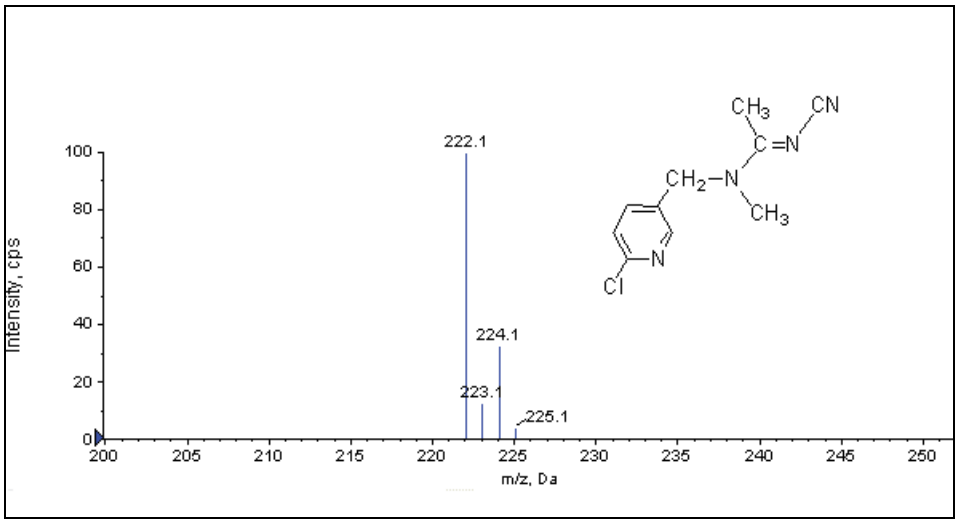


Fig. 7. Expected isotopic distribution of acetamiprid as calculated by the Analyst software.

7.2.1.3 Optimization of the precursor ions

The injection of individual standard of acetamiprid showed in (Fig. 8) and running Q1 scan (MS1 scanning & MS2 static). It is clear that the parent compound has gained a proton to give molecular ion mass at 223 (M+1), also isotopic molecular ion mass at 225 of 33% abundance due to the presence of one chlorine atom (37Cl).

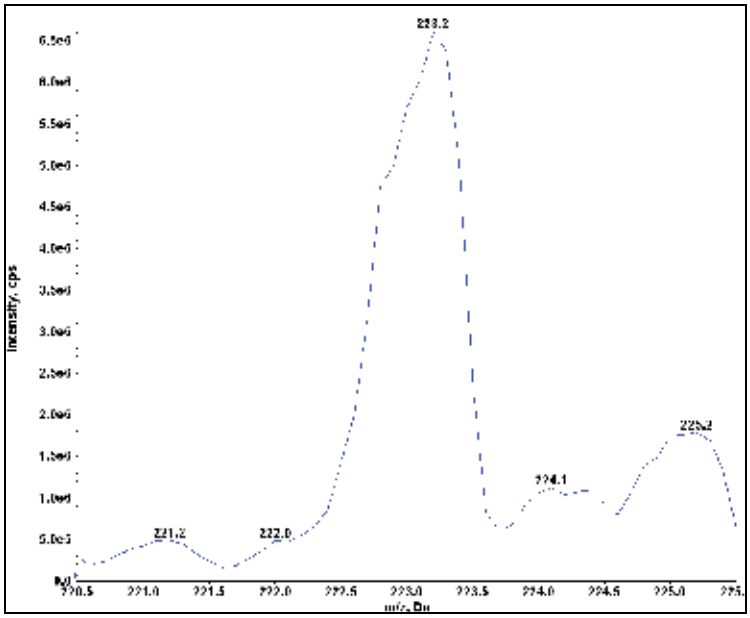


Fig. 8. Injection of individual standard of acetamiprid and running Q1 scan.

7.2.1.4 Optimization of the declustering potential

Q1 scanning (MS1 scanning & MS2 static) of acetamiprid while changing the declustering potential from 0 to 240 volts to get the optimum DP. It is clear that the optimum DP for acetamiprid is 49 volts (Fig. 9).

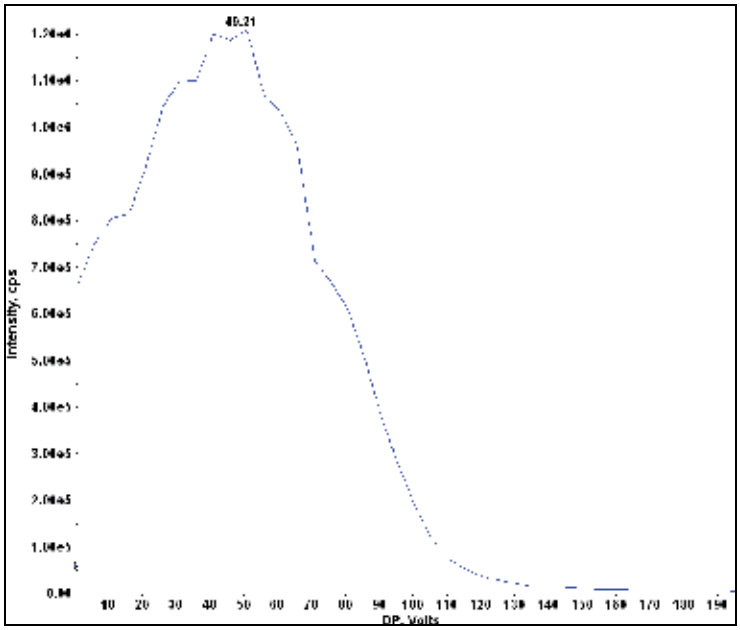


Fig. 9. Optimization of declustering potential (DP).

7.2.1.5 Optimization of the daughter ions

The fragmentation of acetamiprid in the collision cell and the Quadra poles Q1 scanning and Q3 scanning (MS1 scanning & MS2 scanning) (Figs 10, 11).

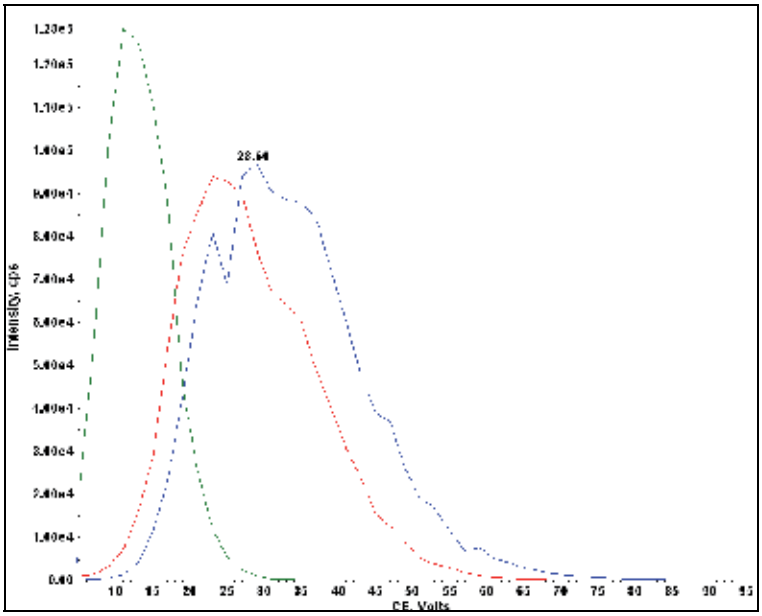


Fig. 10. Optimization of collision energy (CE)

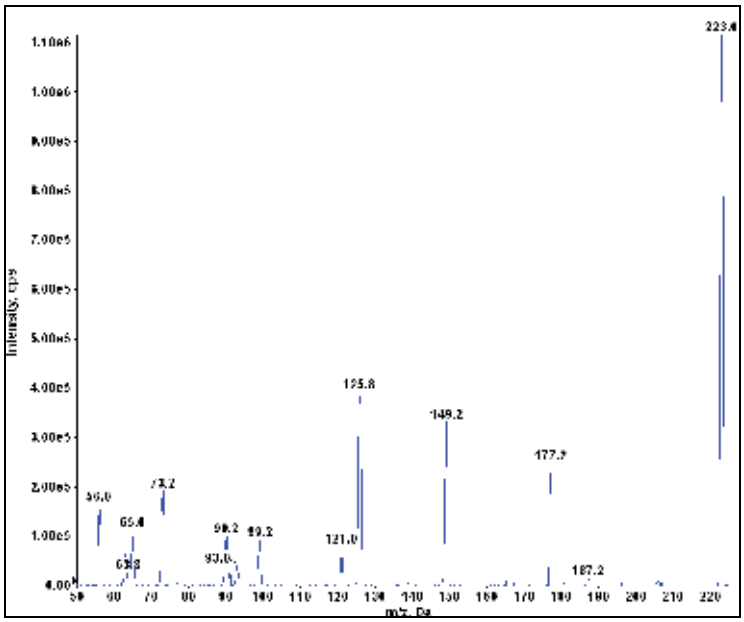


Fig. 11. LC-MS/MS spectrum of acetamiprid.

The following table(9) showed the molecular weight, the calculated molecular weight related to isotopic distribution, isotopic elements of six pesticides compound with their masses, declustring potential (DP) which was very important for the tuning of parent ion and collision energy (CE) necessary for fragmentation .

	Pesticide	Molecular weight/Da	Nominal molecular weight /Da	Isotopic element (Mass)	DP (volt)	CE (volt)
1	Acetamiprid	222.7	222.1, 223.1, 224.1	Cl(35,37) , N(14,15)	49.2	28.6
2-1	Cyhalothrin-L	449.9	449.1, 450.1, 451.1	Cl(35,37) , N(14,15) , O(16,17,18)	–	–
2-2	Cyhalothrin-L-NH4	467.9	467.1, 468.1, 469.1	Cl(35,37) , N(14,15) , O(16,17,18)	52.1	21
3	Malathion	330.4	330, 331, 332	S(32,33,34) , O(16,17,18)	66.1	31.6
4	Methomyl	162.2	162, 163, 164	S(32,33,34) , O(16,17,18) , N(14,15)	41.2	39
5	Propagiter	350.5	350.2, 351.2, 352.2	S(32,33,34) , O(16,17,18)	65.5	24.8
6	Tetraconazole	372.1	371, 372, 373	Cl(35,37) , N(14,15) , O(16,17,18)	56.6	54.4

Da = Dalton.

Table 9. Molecular weight and nominal molecular weight related to isotope distribution.

The conclusion from Table (9) showed that every pesticide compound needs this tuning to get the best conditions for highest sensitivity. It is clear also that each pesticide has different DP and CE to get the best sensitivity; these parameters have been collected to build up the acquisition method for the 150 pesticides

8. Risk assessment based pesticides contamination

8.1 Impact of pesticides contamination to human risk

Human milk the major source of infant food have been studied in detailed about the distribution of pesticides residues in all over 26 Governorate of Egypt . Different types of pesticides have been identifies in milk and the results described as follows:

8.1.1 Chlorinated insecticides levels in human milk

The data in Table 10 shows that the main detected organochlorine insecticides and their metabolites were DDE and lindane .DDT and endosulfan I residues were also detected in some milk samples .Endrin was only detected in one of the milk samples in New valley, while aldrin was not detected in any of the milk samples .However, from the 60 human milk samples, 51% of the samples were free from any detectable DDT level a fact which may suggest that there were no recent sources of pollution by intact DDT (Saleh et al., 1996a,b , 1999).

8.1.2 Hexachlorocyclohexanes HCH isomers

δ-HCH lindane was detected in 95 %of the analyzes human milk samples .The lowest levels were found in governorates between Cairo and Assiut and in Suez 0.00-10.00 ppb while the higher levels 10.00-33.00 ppb (were found in the Delta area and in Alexandria .

The higher levels could be a reflection of the use of lindane in agriculture and in the control of cattle ecto-parasites .Also, this might be due to the human consumption of large quantities of polluted fatty fish (table 10). Kucinski, (1986) have pointed out the presence of organochlorine residues including lindane in different food stuffs meat, dairy products, grain and drinks. Residues of some organochlorine pesticides OCPs, such as HCB and heptachlor as well as some organophosphorus pesticides OPPs, such as methamidophos,thiometon, profenofos, phorate and pirimiphos-methyl were found in a number of potatoes samples produced under different condition (convention, C; organic, O) at concentration levels exceeding their MRLs as reported by Mansour et al., (2009).

The results in table (10) proved that pesticides residue in human milk product depends mainly on the regional area .Pesticides residues do its effect and shows its impact factor through creation a lot of diseases as described by the transportation of the pesticides in biological system to reach its biological function .Transportation of pesticides were carried out through protein binding with the major protein in serum like serum albumin as well as other protein exists in liver such as of α-Synuclein Fibril protein Formation and other organs as described by Afify et al., (2000) and Afify (2010) .Parkinson's disease involves intracellular deposits of α-synuclein in the form of Lewy bodies and Lewy neurites .The etiology of the disease is unknown; however, several epidemiological studies have implicated environmental factors, especially pesticides .Here we show that several

	Lindane		Endosulfane I		4,4'-DDE		4,4- DDT	
Governorate*	Average	Range	Average	Range	Average	Range	Average	Range
Greater Cairo								
Cairo (7)	5.05	2.72-8.98	0.00	0.00 -0.00	11.30	1.40-19.7	0.95	0.00-2.85
Giza (8)	4.96	2.69-6.59	0.69	0.00-2.08	12.02	1.96-19.7	0.00	0.00-0.00
Kaliobia (6)	13.87	1.21-33.20	0.00	0.00-0.00	20.62	8.95-27.3	2.04	0.00-3.87
Delta Region								
Sharkia (5)	6.57	4.15-9.78	0.00	0.00-0.00	54.50	19.7-83.3	2.43	1.7-3.64
Gharbia (1)	4.63	3.47-6.49	10.5	0.00-18.90	25.82	4.06-53.3	1.68	0.00-2.53
Behera (3)	12.82	4.47-22.80	29.98	7.34-57.90	50.95	5.74-117.0	5.51	0.00-10.6
Dakahlia (2)	19.53	13.40-24.70	6.00	0.00-18.00	41.70	7.3-67.2	4.12	0.00-9.38
Menoufia (4)	1.52	4.15-9.78	0.00	0.00-0.00	7.93	2.4-10.9	2.59	0.00-7.76
Upper Egypt								
Fayoum (9)	3.77	0.68-7.82	0.00	0.00-0.00	20.06	4059-37.4	1.84	0.00-4.61
Beny Sweif (10)	2.04	0.95-2.90	3.25	0.00-5.81	27.26	9.17-46.7	1.59	0.00-3.41
Minia (11)	3.11	0.81-6.30	8.03	0.00-20.30	16.36	6.88-21.7	4.83	0.00-14.5
Assuit (12)	10.95	0.78-31.00	1.63	0.00-4.90	30.89	3.47-71.3	5.12	3.51-7.02
Sohag (13)	12.88	6.75-21.30	4.77	0.00-14.3	29.42	2.97-77.5	5.47	0.00-13.6
Aswan (14)	12.84	4.47-28.20	7.02	2.41-12.7	8.95	3.8-18.5	2.46	0.00-7.38
Costal Areas								
Alexandria (17)	12.46	2.37-20.50	0.00	0.00-0.00	9.35	7.32-12.1	0.00	0.00-0.00
Matrouh (16)	11.30	6.41-15.00	25.83	0.00-61.8	15.11	7.04-23.0	0.00	0.00-0.00
North Sinai (18)	11.81	9.92-12.9	0.00	0.00-0.00	8.84	5.17-13.7	14.52	4.77-32.9
Ismailia (19)	3.71	1.36-5.43	0.00	0.00-0.00	19.17	14.2-25.4	0.00	0.00-0.00
Suez (20)	1.44	0.00-2.21	0.00	0.00-0.00	11.35	5.78-20.3	2.39	0.00-7.16
Desert								
New Vallery (15)	13.08	8.93-18.20	0.00	0.00-0,00	5.83	4.13-8.54	1.04	0.00-3.13
Average in Egypt	8.42	0.00-31.00	4.84	0.00-61.8	1.37	1.4-117	2.93	0.00-32.9

*No .of collection samples

Table 10. Distribution of the main organochlorine insecticide residues in Egyptian Mother's milk

pesticides, including rotenone, dieldrin and paraquat, induce a conformational change in α-synuclein and significantly accelerate the rate of formation of -α synuclein fibrils in vitro . They propose that the relatively hydrophobic pesticides preferentially bind to a partially folded intermediate conformation of α -synuclein, accounting for the observed conformational changes and leading to association and subsequent fibrillation .These observations suggest one possible underlying molecular basis for Parkinson's disease .α-Synuclein, a relatively abundant brain protein of 140 amino acids and of unknown function, was first identified in association with synaptic vesicles Maroteaux *et al.*, 1988. α-Synuclein belongs to the class of proteins known as natively unfolded; i.e., the purified protein at neutral pH is substantially disordered (Uversky et al., 2001a,b) .

8.2 Impact of pesticides contamination in potatoes

Table (11) presents a survey for the numbers and percentages of contaminated samples, as well as the violated ones. In case of (C) potatoes contaminated samples with HCB accounted to 41.7%, compared to 16.7%, for (O) potatoes, while 33.3% and 16.7%, of the total samples exceeded the MRL of HCB, for (C) and (O) potatoes, respectively. The highest percentage of insecticide contamination of (C) potatoes reached 58.3% with methamidophos in case of (O) potatoes the highest percentage reached 25% with heptachlor.

Insecticide	Contaminated samples with each insecticide				Violated samples			
	C		O		C		O	
	n	%	n	%	n	%	n	%
HCB	15	41.7	6	16.7	12	33.3	6	16.7
lindane	18	50.0	6	16.7	0	0.0	0	0.0
heptachlor	12	33.3	9	25.0	12	33.3	9	25.0
aldrin	nd	-	3	8.3	-	-	0	0.0
dieldrin	12	33.3	3	8.3	4	11.1	0	0.0
o,p-DDD	6	16.7	3	8.3	-	-	-	-
p,p-DDD	21	58.3	15	41.7	-	-	-	-
o,p-DDT	3	8.3	3	8.3	0	0.0	0	0.0
p,p-DDT	6	16.7	9	25.0	1	2.8	0	0.0
chlorpyrifos	3	8.3	nd	-	0	0.0	-	-
chlorpyrifosmethyl	6	16.7	nd	-	0	0.0	-	-
fenthion	3	8.3	nd	-	0	0.0	-	-
malathion	6	16.7	6	16.7	3	8.3	0	0.0
methamidophos	21	58.3	18	50.0	4	11.1	3	8.3
phorate	3	8.3	9	25.0	3	8.3	3	8.3
pirimiphos-methyl	9	25.0	3	8.3	5	13.9	0	0.0
profenofos	6	16.7	nd	-	4	11.1	-	-
thiometon	6	16.7	6	16.7	6	16.7	6	16.7

Table 11. Numbers and percentages of contaminated and violated samples of different types of potato tubers collected from the Egyptian local markets during 2006/2007 with respect to detected insecticides in the analyzed samples

nd :not detected; na :not available . A Total number of analyzed samples for each type of potatoes =36, B Maximum Residue Limits (MRLs) refer to (Codex 2006a) for potatoes.

Regarding potatoes, risk assessment based on their contamination levels from pesticides presented in Tables 12 and 13 a daily potato consumption of 0.06 kg for an adult person of 60 kg body weight (WHO, 2003) yielded the estimates .
Comparing the estimated dietary doses for the studied pesticides with their Acceptable Daily Intake-ADI; JMPR (Tomlin, 2004), revealed that only phorate residues either in (C) potato (0.001mg/kg b.w/d) or in organic potato, (0.0013 mg/kg b.w/d) pose risks to human health due to consumption of such potatoes since the estimated dietary doses accounted to 2.22 and 2.68 times the WHO-ADI for this pesticide (0.0005mg/kg b.w/d), respectively table (12)(Mansour et al.,2009).

Insecticide	WHO-ADI (mg/kg bw/d)	Estimated dose (mg kg bw/d)		Hazard Index		Risk	
		C	O	C	O	C	O
lindane	0.005	0.0004	0.0002	0.09	0.04	No	No
HCB	-	0.0014	0.0010	-	-	?	?
p,p-DDT	-	0.0002	0.00006	-	-	?	?
chlorpyrifos-methyl	0.01	0.00003	nd	0.003	nd	No	No
fenthion	0.007	0.000004	nd	0.0006	nd	No	No
malathaion	0.30	0.001	0.0002	0.003	0.001	No	No
methamidophos	0.004	0.001	0.0003	0.30	0.09	No	No
phorate	0.0005	0.0011	0.0013	2.22	2.68	Yes	Yes
pirimiphos-methyl	0.03	0.001	0.00001	0.03	0.0004	No	No
profenofos	0.01	0.00007	nd	0.007	nd	No	No
thiometon	0.003	0.0001	0.0003	0.03	0.08	No	No

Table 12. Calculated health risks for systemic effects associated with dietary intake of insecticide residues from potato tubers insecticide WHO-ADI (mg/ kg bw/d) estimated dose (mg kg bw/d) Hazard Index Risk.
Acceptable Daily Intake (ADI) (JMPR), cited from Tomlin (2004). Estimated dose = Residues (mg/kg) Food item / Body weight; where the following was considered in calculations: Residues = the highest mean value for each insecticide over12 months, daily potato consumption = 0.06 kg and body weight = 60 kg (WHO, 2003).Hazard Indices are resulted from dividing estimated doses by ADIs; indices <1 mean no risk and vice versa for indices >1. C: conventionally-farmed potatoes; O: organically-farmed potatoes; nd: not detected; no data available.

9. Pesticides binding to individual proteins

In vitro Binding of three pesticides Trichlorphenol, Fenvalerate and α-Endosulphan to Rat Serum Transferrin and Albumin for Bio-monitoring of Pesticides Pollution were carried out according to Afify et al., (2000). The results of the electrophoresis separation of the protein subunits of rat serum treated with different pesticides concentration 5, 10, 15 and 20 PPm (Table 13) showed that these pesticides have high affinity to albumin as well as high molecular weight proteins .The increase in the intensity of transferring protein was occurred with trichlorophenol and α-endosulphan .On the other hand, the intensity of the

albumin fraction was decreased with fenvalerate, while it is markedly increased with trichlorophenol and α-endosulphan .The individual incubation of each pesticide with transferrin,, albumin or prealbumin showed that trichlorophenol and α -endosulphan was found to cause aggregation of transferring by 49.1 and 43.9%, respectively, while fenvalerate was found to cause marked disintegration of transferrin as compared to controls .The albumin fraction was significantly decreased with the three pesticides .The Pre-albumin was found to markedly increased in its Intensity by 44.8 and 57.3 %with Trichlorophenol 5 ppm and α-endosulphan 15 ppm, respectively. The results concluded that several proteins have responded to pesticides treatment including the known serum proteins, transferrin, albumin, pre-albumin and small molecular weight proteins (Table 14) .However, some of the small molecular weights proteins have been identified as results of pesticides binding which require further characterization .Therefore, the detection of serum proteins after electrophoresis is considered a very good diagnostic parameter for bio-monitoring of pesticides pollution as studies by Saleh et al., (1996b); Afify et al., (1997).

	Rat serum proteins MW (kDa)												
Groups	300	200	160	100	76	70	67	55	52	45	37	35	30
Control	0.1	0.7	0.6	0.8	5.2	5.2	40.1	15.2	14.3	3.4	3.9	5.9	4.6
Trichlorophenol													
5 ppm	2.2	2.9	1.5	1.8	8.9	4.2	58.2	8.5	7.5	2.7	1.6		
10 ppm	2.8	3.3	1.3	1.5	9.6	2.3	56.7	7.9	9.5	2.2	2.9		
15 ppm	1.4	2.9	2.5	4.5	10.6	2.5	56.8	5.7	6.6	4.2	2.3		
20 ppm	2.4	3.1	2.3	2.2	15.2	3.5	56.2	4.5	6.4	5.3	2.2		
Fenvalerate													
5 ppm	0.1	0.3	0.3	0.7	12.5	21.9	38.5	10.9		3.6	3.8	4.2	3.2
10 ppm				1.2	1.4	0.6	45.9	18.1		5.8	4.8	5.5	4.3
15 ppm					10.7		45.3	26.2		6.4	4.2	3.1	4.1
20 ppm							30.6	22.1		25.9	2.4	1.8	17.2
α-Endosulphan													
5 ppm	3.5	2.8	7.2	3.1	7.5	2.9	52.8	16.5	5.6	1.1			
10 ppm	1.5	4.9	8.3	3.4	8.1	3.6	48.3	12.7	5.4	3.8			
15 ppm	2.1	4.4	8.6	3.2	8.3	3.9	52.2	9.2	4.6	3.1			
20 ppm	2.1	6.8	7.1	2.5	8.5	3.8	55.5	9.5	4.5	3.7			

Table 13. Scanning of electrophoretic pattern of rat serum protein subunits treated with different concentration of trichlorophenol, fenvalerate and α-endosulphan pesticides

	Transferrin MW (kDa)									Albumin MW (kDa)						
Groups	76	60	55	52	48	45	35	30	67	55	52	48	45	40	35	30
Control	38.8	22.1	18.1	9.4	6.9	3.4	0.7	0.6	64.1	21.2	4.1	4.2	6.4			
Trichlorophenol																
5 ppm	48.7	13.6	12.6	6.9	13.4	4.8			37.6	44.8	4.3	6.8	6.5			
10 ppm	49.1	13.3	12	6.1	13.8	5.7			49.5	26.1	11.2	6.9	6.3			
15 ppm	46.2	14.4	18.1	7.5	10.1	3.7			49.8	18.4	15.5	9.3	7			
Fenvalerate																
5 ppm	34.8	16.7	16.6	6.6	12.6	7.3	2.3	3.1	41.6	30.6	9.2	12	6.6			
10 ppm	36.5	15.6	15.2	8.9	15.6	8.2			39.8	33.4	12.5	7.8	6.5			
15 ppm	34.1	14.1	16	8.4	17.8	9.6			49.3	30.5	5.8	2.2	12.2			
α-Endosulphan																
5 ppm	42.5	14.2	8.6	8.5	10.9	10.1	2.8	2.4	31.1	29.6	13.4	2.9	7.1	1.6	4.2	10.1
10 ppm	43.9	12.4	8.5	8.5	11.8	7.7	2.3	4.9	30.2	52.1	9.4	3.5	4.8			
15 ppm	43.6	12.6	8.6	8.4	9.1	6.6	5.7	5.4	26.9	57.3	8.2	2.8	3.5	1.3		

Table 14. Showed that scanning of electrophoretic profiles of Transferring and albumin incubated with different concentration of pesticides 5, 10 and 15 ppm.

Investigation was carried out to determine if there are any changes among serum proteins which could be used as a biomarker for pesticides pollution .In addition, during the transport of the pesticides with carrier proteins in blood throughout the organs, do complex cause destruction in macromolecules .The data in table (14) of the present study revealed that the incubated pesticides have, high affinity to the proteins binding sites (Saleh et al., 1996b; Afify et al., 2000). Similar, observations have been recorded for particle mediated uptake of chlorinated pesticides by human, rat and insect lipoprotein (Shalsky and Guthrie, 1975; Larsen et al., 1994) and by serum albumin and α-globulin in rat and rabbit (Shakoori et al., 1996) . The binding of pesticides to proteins is correlated to the binding of DNA .DNA was considered the most important leader of the genetic code in human (Hemminki, 1986)which may induce genetic, risks) (Ehrenberg *et al*., 1974.). Therefore, the binding of pesticides to the macromolecules of rat serum protein could be serve as biomarker in the monitoring of pesticide (Hemminki, 1986). Pahler et al .(1999) showed that the accumulation of some proteins such as alpha 2 macro-globulin has been implicated in the tumorigenicity of many nongenotoxic chemicals to the kidney of the male rat .These chemicals have been shown to bind to alpha 2 macro-globulin and this binding was found to impair the renal degradation of the protein, resulting in lysosome overload, cell death, increased cell proliferation and, presumably renal tumor formation .The present study proved that the major proteins transferrin and albumin are the main sites for the three studied pesticides .The data of incubation of the three pesticides with transferrin and albumin were showed that the destruction of transferrin and albumin with the three pesticides produced a similar but not identical protein profile and the prealbumin was found to represent the major one as recorded by Altland et al .(1981). Dissociation into small MW proteins has been demonstrated in case of in vitro incubation with the tested pesticides .These results are in agreement with the results obtained by prolonged exposure of proteins to pesticides)Nilsson *et al*., 1975) .The changes in the binding of serum acute phase proteins such as transferrin and albumin with some chemicals has been used to detect or identify human breast cancer (Heys *et al*., 1998). Insecticides have been shown to bind to blood protein especially organochlorine compounds which are extensively bound to blood lipoproteins (Shalsky &Guthrie, 1975, 1977) .Dutta et al .(1992) revealed that malathion an organophosphorus pesticide has profound effect on serum protein as other parameters .Therefore, the detection of the prealbumin as well as small MW proteins after electrophoresis is considered a very good diagnostic marker for pesticide pollution .In conclusion the induced destructed proteins by pesticides in-vivo and in vitro may be utilized as biomarkers reliable for pesticides monitoring (Saleh et al., 1996b; Afify et al., 2000).

10. Conclusion

To improve agricultural productivity and control pesticide residues in food and environment; three different methods of extraction for pesticides were applied and methods based on chromatographic separation HPLC with mass spectrometric detection(LC-MS/MS tandem spectroscopy) considered useful methods for determination of pesticide residues in natural products under different types of farming production . Therefore this chapter evaluates the capabilities of mass spectrometry (MS) in combination with liquid chromatography (LC) for the determination of multi-residue pesticides extracted with three different methods. LC–MS/MS using electrospray ionization (ESI) are identified as techniques most often applied in multi-residue methods for pesticides at present in most

labs . Therefore, applicability and sensitivity obtained by LC-MS/MS is evaluated for each of the selected pesticides. A modified multi-residue method for analysis of 150 pesticide residues in green beans and grapes using liquid chromatography-tandem mass spectrometry were evaluated and compared for a wide range of physicochemical properties followed by LC-MS/MS detection. GC systems with three different detectors GC-ECD, GC-NPD and GC-MSD were used to compare between its efficiency and LC-MS/MS in separation and sensitivity.

Multi-residue method of determination of 150 pesticides is developed at 0.01 mg/kg limit of determination which fulfills the EU MRLs for organic agricultural products and baby foods. Grapes and green beans were selected not only for their wide consumption in the local market but also because they are promising exporting products to the international markets. The mass spectrometric parameters were optimized to give the best sensitivity, two MRM's were chosen for quantification and conformation of pesticides. The selected MRM's were based on the optimized declustring potential and collision energy which help improve pesticides selectivity and justification.

Risk associated with consumption of foods contaminated by pesticides has stimulated research to find out their impact to human health risk .Therefore Human milk samples were analyzed for pesticides residues along 26 of Egypt Governorates as well as pesticides residues in potatoes produced under different farming condition. In vitro binding of three pesticides e.g. Trichlorphenol, Fenvalerate and α-Endosulphan to rat serum proteins were studied to evaluate their binding and predict biomarker molecules.

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Pesticide Residues in Natural Products with Pharmaceutical Use: Occurrence, Analytical Advances and Perspectives

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1. Introduction

A pharmaceutical raw material is every active or inactive substance used in the manufacturing process of a pharmaceutical dosage form. According to their function in the medicament, they could be classified into two groups: (i) active ingredients with pharmacological activity; and (ii) excipients which allow the active ingredients dosification and makes suitable the route of administration (de la Rosa et al., 1995). In spite of the high number of synthetically produced pharmaceuticals and the progress in biotechnology and gene engineering, there are a number of raw materials of natural origin, which are still used for manufacturing active pharmaceutical products (Balandrin et al., 1985).

Quality is a mandatory requirement in the materials to accomplish the Pharmaceutical 'Good Manufacturing Practices'. Nowadays, the presence of pesticides in animal and vegetal commodities is a topic of public concern for the potential health hazards derived from them (WHO, 1998). The presence of pesticide residues in animal or vegetal raw materials can be originated in agricultural practices, environmental contamination or cross contamination. For example, oils rendered from whole fish and from fish offal represent an important dietary component in many areas of the world. In addition, fish oils are extensively used as ingredients in food and in dietary supplements for their therapeutic benefits in the treatment of cardiovascular, arthritic and dermatological diseases. Fish oils, however, are susceptible to contamination with lipophilic organic chemicals, in particular many organochlorine chemicals are now ubiquitous contaminants of marine ecosystems, but there is no specification on the pesticide residue allowed (Jacobs et al., 1997).

The number of trace pollutants to be monitored is continually growing and the levels at which these compounds need to be determined are becoming lower. Multi-residue methods (MRMs) are the preferred strategy over single analyte or class residue methods (SRMs). Analytical features of the MRMs allow to cover a large range of pesticides in a single analysis. Many MRMs employing different extraction and clean-up techniques and a variety

of detection methods, have been reported for the determination of pesticide residues in raw materials. The analytical method to be used depends mainly on the matrix composition, the physicochemical properties of the target analytes as well as the chemical nature of interferents from the analyzed matrices and/or the available instrumentation.

Currently, general methods to control pollutants in materials intended to be used in pharmaceutical preparations are described in the United States and European Pharmacopeias (USP 2007, EP 2008). Many herbs and spices that have been included in the Pharmacopeias, like Cinammon and Ginger, even when they were evaluated to detect low contaminant levels using the newest analytical methodologies and procedures available, their specifications remain unchanged. The different classes and wide range of pesticides and agricultural commodities containing them have fostered the development of new methods. However, the difficulty to cover all analytes in one single method and for all the possible matrices, has turned this approach into an almost impossible task, despite the advances in instrumentation in the last decade. Moreover, new pesticides have been introduced in the market which are more polar, non-chlorinated, less persistent or unstable leading to a change in the concept of the analytical and detection features required.

The actual trend in pesticides residue analysis focuses on the use of liquid chromatography coupled to mass spectrometry techniques (LC-MS) instead of gas chromatography (GC) with selective detection as electron capture (ECD), flame photometric (FPD), nitrogen-phosphorus (NPD) and thermal conductivity (TCD) detectors which represented the state-of-the-art at the end of the last century. They are intended for lipophillic (in many cases obsolete) pesticides like most organochlorine (i.e. p,p'-DDT, aldrin, dieldrin, lindane). Moreover, the targeted compounds monitored by such methods sometimes are neither pesticides of toxicological relevance or the most commonly found in a particular sample. Furthermore, MRMs could not be suitable and additional steps or SRMs are required. The wide scope of targeted analytes lies unchanged in the last editions of the most important Pharmacopeias as well as the methods for their anaylsis. The new instrumentation and sample preparation procedures, which allowed to diminish the limits of detection and consequently lowered the maximum residue levels (MRLs) in food commodities to 10 µg/kg for the great majority of pesticides in the European regulation, has not been yet included in the most important Pharmacopeias. The USP includes in its last Edition the detection of impurities in heparin using sophisticated high field NMR (nuclear magnetic resonance) techniques (USP, 2010). Nevertheless, the use of LC-MS/MS for pesticide residues and other contaminants is not mentioned at all. It is worth to consider that, these raw materials could be employed for pharmaceuticals which would be taken by people whose general health situation is jeopardized by their particular condition and therefore, the toxicological risk could be magnified. Therefore, strict regulations should rule the content of noxious substances like pesticides.

There is also a growing market for medicinal plants and their pharmaceuticals that is freely available in markets of Europe and USA: these Over The Counter (OTC) products are actually sold as "panaceas" for a well and long lasting living. There is a grey zone, from the regulatory point of view, with relation to pesticide residue allowable contents where all these kind of products are laid. The self medication and, therefore, uncontrolled use or misuse as well the lack of specific regulation in pesticide residue limits for OTC products deserves attention from the regulatory bodies. Nutraceuticals, natural cosmetics and phytopharmaceuticals share the same kind of regulatory alibi where the risk due to the

presence of pesticides residues has not been yet particularly considered. For many food products like oils or meals, processing factors have been estimated, but which is the processing factor for a specific pesticide in a Hammamelis tincture? Or in propolis tincture? Pesticides are concentrated or diluted? Undoubtely, there are more open questions than answers.

New trends in pesticides residue analysis have been focused on the miniaturization of the sample preparation methodology, moving to the development of straightforward, faster, cost-effective, and environmentally friendly procedures, adaptable for routine use in laboratories. Even with the advent of highly sensitive mass spectrometry (MS) and tandem mass spectrometry (MS/MS) detection techniques, the total knowledge of the contribution of pesticides in pharmaceutical products has not been completely developed yet, as for food and feed commodities or environmental samples. This sitution makes the development of new methods in organic contaminants residue analysis in pharmaceutical raw materials, a non closed and challenging issue.

This chapter addresses the main features of pesticide residue analysis in natural complex matrices frequently used in the pharmaceutical industry and folk medicine. An overview in the literature for methods and new improvements to determine pesticide residues in some critical pharmaceuticals raw materials is here presented. Expected pesticides, strategies in sample preparation procedures, recent applications in detection capabilities about these particular uses of the above mentioned pesticides are critically discussed. The authors are aware of reports in literature that were published in a variety of languages or in restricted access publication that were not included in this chapter. However, we tried to set priority on the most relevant, high impact and recent developments in the area.

2. Natural products of economical importance in the pharmaceutical industry

As above mentioned, some natural products are widely used in pharmacy. Table 1 shows an overview of selected commodities studied in this chapter and features for which are used. Particularly, traditional Chinese medicines are the most used, being more than 1.5 billion people all over the world which trust in their efficacy and safety (Li et al., 2010). The use of medicinal plants in both crude and prepared forms has increased greatly. The WHO has estimated that 80% of the global population relies on traditional medicine for health care. About 51% of all drug preparations in industrialized countries derive from plants, acting as sources of therapeutic agents or models for new synthetic compounds, or as raw material for semi-synthetic production of highly complex molecules (Zuin & Vilegas, 2000).

Aromatic plant drugs including essential oils and natural aromachemicals have been used extensively in the pharmaceutical industry as flavouring, and to mask the foul odour or taste of some pharmaceuticals as excipients.

However, in recent years, many aromatic plant drugs are used as active ingredients of botanicals. The simplest common traditional and modern use of aromatic plant drugs is as herbal tea. In many countries, pharmacies can freely dispense established tea formulations to patients for mild indications (Wichtl et al., 1994). Thus, essential oils could also make their way from the traditional into the modern medical domain (Bakkali et al., 2008).

Beeswax, instead, is constituted by saturated and unsaturated hydrocarbons, long chain saturated, mono and di-unsaturated esters and is being used as a lipid in pill coatings for drug delivery systems from tablets although current cosmetic uses are also known (Üner et al., 2005). Propolis is well known for therapeutic and dermal contact applications due to

its antibiotic and antiviral activities; more activities of interest were widely reported principally citotoxic, antioxidant and antifungal ones (Marcucci, 1995). Lanoline is a complex matrix composed principally by a mixture of esters involving different aliphatic acids combined with different alcohols. Lanolin is the by-product obtained after wool wax purification and it is widely used in cosmetic and pharmaceutical formulations because its unique surfactants properties. It also represents the world first source of cholesterol and lanosterol (Jover et al., 2002).

Commodity	Features/Properties	Pharmaceutical use
Beeswax	Compatibility with skin oils , UV inhibitor	Hand and body creams, ointments and pill coatings
Essential oils	Aromatic	Flavoring
Lanolin	Compatibility with skin oils, water absorbing, surfactant and emulsifying	Creams and ointments
Propolis	Antibiotic, anti-inflamatory, healing, antioxidant	Dermal contact, dietary supplement, cough candies
Medicinal Aromatic herbs	Wide variety of active constituents (i.e. antibiotic, anti-inflamatory)	Depending on active constituents. Dietary supplement

Table 1. Selected commodities and their uses in pharmacy.

3. Pesticide residues occurrence in natural products of economical importance

Since 1963 pesticide residues were detected in natural drug matrices although most monitoring reports have been published in the last 20 years. Organochlorines (OCs) such as DDT, aldrin, dieldrin, endrin, hexachlorocyclohexane (HCH) isomers and hexachlorobenzene (HCB) have been widely used as insecticides since the introduction of pesticides in management strategies for the protection of crops. Due to its well known environmental persistence, most OCs are currently banned worldwide. Synthetic pyrethroids and organophospates (OPs) insecticides were then introduced as replacements, being most of them currently commercially available in several countries. The occurrence of pesticides residues in phytopharmaceuticals and phytomedicines reviewed by Zuin and Vilegas (2000) state evidence that most reports were focused on the detection of OCs and OPs. On the other hand scarce reports have deal with the monitoring of other insecticide families such as carbamates and bridged diphenyls; triazine and urea herbicides; phtalamides and benzimidazole fungicides; a trend which is slowly overcome nowadays. Efforts in stricter regulation in MRLs settling for pesticides was stated by Codex Alimentarious, European and United States legislation for food commodities which has change the concept of contamination with pesticide residues. Therefore, more and more compounds are being targeted in raw materials although also banned OCs are still being found.

3.1 Pesticide residues in citrus essential oils

Depending on the extraction procedures, some oils could concentrate pesticide residues, as the case of citrus oils and extracts, being necessary to control the impact of the different

processing steps from fruit to the essential oil and the storage materials used. Citrus essential oils are by far the most studied essential oil commodity for pesticide residues. Di Bella and co-workers have been extensively reported the occurrence of variety of contaminants in Italian citrus essential oils. From the technological package applied in citrus crops, liphophilic pesticides are suggested to be preferably present in the oil phase (Dugo & Di Bella 2002). Residues of the bridged diphenyls (BDPs) tetradifon and dicofol along with the its metabolite, 4,4'- dichlorobenzophenone, were firstly reported in a five years monitoring campaign in lemon, mandarin, bergamot and orange essential oils (Saitta et al., 2000). On the other hand, Uruguayan lemon essential oils were found containing the OPs methidathion and chlorpyriphos (Dellacassa et al., 1999). More recently, a wide variety of OPs were reported in bergamot essential oil (Di Bella et al., 2004) and biological celementine, lemon, mandarin and orange essential oils (Di Bella et al., 2006).

Finally, a recent study with variety of samples from Argentina, Brazil, Italy, South Africa and Spain, revealed the introduction of more compounds to the target list since residues of the BDP bromopropilate, and OPs fenthion, malathion, phentoate and pyridafenthion were found (Di Bella et al., 2010). From this work, the authors conclude the wide occurrence of chlorpyriphos and dicofol residues in most samples, being Brazilian and Spanish ones the largest contaminated with individual levels up to 4.8 and 8.5 mg/L respectively.

3.2 Pesticide residues in bee's by-products: Propolis and beeswax

The contamination of bee's by-products with acaricides residues was extensively investigated by Bogdanov and co-workers. Important contamination with the most worldwide used synthetic acaricides to control Varroasis such as achrinathrine, amitraz, bromopropilate, coumahos, flumathrin and τ-fluvalinate was depicted in several studies (Bogdanov, 2003, Bogdanov, 2006, Bogdanov et al., 2003, Wallner, 1999). Even in a less extent, studies reporting the occurrence of several lipophilic pesticides from environmental pollution in beeswax were established. Not only acaricide contamination occurs, residues of the OC lindane, and the OCs metabolites p,p'-TDE, endosulfan sulphate and 3-phenoxybenzaldehide were found in beeswax from Spanish beehives (Jiménez et al., 2005). A wide variety of residues of OPs, synthetic pyrethroids and dicarboximide fungicides (procymidone and vinclozolin) were also detected in beeswax from France (Chauzat & Faucon 2007). Recently, Spanish commercial beeswax were found containing chlorimefon, chlorfenvinphos, chlorpyriphos, endosulfan and malathion residues (Serra-Bonvehí & Orantes-Bermejo, 2010). Over 197 samples analysed, the authors reported residues of chlorfenvinphos residues in 96 % of samples with concentrations up to 10.6 mg/kg (pesticide not legally authorised for use in beekeeping) whereas τ-fluvalinate was detected in 93.6% of samples with concentrations up to 88.7 mg/kg (Serra-Bonvehí & Orantes-Bermejo 2010). Currently, a critical situation with pesticide residues in bee's by-products is being found worldwide. A recent study carried out in hive matrices of USA and Canada, showed the occurrence of 121 different pesticides and metabolites (a variety of acaricides but also insecticides, fungicides and herbicides introduced in the hive by the bees from pesticide containing crops) within 887 beeswax samples analyzed (Mullin et al., 2010). The authors found that almost 98 % of the North American beeswax samples were contaminated with up to 204 and 94 mg/kg residues of τ-fluvalinate and coumaphos respectively. With relevant incidence, lower amounts of amitraz degradates and the fungicide chlorothalonil were found while an average of 6 pesticide detection per sample and a high of 39 was stated (Mullin et al., 2010).

On the other hand, since the composition of raw propolis contains high amount of beeswax, pesticide residues occurrence in propolis are expected to be in the same line to beeswax contamination (Bogdanov 2006, Wallner 1999). Recently our group investigated the presence of coumaphos, the most used acaricide, in propolis tinctures from Uruguay. Moreover, minor residues of ethion and chlorpyriphos were also detected (Pérez-Parada et al., 2011).

3.3 Pesticide residues in lanolin

To prevent fleece damage by sheep ectoparasites and to protect wool during storage, OCs, OPs and pyrethroid insecticides were extensively used in sheep sanitary treatments. Because of their lipophilic nature, these pesticides tend to accumulate in wool wax. Nowadays ectoparasite control is achieved exclusively through the application of OP and pyrethnoid insecticides and chitin synthesis inhibitors such as diflubenzuron and triflumuron (Jones 1996). However, few efforts were carried out in the last decade to determine the occurrence of pesticide residues in wool wax and processed wool wax (lanolin). In a previous study, our group found residues of chlorpyriphos, cypermethin, diazinon and ethion in Uruguayan lanolin samples (Pérez et al., 2010). Furthermore, when recently searching for 40 OPs residues in raw sheep wool from Uruguay, the extracted wool wax was found only containing diazinon and ethion residues (Niell et al., 2011).

3.4 Pesticide residues in medicinal plant materials

The interest in pesticide residues in medicinal herbs have been mainly focused on method developments rather than in the publication of results for public concern. However, the main fact can be divided into (i) findings are still OCs and OPs insecticides and (ii) the occurrence of a growing amount of pesticides, mainly fungicides, contained in medicinal products which enlarge the target list of pesticides to search for. Most studies still revealed the high incidence of OCs pesticides in several herbal drugs worldwide (Abhilash & Singh, 2008, Jeon et al., 2007, Leung et al., 2005, Mishra et al., 2007, Sun et al., 2007, Zuin et al., 2003).

The occurrence of banned OCs is accentuated when determining pesticide occurrence in commodities based on roots principally due to the cultivation in contaminated soils with several decades of agricultural use (Hayward & Wong, 2009, Leung et al., 2005, Li et al., 2010, Wong et al., 2010, Wu et al., 2011). Additional interest lies on OCs residues in those commodities which are consumed with dietary supplement purpose such as Ginseng root powders (Wong et al., 2010). Other authors reported residues of carbendazim, cyazofamid, diethofencarb and pyrimethanil in Asian Ginseng (Choi et al., 2007).

A wide variety of pesticides were found in a study carried out with Korean herbs (Nguyen et al., 2010). Chlorfenapyr, chlorfluazuron, λ-cyhalotrin, metalaxyl, pyridalyl, fenvalerate, tebuconazole and tebufenozide residues were found suggesting new strategies in pest control for medicinal plants which include herbicides, fungicides and insecticides. However, residues of p,p′-DDE were also detected as main contaminants in several plants (Nguyen et al., 2010). Residues exceding MRLs were reported (Abou-Arab & Abou Donia, 2001).

Monitoring campaign in Chinese and Korean medicinal drugs showed main contamination with 5 pesticides: methoxychlor, DDT, γ-HCH (lindane), endosulfan and procymidone with residues concentration ranging from 0.044 to 0.501 mg/kg. Intensive monitoring campaigns were suggested since the detection rate of pesticides in 30 different types of drugs was determined as 3.1% from the 229 samples analyzed. On critical observation was the detected amount of procymidone (0.501 mg/kg) and methoxychlor (0.382 and 0.312 mg/kg) (Oh

2009). Other studies showed that, from the 8 detected pesticides (residues between 0.034–0.579 mg/kg), 4 of them were fungicides (captan, chinomethionate, procymidone and tolyfluanid) (Oh 2007). Moreover, major residues of chlorothalonil fungicide were also detected in Brazilian *Passiflora L.* leaves (Zuin et al., 2003).

4. Pesticide residue analysis in natural products

Scientific advances in the employment of natural products with pharmaceutical relevance have focused on the analysis of constituents with the purpose of standardizing the applicability of such commodities. Aware for recent interest in residue analysis is intended to ensure assessment for safety consumption. Main pitfall in the study of pesticide residues in such matrices is the amount and nature of different commodities of interest and pesticides applied. Residue testing in this field is a major challenge because of the wide range of target agrochemicals but also it is an unexplored field, i.e. most medicinal plants are still lacking on analytical studies. This has lead to the development of fit-for-purpose methods since official methodologies are often described for not to face real life conditions (i.e. laboratory infrastructure, variability between different commodities, variability between samples from different geographical origins, residue legislation of different countries and non-harmonized application of used pesticides or commodity consumption) (Zuin et al., 2003b). Therefore, method development for contaminants is currently of paramount interest to determine the quality of these materials as demonstrated for the increasing numbers of publications in the area. Effort has been carried out for the residue analysis of pesticides in medicinal derivatives in the last ten years by modifying sample preparation, the inclusion of unstudied pesticides along with the introduction of more reliable detection and quantitation techniques. In accordance to pesticide residue research in food, the need was directed to more accurate, faster and more sensitive analytical methods. Dynamic sample treatment methodologies are mandatory. Trends were intended for avoiding tedious; time consuming and expensive extraction and clean-up protocols. Generally, classical procedures (as those described in official methodologies) drawbacks were related to the use of liquid-liquid or solid-liquid extraction which uses high amount of hazardous organic solvents. Modern techniques are focused on miniaturization as well as rapid or cost-effective features of the selected sample preparation. This has promote methodologies based on the use of new sorbents (i.e. primary-secondary amine, PSA; graphitized carbon black, GCB) or tailored sorbents (molecular imprinted polymers, nanomaterials, immunosorbents); integrative methods (defined as those which perform simultaneously several steps, i.e. extraction and clean-up); automated or even non-automated methods which can be carried out without the need of specific equipments. Remarkable attention has been conducted since the development of versatile MRMs such as the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) (Anastassiades et al., 2003a) along with its modifications; or MSPD (Matrix Solid Phase-Dispersion) (Barker 2007) which are suitable for large scale residue analysis in a wide variety of matrices.

At the same time, numerous regulations have set MRLs for pesticides and their relevant metabolites in a broad range of commodities (principally for food, feed and environmental matrices). Nowadays, chromatography coupled to MS has emerged as the default tool for residue analysis because of its improved sensitivity and selectivity provided for quantitative and qualitative analysis in comparison to conventional detection systems (i.e. ECD and FPD). The successful coupling of LC-MS and MS/MS have become the most used

configuration for large scale residue analysis according to the offered capabilities such as simultaneous identification, confirmation and quantitative determination for residue analysis. However, natural products were comparatively neglected in such a trend.

4.1 Sample preparation: Extraction and clean-up techniques

Despite the increasing progress in detection, sample preparation still remains as the bottleneck in natural products residue analysis. Current methods involve the use of one or a combination of sample preparation steps. Table 2 shows an overview of reported methodologies in the latest ten years for the sample treatment and chromatographic determination of pesticide residues in pharmaceutical raw materials and medicinal plants. A valuable indicator of the advances in residue analysis scenario for natural products, can be found in the pesticide determination in medicinal plants reviewed by Zuin & Vilegas (2000) years ago and the current situation with the latest improvements concerning miniaturized sample preparation (Zhou et al., 2011) and hyphenated MS techniques (Wong et al., 2010).

4.1.1 Official methodologies and related techniques

Testing for pesticide residues in pharmaceutical raw materials is described in official monographs for pesticides in botanical origin commodities and lanolin (USP 2007, EP 2008). They are based in liquid phase extraction (LPE) and further size-exclusion chromatography (gel permeation chromatography, GPC) as clean-up step. Subsequent clean-up step using SPE (solid phase extraction) in alumina is used in EP prior to gas chromatography (GC) injection. By far, these procedures are rugged and slightly differ in the experimental conditions.

4.1.1.1 Solid-liquid / liquid–liquid extraction

Most sample preparation methods in phytopharmaceuticals are based on LPE procedures either liquid-liquid (L-L) or solid-liquid (S-L) methodologies. Maceration of coarsely powdered solid substances, both fresh and dry materials with acetone is currently employed for extraction of pesticide residues as described in both official documents (EP 2008, USP 2007). The obtained extract, is then purified using GPC and analyzed by GC with selective detectors. Earlier literature was based on this scheme (Lino et al., 1999, Lino et al., 1997, Zuin & Vilegas, 2000). After maceration, further L-L partitioning between an immiscible organic phase (commonly petroleum ether or hexane) was widely assayed as a variation of the official methods. Subsequent clean-up using sorbent based techniques such as column chromatography and SPE was required for GC determination. L-L partitioning between a polar phase such as methanol or acetonitrile (MeCN) and n-hexane was reported as a preliminary clean-up in the determination of variety of lipophilic pesticides from beeswax (Jiménez et al., 2005, Jiménez et al., 2004a, Jiménez et al., 2004b). Likewise, L-L was proposed as the first clean-up stage in order to remove most waxes for the residue determination of 17 OCs in raw propolis (Chen et al., 2009).

Recent literature still uses these procedures because of the offered versatility derived from independence from expensive analytical instrumentation which are limiting requirements for laboratories. Acidic digestion procedures of the obtained organic extracts employing H_2SO_4 were reported for the analysis of OCs pesticides in Ginseng root (Chan et al., 2007, Quan et al., 2004). Similarly, a selected group of OCs and OPs were determined in herbal essential oils by using this technique (Yoon et al., 1999). However this strategy is limited for

the analysis of a reduced group of non-hydrolysable compounds. Moreover, Soxhlet extraction was reported as enhanced extraction methodology for OCs determination in Ginseng root using EtAc (ethyl acetate):petroleum ether (7:3) (Chan et al., 2007) and acetone:petroleum ether (3:1) solvent mixtures (Quan et al., 2008). A selected group of carbamates (metalocarb, isoprocarb, fenobucarb, carbofuran, pirimicarb and carbaryl were extracted with Soxhlet based extraction in CH_2Cl_2 from three different Chinese herbal medicines (Wu et al., 2005). Major pitfall of this strategy is its restriction for thermally stable compounds.

4.1.1.2 Gel permeation chromatography

GPC is a well established method for the fractionation and clean-up of non-fatty and fatty matrices in both plant and animal origin samples. It is generally recommended for purifying extracts obtained from complex samples. GPC is widely extended technique in routine laboratories for the analysis of pesticide residues after a preliminary LPE step and usually followed by GC analysis with different detectors.These conventional methodologies for pesticide residue analysis are often displayed as costly, time consuming and environmentally unfriendly because of the requirement of large amount of samples and greater volumes of hazardous solvents. The classical approach used in described official methodologies merely focus on GC based analysis with selective detection (i.e. ECD, TCD) or MS for confirmatory purposes. Although the inherent ruggedness, such methods cannot be applied to different conditions (target pesticides and different matrices) with the same analytical performance. Consequently several modifications were applied as evidenced for GPC based sample preparation of lanolin (Jones 1996, Jover & Bayona, 2002). The development of more straightforward, cost-effective and environmentally aware procedures while also providing the same analytical performance and fulfilling the requirements of regulatory issues is often suggested to substitute this kind of procedures (Pérez et al., 2010, Zuin et al., 2003a, Zuin et al., 2003b).

4.1.2 Recent advances, trends and achievements for pesticide residue analysis in natural products

Many modern multiresidue procedures employing different clean-up techniques and a variety of detection methods have been reported for the determination of pesticide residues in natural products with pharmaceutical application. Despite advances in the sensitivity of the analytical instrumentation for the end-point determination of analytes a pre-treatment is compulsory required to extract and isolate the target pesticides from the matrix, thus facilitating their determination (Gilbert-López et al., 2009). The sample treatment applied depends heavily in the complexity of the matrix. Modern sample treatment strategies for food and feed use to classify matrices depending on the water, sugar, acidic or fatty content matrices (DG SANCO 2009). Similarly, different natural product matrices can be grouped depending on its water content (low or high, i.e. dry and fresh herbs), waxy content (lanolin, beeswax and raw propolis) and polyphenolic content (propolis). This categorization is helpful because extraction strategies and involved inteferents are of similar nature which is relevant for the development of the sample preparation step. Moreover, the selection of sample preparation methodology is directly related to the detection method available. The more sensitive and specific detection method is used, the less stages of sample treatment will be required (Gilbert-López et al., 2009). In general, selective capability of the detector would compromise the followed sample treatment methodology (i.e. TCD, ECD detectors in GC and diode array detection (DAD) in

LC) require exhaustive and dedicated clean-up stages. In all cases, it is often necessary further clean-up to remove non desired interferents of the matrix such as pigments (i.e. chorlophylls, polyphenols, carontenoids) and waxes for increased sensitivity and reliability of the results along with maintenance of the instrumentation.
It must be highlighted that most recent reports in such matrices focus on the sample preparation instead of the use of modern detection. The advent of advanced MS detectors equipped with high selectivity, specificity and sensitivity capabilities, was recently introduced in the field of pesticide residue analysis of pharmaceuticals raw materials. Another key issue is the nature and number of targeted analytes included in the method. There is a wide range of agrochemicals with different physicochemical properties. For this reason, large scale methods use generic or less specific sample treatment at the expense of the sensitivity and selectivity provided by the MS determination.

4.1.2.1 Solid phase extraction

Nowadays, SPE is by far the most popular technique in the extraction and clean-up of complex extracts. Distinctive purposes can be accomplished depending on the used strategy: to retain target compounds and exclude interferents from the solid-phase or either retain interferents while pesticides are selectively eluted. Several advantages of SPE over liquid partitioning procedures are highlighted in higher sample throughput and lower solvent consumption. Moreover, the formation of emulsions is avoided with concomitant increased repeatability of the sample preparation. The flexibility to work with different commercially available solid phases offer increased features in the application of difficult to handle samples with inexpensive equipment (Gilbert-López et al., 2009). The main issue in method development for SPE lies on the selection of the sorbent material and the elution protocol. As commented above widely reported is the combination of preliminary L-L partitioning clean-up prior to SPE in order to avoid saturation of the material capacity. Methods using combined clean-up steps based on GPC and SPE (Hayward & Wong, 2009, Jones 1996) or sequential SPE (Chen et al., 2009, Jiménez et al., 2004b) are often suggested for better GC maintenance and improved sensitivity. Early methods were focused on the use of silica gel, alumina and Florisil columns but currently there are a wide variety of polymeric phases, carbon based, basic and acidic sorbents or even a combination of phases for particular necessities. A validated multiresidue method was developed for the analysis of a selected group of LC and GC amenable pesticides in lemon essential oils using SPE on Florisil cartridges. The vast majority of terpenic oil is selectively removed in a rinsing step using pentane while elution of the pesticides is performed with CH_2Cl_2 (Barrek et al., 2003). Several applications were developed for the residue analysis of lipophilic insecticides and acaricides in beeswax. In this case, the preferred strategy is based on the retention of waxes in the sorbent whereas pesticides are eluted. Methodologies using Florisil (Adamczyk et al., 2007, Frison et al., 1999, Tsigouri et al., 2000, Tsigouri et al., 2004), C_{18} (Chauzat & Faucon, 2007, Jiménez et al., 2004a, Kamel & Al-Ghamdi, 2006) also combined with HLB (Jiménez et al. 2004b) were reported. Selective eluting step is carried out using non-polar mixtures (n-hexane:diethylether mixtures) to polar ones (based on MeOH and MeCN), respectively. A group of 17 OCs pesticides was determined in raw propolis after L-L extraction in n-hexane:acetone (1:1) mixture with further double column series SPE on graphitized carbon and Florisil with 6mL elution of EtAc:n-hexane (2:8) mixture and GC-ECD (Chen et al., 2009). Perez-Parada et al., (2011) proposed the use of final SiO_2 removal of polyphenolic interferents for increased maintenance of the equipment in OPs routine analysis.

Most relevant works in this matter addresses the use SPE for the clean-up of medicinal plants extracts. OCs residues were determined in three different leafy plants harvested in Brazil using a mixed cartridge containing Florisil and silica-gel and performing elution with n-hexane:CH_2Cl_2 mixture (Rodrigues et al., 2007). Combined salting-out MeCN extraction and SPE based multiresidue determination of pesticides in Ginseng root was firstly introduced for the analysis of 18 insecticides and fungicides by GC-NPD/ECD determination (Park et al., 2007). Compulsory for selective detection was the Florisil based clean-up in which the elution was performed by hexane:acetone (8:2) solvent.

Large scale methods with MS detection are currently the top choice for advanced residue surveys. Nevertheless, these multiresidue methods with increased scope of analytes need to be able to selectively eliminate most in interferents while quantities of pesticides are obtained in the final extract. Initially, 102 multiclass residue determination of medicinal plant extracts was stated using GPC clean-up with further Envicarb based SPE. Residues were determined by GC-MS after elution from the cartridge with acetone:EtAc:hexane (1:2:1) solvent (Huang et al., 2007). In addition, a 170 multiclass method containing PSA-GCB based SPE column was developed for final purification of GPC extracts. The combined capacity of GPC for co-extractives removal is complemented with this further clean-up. Quantitative elution of most pesticides was accomplished using a EtAc:toluene (3:1) mixture (Hayward & Wong, 2009).

4.1.2.2 Matrix solid-phase dispersion

Matrix solid phase dispersion (MSPD) is a procedure usually applied for sample treatment in a variety of matrices. This procedure often referred as a modified SPE, is based on the mechanical disruption of the sample in a proper sorbent material with a mortar and a pestle. Sample homogenization, extraction and clean-up can be accomplished simultaneously by using relatively small sample size, low solvent consumption and minimum amount of sorbent phase. After blending, the sorbent material is packed into a column and the analytes are eluted using a suitable eluting solvent. However, the selection of the experimental conditions is critical in the selective extraction and purification of the sample extract (Barker 2007).

Many MSPD procedures uses co-columns, which is the use of packed sorbent with complementary features of the disrupting phase at the bottom of the column in order to obtain exhaustive removal of interferents. Hence, the selection of proper dispersant phase plus elution volume is mandatory to achieve enhanced extraction of the matrix while giving purified extracts for quantitative analysis of pesticides. The use of co-columns has improved the applicability of MSPD by increasing the versatility of the purification step.

MSPD can also be used for extracting analytes from both solid and liquid samples. The potential of this strategy in tricky matrices is relatively new in the literature. A procedure for the determination of buprofezin, tetradifon, vinclozolin and bifenthrin in raw propolis was reported using MSPD over silica gel and Florsil co-colum cleanup by elution with CH_2Cl_2:EtAc (9:1) mixture and GC-MS determination (Santana dos Santos et al., 2008). Analysis of propolis extracts is a challenging issue because of the high polyphenolic content of this matrix. The appropriate selection of conditions for enhanced extraction of polyphenols in propolis tinctures was recently stated by Pérez-Parada et al., (2011.) Popolis tinctures were dispersed on anhydrous $Al_2(SO_4)_3$ using a MSPD approach with Florisil co-colum clean-up by performing elution with CH_2Cl_2:EtAc (9:1) solvent for the determination of coumaphos, ethion and chlorpyriphos.

On the other hand, various authors have used MSPD for the analysis of pesticides residues in medicinal plants. Firstly, Zuin et al., (2003b) reported the determination of 7 OCs and 4 OPs in *Passiflora L.* leaves using a MSPD on Florisil with a Na_2SO_4 co-column to remove wet by eluting with a n-hexane:EtAc (7:1) mixture and GC-ECD determination. Method performance was evaluated and compared to that described in EP. Moreover, matalaxyl, triadimefon, paclobutrazol, vinclozolin, tebuconazole and fenatimol residues were determined by GC-NPD in *Isatis indoctica* and *Paeonia lactifora* herbs using MSPD on SiO_2 and Na_2SO_4 co-column by eluting with acetone (Tang et al., 2006). Similarly, HCH isomers were determined in *Withania somnifera* and *Ocimum sanctum* by GC-ECD using using Florisil MSPD and $MgSO_4$-NaCl co-column by eluting with n-hexane:EtAC (7:3) mixture (Abhilash et al., 2007, Abhilash et al., 2009, Abhilash & Singh, 2008). Analytical features were in the same line to those official methodologies but with more flexibility to work. Other authors have determined acephate, chlorpropham, pyrimicarb, bifenthrin, tetradifon and phosalone residues in *Cordia salicifolia* leaves by using neutral alumina and peat as dispersant phases plus Na_2SO_4 and C_{18} co-columns and eluting with cyclohexane:CH_2Cl_2 and GC-MS determination (de Carvalho et al., 2009a, de Carvalho et al., 2010). In addition, Qi (2010) proposed sonicated MSPD approach on Florisil sorbent and EtAC:hexane (7:3) elution to determine pentachloronitrobencene, pentachloroaniline, methylpentachlorophenyl sulphide and procymidone in Ginseng extracts by GC-ECD. On the other hand, Pérez et al., (2010) reported the determination of a variety of OCs, OPs and pyrethoids in lanolin using MSPD on C_{18} plus alumina co-column with elution performed by MeCN:n-hexane saturated and further GC-ECD/FPD determination. Method performance was also evaluated to that attainable in USP and EP without significant loss of reliability.

Finally, the applicability of modern MSPD sorbents was assayed for the quantitative residue determination of bifenthrin, tetradifon and phosalone in *Cordia salicifolia* leaves using the two dimensional DPA (di-2-pyr-idylamine) coordination polymer (∞[Gd(DPA)(HDPA)]). Purified extracts were obtained after elution with acetone:petroleoum ether (5:3) while residues were determined by GC-MS (de Carvalho et al., 2009b)

4.1.2.3 Ultrasonic and microwave assisted extraction

A straightforward approach on LPE based extractions is the employment of waves for rapid and enhanced extraction of analytes from solid matrices. Ultrasonic wave assisted extraction (UAE) was reported for the pesticide residue analysis in different medicinal plants materials. Determination of 18 different OPs from *Flos lonicera* herbal material was performed by using UAE extraction and further SPE and GC-FPD analysis (Xiang et al., 2006). Furthermore, UAE in acetone:petroleum ether (5:3) solvent mixture was proposed for rapid extraction of 15 fungicides from *Isatis indigotica* herb and granule formulation. Subsequent clean-up to obtain proper extracts was performed by using L-L extraction in n-hexane (Tang et al., 2005). GC-ECD residue analysis of 20 multiclass pesticides (OCs, OPs, pyrethoids and fungicides) in five different traditional Chinese medicines using UAE in acetone:CH_2Cl_2 (2:1) mixture with subsequent SiO_2 column chromatography clean-up was recently reported (Qian et al. 2010). Most relevant improvement of this strategy was recently reported by Wang et al. (2011) for large scale determination of 195 multiclass pesticides in different traditional Chinese herbs using UAE in acetone with further GPC clean-up and GC-MS determination.

Microwave assisted extraction (MAE) was also employed for extracting pesticides in medicinal plants. Water was used as extracting solvent for MAE of 7 OCs in Chinese herbs

as reported Ho & Hsieh, (2001) with further SPME (solid phase microextraction) and GC-ECD determination. Moreover, a rapid strategy was proposed employing MAE in ethanol for the extraction of 16 OPs in 4 different Chinese herbs with posterior dispersive SPE based PSA clean-up and GC-FPD determination (Wan et al., 2010).

4.1.2.4 Supercritical fluid extraction

Quantitative extraction of pesticides using supercritical fluid extraction (SFE) was less reported in the last years principally due to the need of high cost instrumentation and the difficulties in method development concerning the optimization of the extraction conditions. This methodology is based on the modification of a fluid extractability at the supercritical state by performing variation cycles of temperature and pressure. Main advantages are the use of non-toxic, non-flammable and inexpensive fluids, principally CO_2 (Gilbert-López et al., 2009). Firstly, SFE with CO_2 was assayed to extract a variety of OCs, OPs and pyrethroids from raw wool wax by GC-ECD/TCD determination. Extraction and clean-up was performed simultaneously with good recoveries and repeatability (85-108 %, RSD <8 %) (Jones, 1997). Fist attempts in the extraction and clean up in phytopharmaceuticals by SFE, were made for OCs and OPs pesticide residues in camomile using CO_2 as supercritical fluid and GC-ECD/FPD determination and GC-MS confirmation of residues (Carisano & Rovida, 1995). Other authors reported SFE for residue extraction of a variety of OCs and OPs pesticides in *Passiflora L.* and *Angelica sinensis* leaves by GC-ECD and GC-FPD (Zhao et al., 2002, Zuin et al., 2003a)

4.1.2.5 Accelerated solvent extraction

Pressurized liquid extraction (PLE), so-called accelerated solvent extraction (ASE) is a solvent based methodology working under elevated temperatures (50–200 ^{o}C) and pressure (500–3000 psi) conditions for short time periods (5–10min). Tipically, a solid sample is packed into the extraction cell and analytes are extracted from the matrix with conventional low-boiling solvents or solvent mixtures at elevated temperatures up to 200 ◦C and pressure (30–200 atm) to maintain the solvent in the liquid state. A very interesting feature of this technique is the possibility of full automation and many samples can be extracted sequentially with good repeatability (Gilbert-López et al., 2009). However, the extraction efficiency of ASE is dramatically influenced by the extraction pressure and temperature conditions as well as the nature of the sample and its water content. Therefore, the extraction behaviour of ASE is not plain and the optimization of operating conditions is laborious. Fifteen OPs pesticide residues were extracted from Ginkgo leaves by using ASE in MeCN. Extracts for proper GC-FPD determination were cleaned-up by SPE cartridges (Yi & Lu, 2005). Moreover, also reported was ASE for routine extraction of 74 multiclass pesticides in six traditional Chinese herbs by LC-MS (Mao et al., 2010).

4.1.2.6 Solid phase microextraction and stir bar sorptive extraction

Solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) are solvent free methodologies applied for GC-amenable compounds in liquid samples. Interesting capabilities of these techniques derives from the integration of extraction, pre-concentration and injection of the analytes in the GC inlet by thermal desorption in one single step (Gilbert-López et al., 2009). With this aim, a suspended coated fiber either directly immersed in the liquid sample (normal SPME) or in the headspace (HS-SPME) is introduced in a vial. SPME was successfully assayed in a variety of infusions and extracts. Polydimethylsiloxane (PDMS) coated fibers were reported in the determination of a variety of herbal infusions and

formulations (Hwang & Lee, 2000). HS-SPME was applied for the residue analysis of five pesticides (3OCs and 2OPs) using a PDMS-polyvinylalcohol (PVA) fiber in *Passiflora L.* infusions by GC-ECD determination (Zuin et al., 2004).
SBSE is based on the sorption of the targeted analytes onto a coated stir bar. Solid phase normally used is typically also PDMS material. The analytes are extracted by stirring a magnetic the bar immersed in the aqueous sample and recovered by desorbing the stir bar thermally in the GC inlet. SBSE has successfully been applied to the analysis of 11 different OCs and OPs residues in *Passiflora L.* herbal infusions by GC-ECD/FPD (Bicchi et al., 2003).

4.1.2.7 Dispersive Liquid-Liquid Micro Extraction (DLLME)

Dispersive liquid–liquid microextraction (DLLME) has become a very popular environmentally benign sample-preparation technique, because it is fast, inexpensive, easy to operate with a high enrichment factor and consumes low volume of organic solvent. DLLME is a modified solvent extraction method in which acceptor-to-donor phase ratio is greatly reduced compared with other methods. Its combination with different analytical techniques such GC, HPLC, inductively coupled plasma-optical emission spectrometry (ICP-OES) and electrothermal atomic absorption spectrometry (ETAAS) makes DLLME an intersting tool for contaminant analysis (Rezaee et al., 2010, Fariña et al., 2007).

4.1.2.8 QuEChERS and other means

Since the introduction of the QuEChERS method for fruits and vegetables (Anastassiades et al., 2003a) it has been widely expanded to other matrices principally due to the versatility of a miniaturized solvent based extraction and easy applicability for most laboratories. This fit-for-purpose procedure start with a liquid partitioning or salting-out of MeCN:water followed by a dispersive SPE (dSPE) clean-up step in dedicated sorbents (i.e. primary secondary amine (PSA), graphitized carbon black (GCB)) which is applied depending on the nature of the matrix and requirements for removal of interferents. Main advantages are the low solvent consumption and cost-effectiveness. Several modifications of the original method has been successfully introduced ranging from the modification of the salts, used extraction solvents and dSPE sorbents depending on the nature of the matrix and the targeted species. Few reports were recently stated for the QuEChERS method in these matrices. Firstly, a procedure for American and Asian dried Ginseng residue analysis was described (Wong et al., 2007).
Wu et al., (2011) has proposed QuEChERS sample preparation for the residue analysis of 15 OCs pesticides in American Ginseng root by GC-ECD determination. Excellent extraction rates were obtained with recoveries ranged from 81.4-95.2 % and RSD < 8 %. Major improvement was reported for the high throughput residue analysis in Ginkgo leaves of 81 multiclass pesticides in agreement to those widely accepted method performance requirements stated by DG SANCO (2009) guidelines. Of special interest was the recent proposal of Wong et al., (2010) for the residue analysis of 168 pesticides in Ginseng powder using MeCN o acetone based extraction. The inclusion of C_8 based dSPE step prior to the GCB+PSA dispersive clean-up, showed to be straightforward, relatively inexpensive and fast. Moreover, analytical features were similar to those attained in GPC based ones (Hayward & Wong, 2009). More recently, other miniaturized large scale analytical method based in the same scheme was reported by Nguyen et al., (2010) for the determination of 234 pesticides in Korean herbs, while Mullin et al., (2010) reported the determination of 121 pesticides residues in beeswax using a modified QuEChERS strategy for partitioning of the

wax material between of MeCN:H_2O. Widespread use of QuEChERS based methods for pesticide multiresidue analysis in these matrices is expected in a short term.

4.2 Separation sciences and detection techniques

4.2.1 Gas chromatography

In accordance to official methodologies, capillary GC is the most used separation technique in residue analysis of non-polar and semi-polar pesticides for this kind of matrices. Major attention has paid target determination of OCs, OPs and pyrethroids GC-amenable compounds. As discussed previously, most reports focuses on selective detection of pesticides using ECD, NPD, TCD and FPD detectors. The increased selectivity of FPD allowed direct injection of diluted citrus essential oil with proper internal standard as previously assayed for the determination of OPs residues without the use of clean-up steps (Dellacassa et al., 1999, Di Bella et al., 2004, Saitta et al., 1997). ECD is described as more sensitive than the rest of selective detectors although the lack of specificity needs to be companied with exhaustive clean-up steps. Evidence when performing determination of OCs in citrus essential oil by GC–ECD, was the required silica gel column chromatography for the removal of polyphenolic interferents (Di Bella et al., 2004, Saitta et al., 2000).

Most recent application of ECD still relies on the sensitive detection of OCs. ECD was recently described for the successful determination of variety of OCs, and halogenated OPs, pyrethroids or fungicides using different clean-up steps in several matrices.

Using hydrolysis and derivatization with heptafluorobutyric anhydride (HFBA), total amitraz residues were determined in beeswax with LODs < 0.08 mg/kg by GC–ECD (Jiménez et al., 2004a).

On the other hand, SPE is by far the most reported clean-up strategy prior to GC–ECD analysis. Fluvalinate residues were determined in beeswax by ECD after Florisil SPE (Tsigouri et al., 2000). Regarding medicinal plants, GC–ECD determination was widely used for the determination of HCH isomers with LODs at the ppb level (Abhilash et al., 2007, Abhilash et al., 2009, Abhilash & Singh, 2008). Twenty multiclass pesticides were also determined in variety of medicinal plants using this detection technique (Qian et al., 2010). Pentachloronitrobence, pentachloroaniline, methylpentachlorophenyl sulphide and procymidone residues were determined in Ginseng extracts by μECD with LODs at sub ppb level. Also QuEChERS sample treatment with GC–ECD analysis was used for the determination of 15 OCs in Ginseng (Wu at al., 2011).

Adequate selectivity attained with FPD operated in the phosphorous mode is often referred for proper determination of OPs pesticides. Most relevant report was described by Wong et al., (2007) for the determination of 108 OPs in Ginseng root; similar quantitative performance plus lower LODs for troublesome compounds than when applying GC–MS determination were obtained. Advances in carbamate determination was stated for metalocarb, isoporocarb, fenobucarb, carbofuran, pirimicarb and carbaryl analysis in traditional Chinese herbs by GC–NPD obtaining LOQs ≤ 0.05 mg/kg(Wu et al. ,2005). Moreover, NPD was described for the multiclass determination of metalaxyl, triadimefon, paclobutrazol, vinclozolin, tebuconazole and fenatimol (Tang et al,. 2006).

Dual determination of residues using ECD/FPD was applied to medicinal plants (Tang et al., 2005, Zuin et al., 2003a), lanolin (Pérez et al., 2010) and propolis tinctures (Pérez-Parada et al,. 2011) whereas ECD/NPD was also reported for Ginseng root (Park et al., 2007) and beeswax (Kamel & Al-Ghamdi, 2006).

The use of sample preparation should be considered together with the accessible detection technique. Generally, the most sensitive and selective detection is used; the more generic sample preparation would be required. However the sample features are of major importance independently of the determination technique as exposed for highly polyphenolic content matrices. As an example, exhaustive clean-up using SPE series was mandatory for the determination of OCs pesticides in raw propolis by GC–ECD (Chen et al., 2009). Selective features of ECD for the determination of OCs are not efficiently replaced when using other MS based detectors; therefore it is widely being used in laboratories dealing with the determination of OCs in Ginseng root as reported for proficiency testing results in Asia Pacific region (Chan et al., 2010).

In spite of the increased sensitivity of ECD for the determination of organohalogen pesticides, or increased selectivity of FPD and NPD, the use of MS is being compulsory introduced in order to obtain reliable identification and confirmation of residues. Commonly used in routine analysis, is to combine selective detection with MS confirmation. In this sense, our group has used combined ECD and FPD for the determination of OCs, OPs and pyrethroids in lanolin whereas confirmation of residues was carried out by GC–MS (Pérez et al. 2010). After selective detection, confirmation of residues by GC–MS was also described in citrus essential oils (Saitta et al., 2000), medicinal plants (Quan et al., 2004) and beeswax (Kamel & Al-Ghamdi, 2006).

Concerning GC–MS, fingerprinting features of EI (electron impact) mass spectra coupled to commercially available mass spectral libraries is widely used for identification purposes of small organic molecules. Nevertheless, this approach is insufficient when assessing residues due to the loss of ion specificity from the co-eluted matrix. The approach of enhanced selectivity of the selected ion monitoring (SIM) mode of single quadrupole (Q-MS) mass spectrometer results in improved sensitivity and is widely used in the determination of residues as seen in Table 2. In the same line, EI has been used as ionization technique in almost all of the studies described in these matrices although few reports based on chemical ionization (CI) were reported. Increased selectivity and enhanced detectability is achieved when using negative chemical ionization (NCI) for electron-capturing compounds (mainly organohalogens) because the co-eluted matrix does not efficiently ionize. Consequently, few ions with high abundance are usually observed in the relevant mass spectrum resulting in improved sensitivity. NCI was recently used for the analysis of 56 multiclass pesticides (Tagami et al., 2008) as well as in 10 pyrethoids residues (Tagami et al., 2006) and 10 OCs (Guo et al., 2010) in herbal medicines. The advent of MS has opened new perspectives in order to increase the scope of targeted analytes and reliability of the findings. EI-GC–(Q)MS working in the SIM mode was employed in the determination of 12 GC-amenable pesticide residues in lemon essential oil (Barrek et al. 2003).

Regarding bee's byproducts, Jiménez et al. (2005) and Santana dos Santos et al. (2008) have determined by GC–MS pesticide residues in beeswax and raw propolis, respectively. Significant attention in large scale methods using GC–(SIM)MS was recently described for the target trace analysis of 54 (Miao el al., 2010), 81 (Zhou et al., 2011), 102 (Huang et al., 2007), 108 (Wong et al., 2007), 168 (Wong et al., 2010), 170 (Hayward & Wong, 2009), 195 (Wang et al., 2011) and 234 (Nguyen et al., 2010) pesticides in plant matrices with medicinal use.

Less described in the literature is the use of advanced GC–MS techniques although some improvements were made in the last years. Major advantages of modern MS configurations, both full scan MS or MS/MS over conventional MS, are the increased specificity and

sensitivity to quantify residues principally when dealing with concentrations approaching the detection limit. Furthermore, performance in terms of selectivity with confirmation capability is improved remarkably when MS/MS is used. Multiple reaction monitoring mode of triple quadruple (QqQ) MS/MS is currently the more appropriate determination technique for target analysis since it achieves lower LODs but also offers high dynamic ranges (almost four orders of magnitude). Selected qualifiers and quantifiers transitions ions are focused on quantitative and qualitative purposes respectively. Firstly, GC–(QqQ)MS/MS was employed for the determination of 18 pesticides (acaricides, insecticides and fungicides) in beeswax (Chauzat & Faucon, 2007). Other authors, have recently introduced comparative studies of multiresidue determination performance by GC–MS working in SIM mode and GC–(QqQ)MS/MS in Ginseng powders (Wong et al., 2010). Improved reliability in confirmation and sensitivity was stated which even allowed dilution of the extracts for better maintenance of the instrument but also decreasing the matrix effect and improving accuracy of results. On the other hand, pentachloronitrobenzene and its metabolites (pentachloroaniline and pentachloroanisole) were determined by GC–quadrupole-ion trap (QIT)MS/MS configuration (Li et al., 2009).

Target analysis is frequently referred to nominal Q-MS and QqQMS/MS (less often to ion traps) in which the need to select ions (SIM) or transitions limits the number of compounds that can be analyzed. Time-of-flight (TOF) analyzers have overcome these limitations thanks to the ability of accurate mass measurements of the ions by offering resolution powers at full width al half medium (FWHM) in the 10,000-20,000 range. These instruments are typically mentioned as high resolution mass spectrometers (HRMS) which have recently found application in this topic. Mass accuracy with mass deviation errors < 5 ppm, permits the elucidation of the elemental composition of the ions by the coupling to an adequate software tool. Identification and confirmation capability of the compounds via parent and fragment ions recognition is highly improved. A comparative study was carried out using two different GC–MS instruments such as classical Q-MS operated in the SIM mode and TOF-MS for the analysis of 170 organohalogen and OPs pesticides (Hayward & Wong, 2009). In this case, although no significant differences were found in quantitative results, identification of incurred troublesome pesticides was only unambiguous when using accurate mass measurements provided by TOF-MS.

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
9 OPa, 10 OCs, 4 Pys	Lanolin	MSPD on C_{18} plus neutral alumina co-column. Elution MeCN saturated in n-hexane	GC-ECD/FPD GC-MS confirmation	83-118 % (<20 %)	LOQs OPs ≤ 0.070 mg kg^{-1} LOQs OCs & Py ≤0.087 mg kg^{-1}	(Pérez et al., 2010)
Simazine and cypermethrin	Orange essential oil	Extraction in methanolic phosphate buffer for simazine. Cypermethrin was extracted in hexane-acetonitrile partitioning followed by silica SPE	ELISA	-	LOQs simazine ≤ 0.04 mg kg^{-1} LOQs cypermethrin ≤ 0.5 mg kg^{-1}	(Nichkova et al., 2009)

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
22 Multi-class (12 GC amenable; 10 LC amenable) pesticides	Lemon essential oil	SPE (C_{18} and Florisil). Pentane rinsing, elution with CH_2Cl_2	LC-(Q)MS and GC-MS	57-114.6 % (<6.6 %)	LC amenables LODs ≤ 0.06 mg L^{-1} GC amenables LODs ≤ 0.4 mg L^{-1}	(Barrek et al., 2003)
70 Multi-class pesticides	Lavandin essential oil	10 fold dilution and direct injection	LC-(QTRAP)MS/MS	-	LOQs: ≤1 µg/L for 9 pesticides, ≤5 µg/L for 44, ≤10 µg/L for 9, and ≤20 µg/L for 5	(Fillâtre et al., 2011)
Synthetic acaricides (fluvalinate, coumaphos, bromopropylate and its metabolite 4,4'-dibromobenzophenone)	Beeswax	Dissolution in isooctane. SPE (Florisil) clean-up with elution by MeOH	LC-DAD	70-110 % (<15 %)	LODs ≤ 0.2 mg kg^{-1}	(Adamczyk et al., 2007)
16 insecticides and acaricides 2 fungicides	Beeswax	SPE C_{18}, elution with MeCN	GC-(QqQ)MS/MS	N.D.	LODs ≤ 0.05 mg kg^{-1}	(Chauzat & Faucon, 2007)
Total amitraz (amitraz and its degradation products)	Beeswax	Dissolution in MeOH and n-hexane L-L extraction. Hydrolysis to 2,4-dimethylaniline (DMA) and derivatization with Heptafluorobutyric anhydride (HFBA)	GC-ECD	~80 % (<15 %)	LOD = 0.08 mg kg^{-1}	(Jiménez et al., 2004a)
Chlorfenvinphos, fluvalinate, amitraz, bromopropylate, acrinathrin, flumethrin, coumaphos, chlorpyrifos, chlordimeform, endosulfan andmalathion	Beeswax	Dissolution in n-hexane plus heating : freezing cycles. Further SPE (Florisil) clean-up with elution by acetone:hexane (1:1)	GC-µECD/NPD/MS	86-108%	LODs ≤ 0.3 mg kg^{-1} (GC-µECD/NPD) and ≤ 0.085 (GC-MS)	(Serra-Bonvehí & Orantes-Bermejo, 2010)
Total amitraz	Beeswax	Hydrolysis in acidic buffer and HS-SPME	GC-ITD	-	LODs = 0.01 mg kg^{-1}	(Leníček et al., 2006)
Up to 15 lipophilic pesticides (including acaricides, PCBs, OPs, Pys and OCs)	Beeswax	A) Dissolution in n-hexane and L-L extraction with MeCN. B) Subsequent SPE in HLB cartridges and additional SPE of C_{18}.	A) GC-MS (SIM) B) GC-ECD	A) 93-108 % (<16 %) B) 94-107 % (<20 %)	A) LOQs ≤ 0.065 mg kg^{-1} B) LOQs ≤ 0.12 mg kg^{-1}	(Jiménez et al., 2005, Jiménez et al., 2004b)

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
121 pesticides	Beeswax	Modified QuEChERS: addition of MeCN:H_2O, NaAc, $MgSO_4$. Organic phase is clean-up by dSPE using PSA, C18 and $MgSO_4$. For GC further SPE in GCB+PSA was used eluting with acetone:toluene (7:3).	LC- (Q)MS/MS and GC-MS (SIM)	-	LOD ≤ 0.03 mg kg^{-1}	(Mullin et al., 2010)
Flumethrin, tau-fluvalinate, coumaphos, and amitraz	Beeswax	Dissolution in MeCN and SPE of C_{18}.	GC-NPD/ECD GC-MS	90-102 %	B) LODs ≤ 0.05 mg kg^{-1}	(Kamel & Al-Ghamdi, 2006)
Fluvalinate	Beeswax	Dissolution in n-hexane, and the solutions is sonicated and transferred to Extrelut columns. The fluvalinate was extracted with MeCN, and a portion of the extract was cleaned-up on a Florisil cartridge. Elution with diethyl ether-n-hexane (1:1)	GC-ECD	77.4 to 87.3 (<8.31 %)	LOQs = 0.1 mg kg^{-1}	(Tsigouri et al., 2000)
Coumaphos, ethion and chlorpyriphos	Propolis tinctures	MSPD on $Al_2(SO_4)_3$ and Florisil co-column. Elution with CH_2Cl_2:EtAc (9:1). Subsequent silica gel clean-up eluting with. CH_2Cl_2	GC-FPD and GC-MS confirmation	87.4 -115.0 % (<12.8 %)	LODs ≤ 0.0143 mg kg^{-1}	(Pérez-Parada et al., 2011)
17 OCs	Raw propolis	L-L extraction n-hexane-acetone (1:1) plus tandem SPE graphitized carbon and Florisil cartridge clean-up with elution by EtAc and hexane (2:8)	GC-ECD	62.6-109.6 (<9.4 %)	LODs ≤ 0.038 mg kg^{-1}	(Chen et al., 2009)
Buprofezin, tetradifon, vinclozolin, and bifenthrin	Raw propolis	MSPD on SiO_2 and Florisil clean-up. Elution with CH_2Cl_2:EtAc (9:1).	GC-MS (SIM)	67-175 % (<12.1 %)	LOQs ≤ 0.25 mg kg^{-1}	(Santana dos Santos et al., 2008)
7 OCs	Radix et Rhizoma Rhei	SiO_2 hollow fiber sorptive microextraction	GC-MS (SIM)	63-115 %- (<10 %)	-	(Li et al., 2010)
16 OPs	4 Chinese herbs	MAE in ethanol and dSPE in PSA	GC-FPD	73.8–123% (<15.2 %)	LODs ≤ 0.09 mg kg^{-1}	(Wan et al., 2010)

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
20 OCs	Herba epimedii	Maceration in EtAc and further SPE in Florisil+Na_2SO_4 clean-up. Elution with diethylether: hexane (15:85).	GC-MS (SIM)	75.4-90.7 % (<18.3%)	LODs ≤ 0.055 mg kg^{-1}	(Guo et al., 2010)
7 OCs 4 OPs	Passiflora L. leaves	MSPD on Florisil. Co-column of neutral alumina and Na_2SO_4. Elution with n-hexane:EtAc (7:1)	GC-ECD	75.2-110.6 (<17.4 %)	LODs ≤ 0.038 mg kg^{-1}	(Zuin et al., 2003b)
A) 7 OCs & 6 OPs B) 12 OCs	A) Passiflora L. leaves B) Angelica sinensis leaves	SFE using CO_2 as mobile phase	A) GC-ECD/FPD B) GC-ECD	A) 69.8-107.1 (<14.7 %) B) 83.3-98.1 (<6.1 %)	A) LODs ≤ 0.014 mg kg^{-1} B) LODs ≤ 0.010 mg kg^{-1}	A) (Zuin et al., 2003a) B) (Zhao et al., 2002)
3 OCs 2OPs	Passiflora L. infusions	Headspace solid-phase microextraction (HS-SPME) with polydimethylsiloxane- poly(vinyl alcohol) (PDMS/PVA) fiber	GC-ECD	78.7-91.5 (< 14.2 %)	LODs ≤ 0.015 mg L^{-1}	(Zuin et al., 2004)
102 multi-class pesticides	Traditional Chinese herbal infusions	L-L extraction acetone:EtAc:hexane (1:2:1) plus GPC and SPE (Envicarb) elution with the same solvent mixture	GC-MS (SIM)	59.7-120.9 ((< 20.8 %)	LOQs ≤ 2.5 mg L^{-1}	(Huang et al., 2007)
15 OPs	Ginkgo leaves	ASE (accelerated solvent extraction)in MeCN and further SPE (Envicarb) eluting with MeCN:toluene (3:1)	GC-FPD	95.2 % (< 4.6 %)	LODs ≤ 0.044 mg kg^{-1}	(Yi & Lu, 2005)
18 OPs	Flos lonicerae	Ultrasonic wave assistant extraction (UAE) in acetone and further SPE clean-up.	GC-FPD	83.6-88.7 % (< 6.0 %)	LODs ≤ 0.018 mg kg^{-1}	(Xiang et al., 2006)
6 Carbamates (metolcarb, isoprocarb, fenobucarb, carbofuran, pirimicarb, and carbaryl)	Traditional Chinese herbs	Soxhlet extraction with CH_2Cl_2	GC-NPD (SIM)	80.8-154.6	LOQs ≤ 0.05 mg kg^{-1}	(Wu et al., 2005)
Multi-class 195 pesticides	Traditional Chinese herbs	UAE in acetone and further GPC clean-up.	GC-MS (SIM)	Normally 80-120 %	LOQs ≤ 0.05 mg kg^{-1}	(Wang et al., 2011)

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
Acephate, chlorpropham, pirimicarb, bifenthrin, tetradifon, and phosalone	Cordia salicifolia	MSPD using the 2 dimensional coordination polymer (∞[Gd(DPA)(HDPA)])	GC-MS (SIM)	20-107.7 % (< 29.1 %)	LOQs ≤ 0.25 mg kg^{-1}	(de Carvalho et al., 2009b)
Multi-class 15 pesticides (OCs, OPs, Pys and fungicides)	Isatis indigotica raw material, granule formuation and infusion	Twice UAE extraction in acetone: petroleum ether (5:3) mixture and L-L clean-up with hexane for raw material or granule. Infusion L-L extraction with petroleum ether.	GC-ECD/FPD	70.2-119.5% for raw material, 73.2-105.1% for granule formulation, and 72.8-113.3% for infusion formulation	LODs ≤ 0.035 mg kg^{-1}	(Tang et al., 2005)
6 multi-class (metalaxyl, triadimefon, and paclobutrazol, vinclozolin, tebuconazole, fenatimol)	Isatis indigotica Fort and Paeonia lactiflora	MSPD on silica gel. Packing on a column with Na_2SO_4 co-column. Elution with acetone.	GC-NPD	80.6-106.1% (< 17.7 %)	LOQs ≤ 0.05 mg kg^{-1}	(Tang et al., 2006)
A) 9 OCs B) 19 OCs	A) Plant infusions B) Chinese herbal formulations	SPME with PDMS coated fiber	GC-MS (SIM)	A) 90-108 % (< 17.0 %) B) – (< 31.0 %)	A) LOQs ≤ 0.012 mg kg^{-1} B) LODs ≤ 0.001 mg kg^{-1}	A) (Rodrigues et al., 2005) B) (Hwang & Lee, 2000)
7 OCs	Atractylodis rhizoma, Glycyrrhizae radix and poria	MAE in water and SPME with PDMS coated fiber	GC-ECD	-	LODs ≤ 0.00013 mg kg^{-1}	(Ho & Hsieh, 2001)
11 pesticides (hexachlorobenzene, lindane, chlorothalonil, parathion methyl, parathion ethyl, fenitrothion, malathion, dieldrin, α- and β-endosulfan, and tetradifon)	Passiflora L.	SBSE with PDMS coated fiber	GC-ECD/FPD	30-90 %	B) LOQs ≤ 0.117 mg kg^{-1}	Bicchi et al., 2003)
9 OCs	Mikania laevigata, Maytenus ilicifolia and Cordia	Solid-liquid extraction (SLE) with n-hexane: CH_2Cl_2 (4:1), followed by clean-up in solid phase mixed	GC-MS (SIM)	70-124 % (< 7.3%)	LOQs ≤ 0.03 mg kg^{-1}	(Rodrigues et al., 2007)

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
	verbe-nacea	cartridge (Florisil and silica-gel) eluting with n-hexane- CH_2Cl_2 (3:2)				
A) 9 OCs B) 18 multi-class (fungicides, insecticides)	Ginseng root	A) Soxhlet extraction with acetone: petroleum ether (3:1) and cleanup with 5 mL H_2SO_4. The supernatant is separated. B) Chopped into small portions, add MeCN and NaCl and centrifugation for L-L extraction. The organic phase is separated, concentrated and directly injected for GC-NPD. Further clean-up is carried out using SPE (Florisil) elution with hexane:acetone (8:2) for GC-ECD.	A)GC-ECD and GC-MS confirmation B) GC-ECD/NPD	A) - B) 72.3-117.2 % (< 7.3%)	A) - B) LOQs ≤ 0.2 mg kg^{-1}	A) (Quan et al., 2004) B) (Park et al., 2007)
5 OCs (hexachlorobenzene and hexachlorocyclohexanes (α-, β-, δ-, and γ-isomers))	Ginseng root	Soxhlet with EtAc: petroleum ether (7:3) mixture. H2SO4 cc. digestion and the supernatant analyzed	Isotope dilution GC-MS (SIM)	-	Repeatability ≤ 1.4 % Expanded relative uncertainty ranging from 4.0-6.5 %	(Chan et al., 2007)
170 multi-class (OPs and OCs)	Ginseng root	S-L extraction with EtAc and combined clean-up by GPC plus (PSA)/graphitized carbon black (GCB) SPE column eluting with EtAc:toluene (3:1).	A) GC-(Q)MS (SIM) B) GC-TOF MS (HRMS)	A) Mean = 83; 79; 75 for 25, 100, and 500 µg kg^{-1} levels (< 5.0%) B) Mean = 93; 85; 81 for 25, 100, and 500 µg kg^{-1} levels (< 8.0%)	A) Geometric mean LOQ = 0.004 mg kg^{-1} B) Geometric mean LOQ = 0.003 mg kg^{-1}	(Hayward & Wong, 2009)
168 multi-class (OPs and OCs) pesticides	Ginseng root	S-L extraction with A) MeCN or B) acetone/cyclohexane/EtAc (2:1:1) mixture. Further SPE (C8 + GCB/PSA) sorbents clean up and toluene elution.	1) GC-MS (SIM) 2) GC-MS/MS (QqQ)	86-88 % (< 14.0%)	Geometric mean LOQ A1)=0.053; A2)=0.006; B1)=0.048; B2)=0.007 mg kg^{-1}	(Wong et al., 2010)

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
20 multi-class (OCs, OPs, Pys and fungicides)	Radix paeoniae, Isatis indigotica Fort, Pltycodon grandiflorum, Cotex mouta and Poria cocos	Powdered and UAE in acetone/ CH_2Cl_2(2:1) with subsequent clen-up on silica gel column chromatography eluting with ether:acetone (40:60).	GC-ECD	72.5-113.5 % (< 14.0 %)	LOQs ≤ 0.082 mg kg^{-1}	(Qian et al., 2010)
Pentachloronitrobenzene, pentachloroaniline, methylpentachlorophenylsulphide and procymidone	Ginseng extract	Sonicated MSPD. Dispersion on Florisil and extracted twice in EtAc-hexane (70:30)	GC-μECD	83.5-97.4 % (< 10.0 %)	LODs ≤ 0.004 mg kg^{-1}	(Qi, 2010)
54 multi-class pesticides	6 traditional Chinese herbs	Extraction with MeCN and clean-up on SPE (C18/Envicarb/ PSA).	GC-MS (SIM)	70-120 % (< 20.0 %)	LODs ≤ 0.01 mg kg^{-1}	(Miao et al., 2010)
74 multi-class pesticides	6 traditional Chinese herbs	ASE and combined purification on GPC and SPE.	LC-(QqQ)MS/MS	70-110 % (< 15.0 %)	LODs ≤ 0.01 mg kg^{-1}	(Mao et al., 2010)
Acephate, chlorpropham, pyrimicarb, bifenthrin, tetradifon, and phosalone	Cordia salicifolia leaves	MSPD on A) neutral alumina or B) peat, using Na_2SO_4 and C_{18} co-column.and eluting with cyclohexane:CH_2Cl_2 (3:1).	GC-MS (SIM)	67.7-129.9 % (< 15.0 %) 64-118.0% (< 26.4 %)	LOQs ≤ 0.25 mg kg^{-1}	A) (de Carvalho et al., 2009a) B) (de Carvalho et al., 2010)
4OCs: hexachlorocyclohexanes (α-, β-, δ-, and γ-isomers))	Withania somnifera and Ocimum sanctum	MSPD with Florisil and blended with MgSO4 and NaCl and eluting with f n-hexane:EtAc mixture (70:30).	GC-ECD	93-103 % (< 10%)	LODs ≤ 0.005 mg kg^{-1}	(Abhilash et al., 2007, Abhilash et al., 2009)
Aldrin, endrin, dieldrin and HCH isomers	Withania somnifera, Ocimum sanctum L. and Achyranthes aspera.	Modified EP method: maceration in acetone: CH_2Cl_2 (3:1). Further MSPD (Florisil) eluting with the same solvent mixture.	GC-ECD	88-98 % (< 9.85%)	LODs ≤ 0.001 mg kg^{-1}	(Abhilash & Singh, 2008)
108 OPs pesticides	Ginseng root	QuEChERS based MeCN:water extraction. dSPE with PSA:GCB	1) GC-MS (SIM) 2) GC-FPD	> 90 % for most compounds (< 37 %)	1) LODs ≤ 0.50 mg kg^{-1} 2) LODs ≤ 0.05 mg kg^{-1}	(Wong et al., 2007)

Compounds	Matrix	Sample treatment and clean-up step	Determination	Recovery rates (%) (RSD (%))	Analytical features	Reference
15 OCs	American Ginseng	QuEChERS based MeCN:water. dSPE with PSA:GCB	GC-ECD	81.4-95.2 (< 8%)	-	(Wu et al., 2011)
234 pesticides	Korean herbs	QuEChERS based MeCN(HAc):water extraction. dSPE with PSA:GCB	GC-MS (SIM)	62-119 % (< 21%)	1) LODs ≤ 0.40 mg kg^{-1}	(Nguyen et al., 2010)
81 multiclass pesticides	Ginkgo leaves	QuEChERS based MeCN:water. dSPE with PSA:GCB	GC-MS (SIM)	70-110 % for most compounds (< 20%)	LOQs ≤ 0.058 mg kg^{-1}	(Zhou et al., 2011)

Table 2. Analytical features of most recent reports in pesticide residue analysis in pharmaceutical and medicinal plants.

4.2.2 Liquid chromatography

The trend, in which these matrices were not excluded, was the introduction of non-persistent and biodegradable pesticides which have lead to the introduction of more polar (and less volatile) agrochemicals. In agreement to food and environmental matrices, such compounds have prompted the use of LC–MS, which at the moment is widely accepted technique for monitoring purposes of polar and most semi-polar pesticides as well as for regulatory issues. Concerning this matter, few attempts were made in pharmaceutical matrices although major advances are expected in a short term.

LC-DAD was used in beeswax (Adamczyk et al., 2007) and medicinal plants (Choi et al., 2007, Peng et al., 2007, Tuzimski 2011) to determine pesticide residues. Main pitfall is the attained selectivity and sensitivity for proper and unambiguous trace determination.

Due to its versatility for wide variety of organic molecules, electrospray ionization (ESI) operating in positive mode is the preferred interface for most studies that uses LC-MS. In general, ESI can be applied for polar, ionized, and semi-polar analytes if adequate mobile phases are selected. Depending on the polarity, ionization process in ESI usually gives protonated or deprotonated molecules ($[M+H]^+$, $[M-H]^-$) although adducts can also be obtained (i.e. $[M+Na]^+$, $[M+NH_4]^+$).

Residues of methamidophos, imidacloprid, benomyl, thiophanate-methyl, bendiocarb, diflubenzuron, chlorpyrifos, flufenoxuron, carbosulfan and bifenthrin were determined in citrus essential oils by using LC–ESI(+)–(Q)MS with LODs ≤0.05 mg/kg (Barrek et al. 2003). The authors noted high matrix effect for the analyzed extracts which cannot be overcome by simple LC−MS but also suggest the use of MS/MS experiments in further studies. Unfortunately, LC−MS/MS has not been reported until recently in the literature for the determination of pesticides residues in this topic. Nevertheless, advances in the application of LC−MS/MS for testing pesticides in pharmaceutical matrices are expected in a short term since the use of ESI sources and conventional QqQ analyzer is currently the most reported technique for target screening of pesticide residues in food and environmental samples.

Several analytical features are provided when using LC−MS/MS ranging from excellent performance for quantitative analysis when working in the selected reaction monitoring (SRM) mode; allowing the selection of two specific SRM transitions with subsequent confirmation of the analyte in the sample. A combined QqQ scanning functionality with a

sensitive linear ion trap (LIT) is offered in the QLIT (quadrupole-linear ion trap) system. Working in the LIT mode, the hybrid QLIT provides improved performance, higher versatility as well as enhanced sensitivity, both in full scan MS (EMS) and product ion (enhanced product ion; EPI) scan modes. Improvements in this hybrid system are also related to software developments as seen in the sSRM (schedule SRM mode) or the combination of SRM and EPI scans by the built-in information dependant acquisition (IDA) software tools used for confirmation purposes of the residues. Direct injection analysis of 10 fold diluted lavandin essential oil was recently reported for the determination of 70 multiclass pesticides using sSRM acquisition mode in a new generation LC−(ESI)−QLIT MS/MS instrument in both positive and negative ionization (Fillâtre et al., 2011). Obtained LODs were all below 20 µg/L. Major technological improvements in QLIT were focused on enhanced sensitivity. Nowadays the impressively low instrumental detection limits (IDLs) are offering new workflows and providing independence from time consuming sample preparation steps. Last but not least, LC−HRMS is expected to find application in large scale screening of contaminants and reliable conformation of residues using accurate mass measurements. An interesting example was stated by Schürmann and co-workers in the correct identification of false positive sebuthylazine residues in tarragon. The situation lies on the fact that nominal LC−MS/MS confirmation is accomplished by the use of 2 specific transitions plus retention time matching with standards. However, sometimes this approach is not possible to apply for troublesome analytes which even could lead to inadequate report of residues. However, it was found that false positive findings can be discarded by the use of resolving power provided by TOF-MS for the identification of the endogenous matrix compounds (Schürmann et al., 2009).

4.2.3 Matrix effect

Matrix effect is considered to be a suppression or enhancement of the analyte response due to the influence of the matrix. However, when dealing with MS determination, either in GC and LC, matrix effect is normally referred to ion suppression/enhancement. Compensation of the matrix effect is then compulsory for accurate quantitation. Several strategies were employed to reduce the matrix effect such as the use of external calibration, matrix matched calibration, isotopically labeled surrogate compounds, analyte protectants (APs) or internal standard addition. Matrix effect can be estimated by comparing the response obtained from the standard solution and that from the spiked sample extracts. Matrix effect may be partially solved before the detection technique for instance by exhaustive clean-up through the reduction of co-extractives, improved separation (comprehensive two dimensional gas chromatography (GCxGC) or ultra high pressure liquid chromatography (UPLC) by using sub 2µm particle size), sample dilution if enough sensitivity is obtained or reduction of the injected sample (Gilbert-López et al., 2009).

In GC this effect was widely investigated for food matrices principally when GC–(SIM)MS was used (Anastassiades et al. 2003b, Poole 2007). On the other hand, in LC this effect is typically faced at the expense of the sensitivity of MS/MS applying sample dilution (Gilbert-López et al. 2009). Among others, in GC, matrix-induced effect is mainly related to the silanol active sites present in the liner as well as in the chromatographic column which might interact with the analyte, resulting in analyte loses and distorted peak shapes (Anastassiades et al. 2003b). Chan and co-workers introduced the use of isotope dilution (ID) for the determination of HCB and HCH isomers by GC–MS in Ginseng root. ID-GC–MS showed to be a good quality assurance approach since inaccuracy and uncertainty were

significantly reduced in a difficult matrix (Chan et al., 2007). However, this strategy is generally expensive, time consuming for routine analysis and is intended for MS only.
The use of matrix matched calibration (MMC) or APs is being increasingly used since most reports still face determination of semi-volatile compounds by GC with different detection techniques. Moreover, note that when using selective and non specific detection such as ECD, matrix effect could involve co-eluted compounds which can increase the noise while negatively affect the peak area and reducing the quantitative performance. Jiménez et al., (2004b) reported MMC for the multiresidue analysis of pesticide residues in beeswax by GC–ECD. Moreover, Pérez et al., (2010) used MMC for the quantitative determination of pesticides in lanolin by GC–ECD/FPD when using MSPD as sample treatment. On the other hand, several authors used MMC in plant origin samples with medicinal purposes by GC–ECD (Zuin et al. 2003), GC–MS (Hayward & Wong 2009, Wong et al. 2007) and even when more sophisticated GC–(QIT)MS/MS (Li et al., 2009) and GC–(QqQ)MS/MS (Wong et al., 2010) were used. It should be highlighted that MMC is not referred in USP and EP.
Since the possibility of not getting reference matrices or representative materials to perform MMC, the use of alternatives such as APs is of great concern. Ideally, APs overcome these limitations by interacting with the active sites and conducting reliability in the response of the analyte. Dealing with real samples the compensation of the matrix effect cannot be solely related to new method developments, since it could be easily applied to official methodologies to obtain more accurate results. Recently, in accordance to food residue analysis, eight different APs were evaluated for GC–MS determination of an extensive group of 195 pesticides in medicinal plants (Wang et al., 2011). Troublesome and early eluting pesticides such as acephate and omethoate were succesfully determined using d-ribonic acid-γ-lactone (2 mg/mL) whereas sorbitol showed the best compensation effect for late eluted compounds such as fenitrothion and methidation. From the results obtained, the authors concluded a mixture of d-ribonic acid-γ-lactone and d-sorbitol for the reliable determination of most pesticides by GC–MS although there is neither an ideal AP nor mixture of APs that can completely resemble these complex matrices in order to compensate the matrix-induced enhancement for such an amount of pesticides (Wang et al. 2011).
The need for sample treatment step in LC–MS determination encompasses the undesired effects of interferents on analytical performance to requested LODs for proper trace analysis of pesticides. In LC–MS instead, matrix effect is usually referred to co-elutants during the ionization process when using atmospheric pressure interfaces (such as ESI) which are prone to ion suppression and enhancement effects. The approach of MMC and sample dilution is widely assayed in LC–MS. A combined sample dilution and MMC strategy was also employed in QLIT-MS/MS determination of 70 pesticide residues in lavandin essential oil. The authors reported that using this approach, weak matrix effect (≤20%) for 70 % of the compounds was obtained (Fillâtre et al. 2011).

5. Conclusions

This chapter has outlined latest improvements concerning pesticide residues in natural products commodities employed as raw materials used in the pharmaceutical industry or for medicinal purposes. Occurrence of pesticide residues is demonstrated to be moving from classical compounds, in which official methodologies still focus their interest, to more specific compounds and crop treatments. As demonstrated, environmental pollution plays an important role in the occurrence of unexpected pesticides, principally for roots materials

or lipophilic matrices such as beeswax. Current occurrence of previously unstudied pesticides in these matrices corresponds to trends in the use of different pesticide classes and biological target actions (fungicides were also integrated to the sanitary packages) to more environmentally-friendly, more polar, less toxic but also troublesome to analyze. Notably, monitoring trends and exposure monitoring for risk assesment are reavealing lack of legislation as well as dedicated studies in these matrices.

Scientists and regulators should recognize the current pesticide reality about residues occurrence in matrices with dermal contact or direct ingestion applications.

Last but not least, pest control is nowadays a matter of critical concern for bee's by-products which shows the higher contamination levels with pesticides from the studied matrices.

Advances in pesticide residue analysis were stated and discussed. Nowadays, it is being carried out an update in techniques for residue analysis as experimented for foodstuffs and environmental samples years ago. Both newer sample preparation and determination techniques based on chromatography coupled to mass spectrometric detection, are offering improved knowledge and reliability for residue analysis. However, since the magnitude of this problem, several strategies should be necessary to employ in order to cover more target analytes and matrices of interest.

Perspectives in this field are expected to be focused on the implementation of miniaturized, high throughput sample preparation methodologies and widespread use of advanced mass spectrometric techniques. This will help for the comprehensive assessment on pesticide occurrence in matrices particularly not yet well studied and difficult to handle. An update on residue methodologies is a necessary step in further Pharmacopeias along with urgent specific regulations for pesticide residues which are currently being found. Such a lack of information will encourage investigation in this topic in the years ahead.

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15

Non-Targeted Analyses for Pesticides Using Deconvolution, Accurate Masses, and Databases – Screening and Confirmation

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1. Introduction

Technical innovations in crop protection have been a key component in the globalization of food production and distribution over the past several decades. The ease of access to foodstuffs from distant growing regions has depended, to a large extent, on the new pesticides that combat the historic foes of food sufficiency: fungi, insects, and weeds. Yet the same public that has come to expect and demand the ready availability of food products also has high expectations of regulators to ensure the safety of their food supply. Regulators in the United States, Europe, and Asia have, therefore, had to grapple with the task of ensuring that exposure levels to agricultural chemicals in the food supply remain within strictly determined parameters. In each region, regulatory agencies have, during the past decade, produced pesticide regulations that are increasingly stringent both in terms of the number of pesticides tracked and the allowable tolerances of those pesticides in the food supply.
Because over a thousand pesticides are used globally to protect crops and improve food production, regulatory stringency translates into new challenges for the refinement of residue data generation and analysis. Because pesticide contamination of foodstuffs and environmental matrices could provide significant risk to consumers, vigilant monitoring is required; but the number of targeted and non-targeted pesticides that must be monitored in a given global region has risen significantly. Therefore, simple, rapid methods for screening hundreds of pesticides at trace levels in various matrices must be established. The widely divergent chemical properties (polarity, thermal stability, etc.) of these non-targeted pesticides call for the application of different analytical methods. Both GC and LC approaches are available, with each using a different technique for data analysis.

1.1 Gas phase analyses

For samples that are amenable to GC analysis, i.e., nonpolar and thermally stable, retention time locking (RTL) can be used. RTL is a technique developed by Agilent Technologies (Santa Clara, CA) that allows analysts to match analyte retention times (RTs) on any Agilent GC instrument in any laboratory in the world, provided that the same nominal GC method and

capillary column are used (Giarocco et al., 2000). Using RTL, Agilent has developed several retention time-locked databases for GC and GC/MS that include the locked retention time, compound name, CAS number, molecular weight, and mass spectrum (RTL URL). The Agilent RTL Pesticide Library contains this information for 927 compounds, including pesticides, metabolites, and endocrine disruptors along with important polychlorinated biphenyls, polybrominated biphenyls, polycyclic aromatic hydrocarbons, synthetic musk compounds, Sudan dyes, and organophosphorus fire retardants. Another database contains all of the analytes specified for GC/MS analysis in the new Japanese "Positive List" regulations.

While data analysis based on retention time is extremely productive and reliable, additional power and speed of analysis is obtained by deconvoluting the spectra. In GC/MS, deconvolution is a mathematical technique that "separates" overlapping mass spectra into "cleaned" spectra of the individual components. As seen at the top left of Figure 1, a total ion chromatogram (TIC) might consist of several overlapping components.

The deconvolution software utilized in the Agilent Deconvolution Reporting Software (DRS) and discussed throughout this article is the Automated Mass Spectral Deconvolution and Identification System (AMDIS) developed by National Institute of Standards and Technology (NIST) (AMDIS URL). In addition, Agilent's RTL Pesticide Library also includes the AMDIS format for use with DRS. A filter can be set in AMDIS that requires the analyte's RT to fall within a user-specified time window at the expected RT.

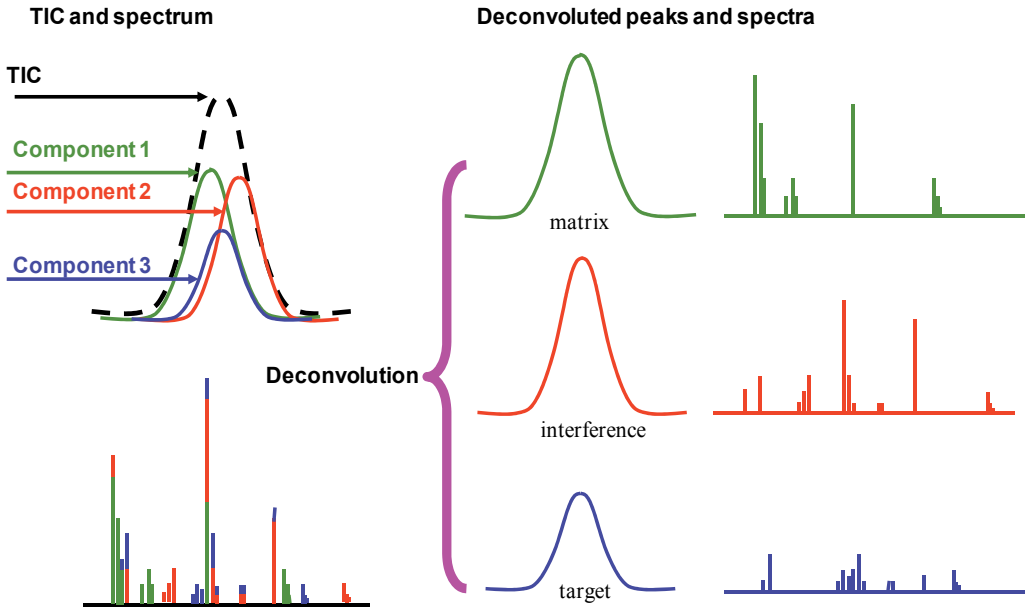


Fig. 1. The mass deconvolution process

The spectra at any particular retention time can be deconvoluted (cleaned) by reporting only those ions whose chromatographic apexes are reached at that particular retention time; all other ions that may elute slightly earlier or later, may be present as general background and are discarded. In addition to the chromatographic apex at a specific RT, peak shape is an additional factor considered in deconvolution. That is, ions with both the same apex location

and similar peak shape are clustered into a component for searching. In this way, the deconvolution process finds those ions whose individual abundances rise and fall together within the spectrum. As illustrated in Figure 2, deconvolution produces "clean" spectra that are the composite of only those ions at the same apex location and with similar peak shape. Deconvolution finds the components (a component is a group of related ions) from a complex TIC. Each component is searched against a retention time locking (RTL) library in AMDIS format. In addition to spectral matching, the locked RT can also be used as a criterion for hits. Depending on the match factor from the search, target compounds can be identified or flagged in a complex TIC.

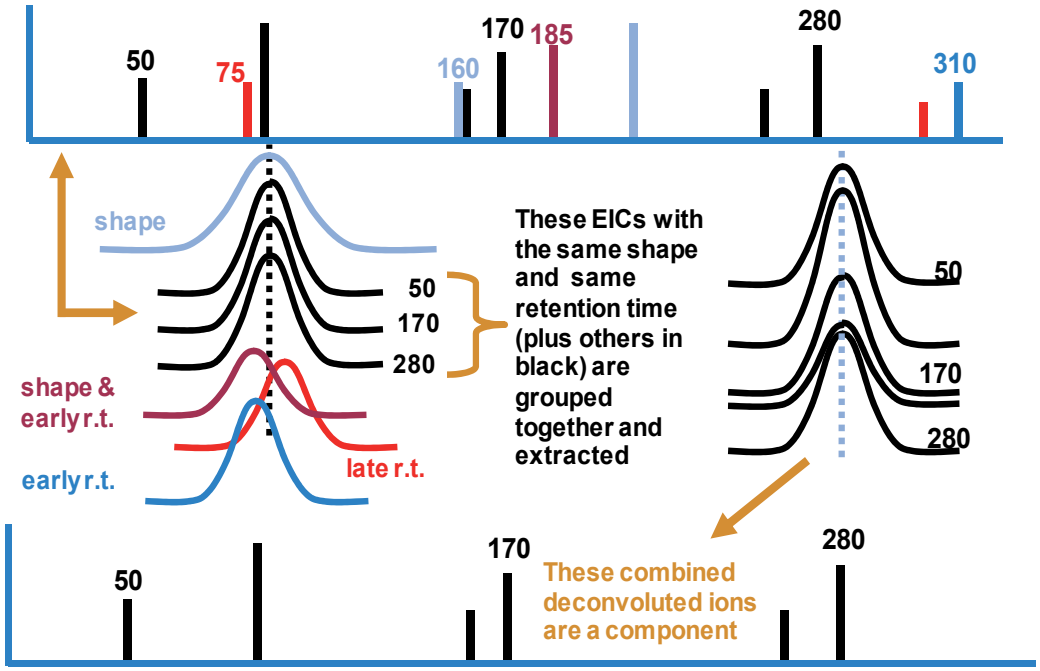


Fig. 2. Graphical representation of the deconvolution process

Because RTL is used to reproduce the RTs with high precision, this window can be quite small—typically 10 s or less. This "RT qualifier" is very useful for blind study/screening (Sandy, 2004). The components identified by deconvolution and Agilent's Pesticide Library can be further confirmed against the NIST library containing pesticides and numerous other compounds. Note that the NIST reverse search is based solely on the ion pattern for the compound and does not incorporate retention time matching.

1.2 Liquid phase analyses

For samples that are more amenable to LC analysis, i.e., polar and thermally labile, a triple quadrupole (QQQ) is often used for trace level target analysis of complex matrixes. The multiple reaction monitoring process removes chemical background from the sample matrix, thereby providing superior selectivity and sensitivity for pesticide analysis. But LC-single quadrupole and QQQ instruments cannot readily be used for nontarget

identifications for two reasons. First, due to lack of selectivity and sensitivity, LC-single quadrupole and QQQ instruments do not usually operate in the full-scan mode for pesticide screening. Second, common LC/MS spectral libraries are unavailable due to difficulties in standardizing and reproducing fragmentation energies among instruments from different vendors. Therefore, when screening for a broad range of pesticides, a TOF (time-of-flight) or Q-TOF is the instrument of choice (Mezcua et al, 2009). A high-resolution TOF instrument always acquires full spectra and gives accurate masses.

Recent experience has demonstrated that LC/TOF-MS has the capability and sensitivity to obtain full spectra with a mass accuracy of less than 1 ppm (Ferrer & Thurman, 2005). This level of accuracy in mass allows the analyst to distinguish between compounds whose molecular weights are extremely similar. Table 1 lists several pesticides whose exact mass varies by a fraction of an amu.

Element	**Atomic Number**	**Exact Mass**
H	1	1.007825
C	6	12.000000
N	7	14.003074
O	8	15.994915
Formula	**Exact Mass**	**Compound Name**
$C_6H_6Cl_6$	287.8600665	Lindane
$C_{10}H_{12}N_2O_6S$	288.0416000	Carbasulam
$C_9H_{21}O_2PS_3$	288.0441285	Terbufos
$C_{13}H_{21}O_3PS$	288.0949000	Iprobenfos
$C_{15}H_{17}N_4Cl$	288.1141743	Myclobutanil
$C_{12}H_{21}N_2O_4P$	288.1238937	Diazoxon
$C_{11}H_{20}N_4O_3PS$	288.1256000	Epronaz
$C_{11}H_{21}N_4O_3P$	288.1351000	Pirimetaphos
$C_{16}H_{20}N_2O_3$	288.1473925	Imazamethabenz

Table 1. Exact masses of elements and pesticide compounds

LC/Q-TOF instruments with the capability of measuring 2 ppm (i.e., 0.000576 amu for mass 288) accuracy can easily distinguish between these molecules. A tool called Molecular Feature Extractor (MFE), which is similar to deconvolution used in GC/MS, finds all ions in an LC/TOF data file that represent ions of real compounds in the sample analyzed. Noise and other extraneous ions are excluded. The resulting list of anion or cation masses is then searched against a database of theoretical monoisotopic exact masses of compounds based on their molecular formula and selected adduct ions (H+ or Na+, etc). Then, by comparison to the

Exact Mass Database of hundreds of pesticides, the normally tedious manual process of matching can be done rapidly and reliably (Thurman & Ferrer, 2005) using mass accuracy and RT as the criteria. After identification, the sample can be reanalyzed in the MS/MS mode using a QQQ or Q-TOF instrument to confirm any hits from the database search.

2. Experimental

2.1 GC: Analysis of surface water

The California Department of Food and Agriculture (CDFA) prepared and analyzed surface water samples using an Agilent 6890N gas chromatograph equipped with an Agilent 5973 inert mass selective detector (MSD) (Siegel et al, 2004). The sample collection and preparation procedure is the following: A 1 L water sample is delivered to the laboratory in an amber glass bottle. Samples are stored under normal refrigerated conditions (approximately 4°C) until extraction within 7 days.

2.1.1 Surface water samples

2.1.1.1 Sample preparation

a. Weigh and record the 1 L water sample, including sediments.
b. Pour the water sample, including sediments, into a 2 L separatory funnel. Do not filter since pesticides would stick to humic materials.
c. Spike with 1 mL surrogate spiking solution: 0.5 ng/mL Chlorpyrifos-methyl (0.5 ng). Shake the separatory funnel gently to mix.
d. Add 10–15 g granular Sodium Chloride (NaCl) for salting-out purposes. Shake gently to dissolve salt.
e. Rinse water sample container with 60 mL Methylene chloride and add to the separatory funnel. Weigh and record the empty water sample container. Subtract and record the water sample weight.
f. Shake and release pressure several times. Shake well for 3 min. Let settle until the lower Methylene chloride layer is completely separated from the above water layer. If there is too much emulsion in the funnel, use a sonicator to break up the emulsion.
g. Filter the bottom organic layer through a bed of granular anhydrous sodium sulfate (approximately 20 g) into a 250 mL round-bottom flask. The sodium sulfate is supported on glass wool and is prewashed with 30–40 mL Methylene chloride.
h. Add 60 mL Methylene chloride into the funnel and repeat steps (f) and (g) two more times.
i. The round-bottom flask should now contain about 180 mL methylene chloride. Place the round-bottom flask on a Rotavapor evaporator (at about 100 rpm) and evaporate down to 5–7 mL at 40°C.
j. Transfer the contents of the round-bottom flask to a 15 mL collection tube. Rinse the round-bottom flask with 5 mL methylene chloride and add to the collection tube.
k. Place the 15 mL collection tube on an N_2-Evaporator with water temperature set at 40°C. Evaporate the sample to near dryness.
l. Remove the tube from the N_2-Evaporator and carefully add 1.0 mL methylene chloride and 10 mL 0.5 pg/mL internal standard (ISTD) solution into the collection tube.
m. Vortex and transfer the solution into an autosampler vial.
n. Cap and store the vial in a –5°C freezer until analysis.

2.1.1.2 Reagents and supplies

a. *Methylene chloride.*—Pesticide grade (Sigma-Aldrich, St. Louis, MO).
b. *Anhydrous sodium sulfate.*—Certified American Chemical Society (ACS) 10–60 mesh (Sigma-Aldrich).
c. *Glass wool.*—Pyrex brand fiber glass (Sigma-Aldrich).
d. *Sodium Chloride (NaCl).*—Certified ACS (Sigma-Aldrich).
e. *Nylon filter (0.45* m*m).* – Sigma-Aldrich.
f. *Surrogate spiking solution.*—0.5 ng/mL Chlorpyrifos-methyl in acetone (Ultra Scientific, N. Kingstown, RI).
g. *ISTD solution.*—Anthracene-d10, pyrene-d10, and chrysene-d12 at 0.5 ng/mL (500 ppt); Ultra Scientific ISM-520.

2.1.1.3 Agilent 6890N GC parameters

- *Column.*—30 m x 0.25 mm x 0.25 µm HP-5MS (Agilent Technologies, Santa Clara, CA).
- *Inlet temperature.*—230°C.
- *Injection volume.*—2 µL (splitless).
- *Oven ramp.*—Initial temperature at 70°C, hold for 2 min ramp at 25°/min to 150°C, hold for 0 min, ramp at 3°/min to 200°C, hold for 0 min, ramp at 8°/min to 280°C,hold for 12 min.

2.1.1.4 Agilent 5973 MSD parameters

- Full scan mode.
- Maximum sensitivity Auto Tune.

2.2 Pear and peach samples

2.2.1 Sample preparation

Samples were extracted using the quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method (Anastassiades et al, 2003; Lehotay et al, 2005).

a. 15 g homogenized sample + 15 mL acetonitrile + internal standard
b. Add 1.5 g Sodium Chloride (NaCl) and 6.0 g Magnesium Sulfate Anhydrous ($MgSO_4$)
c. Shake and centrifuge
d. Transfer 9 mL extract to tube containing 0.4 g Primary and Secondary Amine (PSA) + 0.2 g Graphitized Carbon Black (GCB) + 1.2 g Magnesium Sulfate Anhydrous ($MgSO_4$) and vortex
e. Add 3 mL toluene
f. Shake and centrifuge
g. Reduce 6 mL to ~100 µL
h. Add 1.0 mL toluene + QC standard + Magnesium Sulfate Anhydrous ($MgSO_4$) and centrifuge
i. Transfer to Automatic Liquid Sampler (ALS) vials for GC-MS analysis

2.2.2 Agilent 7890 GC parameters

- Autoinjector: 7693A
- Retention gap: 2 m × 0.25 mm id Siltek capillary tubing
- Column: HP-5MS UI (ultra inert), 15 m × 0.25 mm, 0.25 µm (from inlet to Purged Union) Agilent p/n 19091S-431 UI

- Oven ramp: Initial temperature at 100°C, hold for 1.6 min, ramp at 50°/min to 150°C, hold for 0 min, ramp at 6°/min to 200°C, hold for 0 min, ramp at 16°/min to 280°C, hold for 5 min.
- Run time: 20.933 min
- Inlet: Multimode Inlet (MMI) at 17.73 psi (Retention Time Locked), constant pressure mode
- RT locking: Chlorpyrifos-methyl locked to 8.297 min
- Liner: Helix double taper, deactivated (Agilent p/n 5188-5398)
- Injection mode: 2-µL cold splitless (fast injection)
- Inlet temp. Initial temperature at 50°C, hold for 0.01 min ramp at 720°/min to 300°C, hold
- Septum purge: 3 mL/min
- Purged Union: 4 psi (pressure supplied by a pneumatic control module, PCM)
- Split vent: 50 mL/min at 0.75 min
- Gas saver: 20 mL/min after 4 min
- Cryo on: Cryo use temperature 150 °C; time out at 15 min (Liquid CO_2)

2.2.3 Backflush parameters

- Postrun: 5 min
- Oven: 280 °C
- Purged Union: 70 psi
- MMI: 2 psi
- Restrictor: 0.7 m × 0.15 mm deactivated fused silica tubing (from Purged Union to MSD)

2.2.4 Agilent 5975 MSD parameters

Solvent delay: 2.5 min

- EMV mode: Gain Factor = 2
- Mass Range: Full scan, 45-550
- Threshold: 0
- Sample number: 2 A/D Samples 4
- Transfer Line: 280 °C
- Source: 300 °C
- Quad: 200 °C

2.3 LC/Q-TOF: Analysis of grape sample

An acetonitrile extract of a grape sample was prepared by the QuEChERS extraction method and analyzed by 1200 LC and 6510 Q-TOF (Agilent Technologies; 10). The QuEChERS sample extraction and cleanup procedure for both GC and LC is the following:

2.3.1 Extraction

a. Chop samples into small pieces and freeze in a bag overnight before grinding. Dry ice should be added during grinding.
b. Weigh 10 g homogenized sample into a 50 mL Teflon centrifuge tube.
c. Add 10 mL acetonitrile (and ISTD solution, if used).
d. Add 4 g anhydrous magnesium sulfate, 1 g sodium chloride, 1 g trisodium citrate dehydrate, and 0.5 g disodium hydrogen citrate sesquihydrate to the tube.
e. Adjust the pH to 5–5.5 using 5 M sodium hydroxide (NaOH).

f. Shake the sample vigorously for 1 min using a vortex mixer at maximum speed or by hand shaking.
g. Centrifuge for 5 min at 3000 rpm.

2.3.2 Cleanup

a. Transfer 6 mL supernatant into a 12 mL polypropylene centrifuge tube that contains 150 mg primary-secondary amine adsorbent and 900 mg Magnesium Sulfate Anhydrous ($MgSO_4$).
b. Shake for 30 s.
c. Centrifuge for 5 min at 3000 rpm.
d. Adjust the pH of the cleaned extract to 5.0 for analysis, if necessary.

2.3.3 Agilent 1200 LC parameters

Column	2.1 x 100 mm, 1.8 μm ZORBAX XDB PLUS C18	
Flow Rate	0.3 mL/min	
Injection Volume	10 μL	
Solvent A	0.1% Formic Acid in water	
Solvent B	100% acetonitrile	
Gradient	Time	Solvent B
	0	10%
	20	95%
	25	95%

2.3.4 Agilent 6510 QTOF parameters

Ion Source	ESI			
Drying Gas	325 C			
Drying Gas Flow	10 L/min			
Nebulizer	50 psi			
VCap	4000 V			
Fragmentor	175 V			
Reference Masses	121.050873 and 922.009798			
Acquisition Mode	MS1			
	Min Range	100		
	Max Range	1000		
	Scan Rate	1		
Acquisition Mode	Targeted MS2			
	MS Min Range	100	MS/MS Min Range	100
	MS Max Range	3000	MS/MS Max Range	3000
	MS Scan Rate	1.4	MS/MS Scan Rate	0.7
		Max Time Between MS	10	
		Ramped Collision Energy		
		Slop	5	
		Offset	5	

3. Results and discussion

In a GC/MS scan analysis, it is always very difficult to identify compounds from high matrix background because the matrix ions overwhelm the compound signal. To be certain

of the results, spectral averaging and background subtraction are often practiced. It is therefore a very time-consuming process to confirm compounds in a complex matrix.

3.1 Analysis of GC surface water sample data with DRS

Data files acquired by the CDFA on 17 surface water extracts were compared using two approaches (Wylie et al, 2004). Three example TICs are shown in Figure 3.

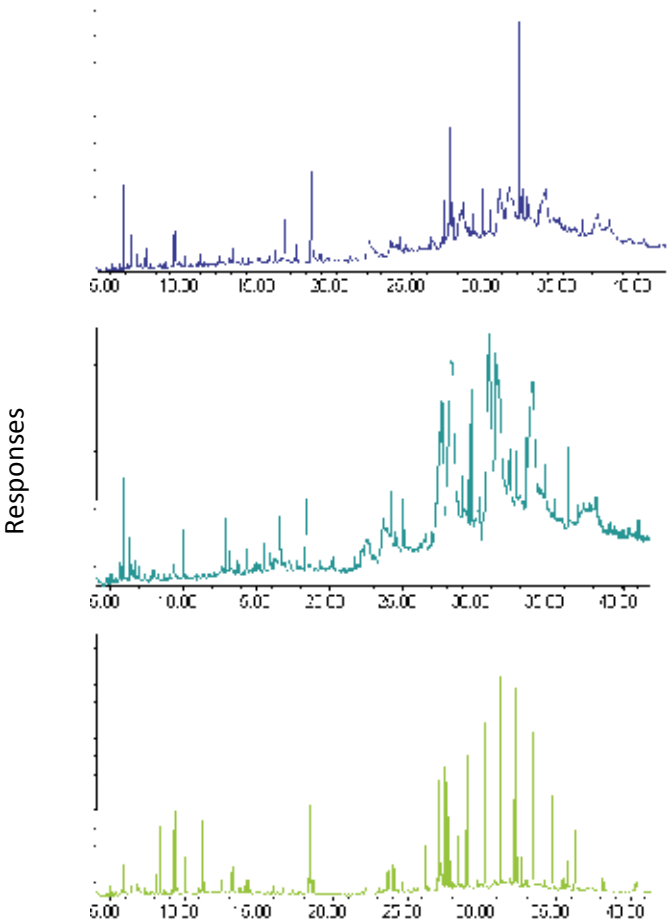


Fig. 3. TICs of surface water extracts showing the complexity of the matrix

The TICs show that the extracts were very complex. Approximately 8 h of a skilled analyst's time were required to process and confirm the results generated by ChemStation and searching the NIST library without the benefit of deconvolution. This workflow resulted in the identification of 37 pesticides plus one false positive. The same data files for the 17 TICs were processed using DRS without re-running the samples. The DRS reports were rapidly generated on all 17 TICs using batch processing. The display screen depicting this option is shown in Figure 4.

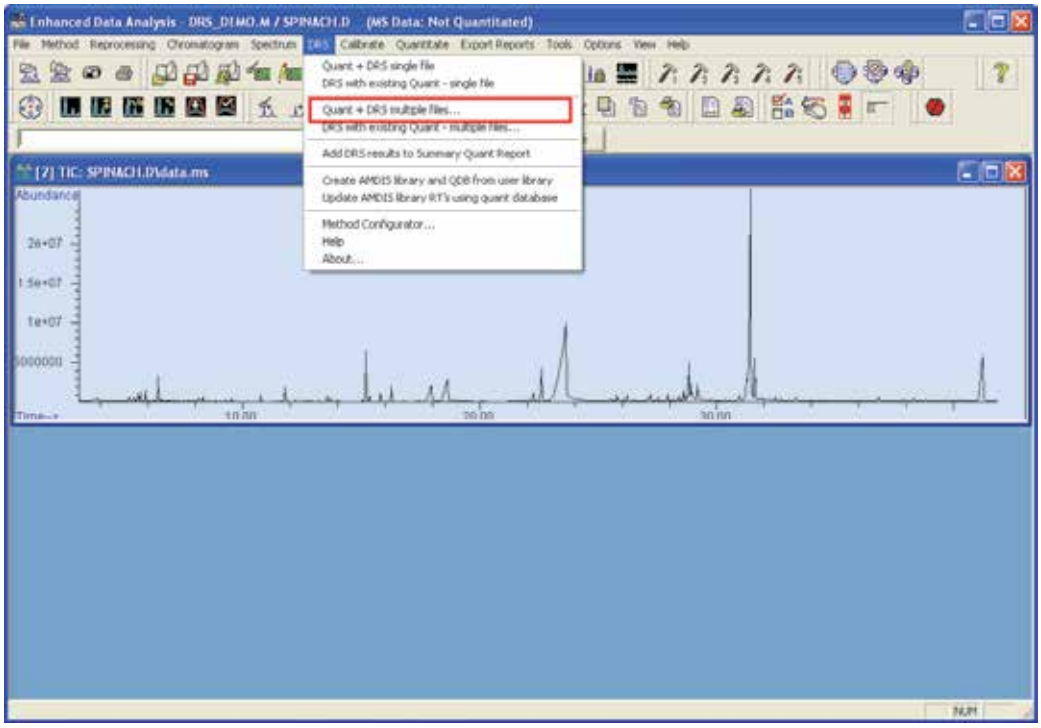


Fig. 4. Selecting simultaneous processing for all TICs in the Deconvolution Reporting Software (DRS)

Figures 5 and 6 show an example chromatogram and the corresponding DRS report for that chromatogram, respectively. The compounds listed in the report are only compounds in the specific DRS library. Therefore, the non-targeted compounds found in any sample are

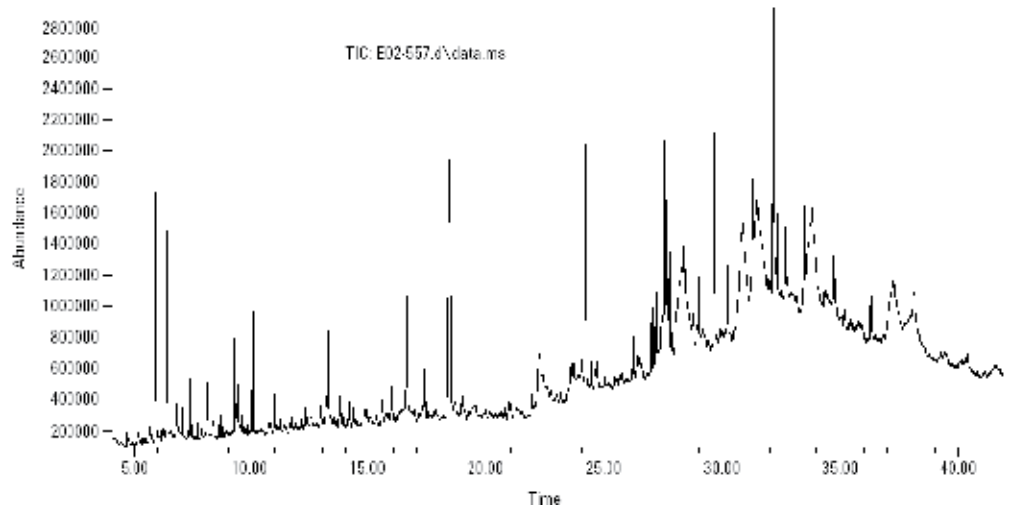


Fig. 5. Example total ion chromatogram (TIC) for pesticide analysis using GC/MS and DRS

			Agilent	AMDIS		NIST	
R.T.	Cas #	Compound Name	ChemStation Amount (ng)	Match	R.T. Diff sec.	Reverse Match	Hit Num.
4.8840	104121	4-Chlorophenyl isocyanate		86	-1.8	86	2
6.3888	102363	Diuron Metabolite [3,4-Dichlorophenyl isocyanate]		99	3.1	95	1
6.8136	54115	Nicotine		72	2.9	64	3
6.8357	759944	EPTC		84	2.0	85	1
7.6988	95761	3,4-Dichloroaniline		93	2.1	89	2
7.9342	131113	Dimethylphthalate		64	1.7	81	4
8.0431	85416	Phthalimide		72	3.8	81	1
8.1112	25013165	Butylated hydroxyanisole		63	-7.7	59	1
8.941	29878317	Tolyltriazole		74	5.9	68	4
9.7859	134623	N,N-Diethyl-m-toluamide		82	2.0	85	2
10.0019	84662	Diethyl phthalate		98	2.6	92	1
10.7109	119619	Benzophenone		88	2.6	85	2
10.9684	126738	Tributyl phosphate		96	3.0	92	1
11.6465	1582098	Trifluralin		84	0.6	76	1
12.9290	122349	Simazine		87	1.2	86	2
13.4460	115968	Tris(2-chloroethyl) phosphate		83	1.9	84	1
13.7451	1517222	Phenanthrene-d10		93	1.2	85	1
14.8285	4147573	Aziprotryn metabolite		64	7.1	79	1
15.3906	58082	Caffeine		61	0.7	78	1
15.9474	84695	Diisobutyl phthalate		89	3.2	89	2
16.5988	5598130	Chlorpyrifos Methyl		97	0.4	91	1
17.3680	7287196	Prometryn		90	1.7	84	1
18.4213	84742	Di-n-butylphthalate		99	0.4	93	1
18.9223	51218452	Metolachlor		94	0.8	91	1
20.5633	121552612	Cyprodinil		72	-0.1	64	1
26.4194	23576241	Norflurazon, Desmethyl-		86	-4.8	73	2
26.9700	27314132	Norflurazon		88	1.5	82	1
27.0010	85687	Butyl benzyl phthalate		94	-0.4	93	1
27.3984	51235042	Hexazinone		89	0.8	83	1
28.0171	78513	Tris(2-butoxyethyl) phosphate		76	3.6	82	1
29.6537	117817	Bis(2-ethylhexyl)phthalate		98	0.3	90	3
13.739		Phenanthrene-d10	10				

Fig. 6. MSD Deconvolution Report for the chromatogram in Figure 5

associated to the specific DRS library used (commercially available or user-built). Using DRS in conjunction with the 927 compound Agilent Pesticide Database, the same 37 pesticides were identified along with an additional 99 new identifications and no false positives. In addition to the improved results, the speed of the analysis was reduced from 8 h to 32 min, thus representing a 15-fold gain.

3.2 Analysis of pear data with DRS: DRS following the GC/MS analysis

The power of deconvolution is appreciated while comparing the top two spectra in Figure 7. The raw scan or original nondeconvoluted scan is shown on top. The clean scan, that is the deconvoluted component, is shown in the middle. The bottom scan is the identified compound in the AMDIS library.

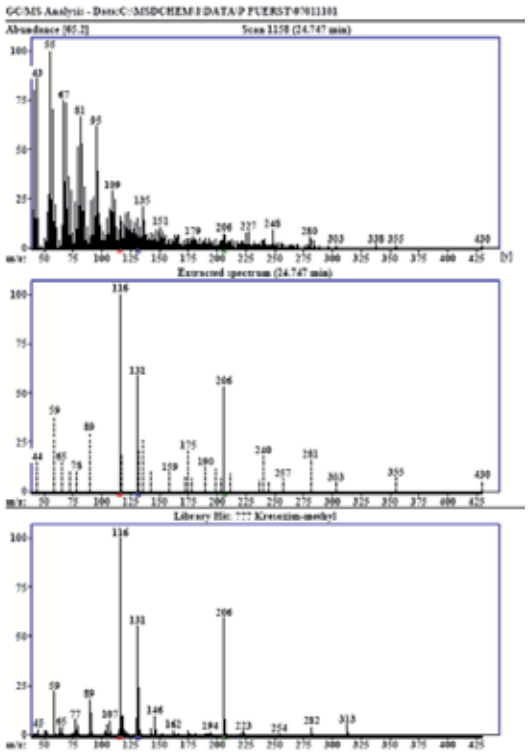


Fig. 7. Analysis results of pear extract using GC and deconvolution (DRS)

Without deconvolution, the analyst would visually compare the background subtracted raw scan and library scans for confirmation. It would be very difficult, if not impossible, to say that Kresoxim-methyl, the target compound in this example, is present using that type of comparison. The top mass spectrum is the raw scan from the TIC at 24.747 min and is clearly unsuited for library-searching purposes. In contrast, the middle spectrum (dashed lines are uncertain ions) presents the deconvoluted scan extracted from the raw scan. This deconvoluted (cleaned) scan is now easily and confidently matched against the library spectrum for Kresoxim-methyl presented in the bottom panel. For discussion on deconvolution parameter settings and additional advantages of deconvolution, reference the Agilent Application Note (Meng & Szelewski, 2010)

3.3 Analysis of peach data with DRS: DRS following the GC/MS analysis

An example DRS report for a peach extract is shown in Figure 8. As shown, Carbaryl appears with an AMDIS match factor of 74 versus the theoretical best match score of 100.

MSD Deconvolution Report
Sample Name: peach
Data File: C:\msdchem\1\DATA\FDA\08_03_07
FDA_S_CI\peach1_S_CI.D
Date/Time: 10:54:30 AM Monday, July 27, 2009

Adjacent Peak Subtraction = 1
Resolution = Medium
Sensitivity = High
Shape Requirements = Medium

The NIST library was searched for the components that were found in the AMDIS target library.

			Amount (ng)		AMDIS		NIST	
R.T.	Cas #	Compound Name	Chem station	AMDIS	Match	R.T. Diff sec.	Reverse Match	Hit Num.
2.2748	97530	Eugenol		0.09	77	2.5	72	7
3.3036	86737	Fluorene			76	0.5	63	16
3.3242	84662	Diethyl phthalate	0.2	0.17	89	1.4	90	1
3.5084	877098	2,4,5,6-Tetrachloro-m-xylene	0.36	0.33	97	3.1	91	1
3.558	119619	Benzophenone		0.06	73	0.9	81	2
3.6458	126738	Tributyl phosphate	1.29	1.09	88	1.5	91	1
5.316	84695	Diisobutyl phthalate		1.59	99	3.1	89	8
5.609	63252	Carbaryl		0.07	74	1.1	81	10
6.142	84742	Di-n-butylphthalate		2.31	97	0.6	93	1
7.083	133062	Captan		0.11	82	1.5	85	1
7.5447	959988	Endosulfan (alpha isomer)	0.04	0.04	91	-0.0	86	2
9.0198	85687	Butyl benzyl phthalate		0.07	81	3.5	85	1
9.070	999048032	Propiconazole-II		0.03	75	4.3		
9.0702	60207901	1H-1,2,4-Triazole, 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-					75	1
9.528	732116	Phosmet		1.33	96	5.4	93	1
10.7788	119611006	Fenbuconazole		0.42	76	6.9		
10.7788	0000	Piperazine-2,5-dione, 3-hydroxy-6-isopropyl-3-trifluoromethyl-					59	1

Fig. 8. DRS report for analysis of peach extract in full scan mode.

In general, an AMDIS match factor of 80 or higher is enough to confirm a hit. The RT difference of 1.1 s between the actual and expected RTs also indicates excellent agreement, i.e., high confidence in the results. RTL and a suitable time window (e.g., ±10 s) provide this added qualification function in screening. Some missing quantitation results in the ChemStation column were due to some out-of-range qualifier ion ratios. Qualifiers out-of-range are very typical for complex matrixes. The last two columns of the DRS report show the results from searching all the AMDIS hits against the NIST08 mass spectral library, which contains information on mass spectra only (not on RT). When the NIST library search finds a compound in the top 100 matches (a user-settable value) that agrees with the AMDIS results, its match factor is listed in the Reverse Match column. The Hit Number is shown in the last column, with 1 being the compound with the best match (highest match factor) in the NIST database. The compounds listed in the figure are all in the top 20 hits, with many as the number one hit. The further confirmation of hits found by DRS can easily be done with another injection in selected ion monitoring (SIM) mode or using GC detectors. In Figure 8, four compounds of interest with AMDIS match factors close to or less than 80 (Carbaryl, Captan, Propiconazole-II, and Fenbuconazole) were highlighted and further confirmed using SIM and GC detectors. Figure 9 is a DRS report of the same peach extract analyzed in SIM mode.

MSD Deconvolution Report
Sample Name: peach
Data File: C:\msdchem\1\DATA\FDA\08_03_07 FDA_S_CI\peach_SIM.D
Date/Time: 10:35:43 AM Monday, July 27, 2009

Adjacent Peak Subtraction = 1
Resolution = Medium
Sensitivity = High
Shape Requirements = Medium

The NIST library was not searched for the components that were found in the AMDIS target library.

			Amount (ng)		AMDIS		NIST	
R.T.	Cas #	Compound Name	Chem station	AMDIS	Match	R.T. Diff sec.	Reverse Match	Hit Num.
5.616	63252	Carbaryl	0.1	0.07	94	2.5		
7.0882	133062	Captan	0.2	0.14	96	2.3		
9.003	60207901	Propiconazole-I	0.03	0.02	93	4.6		
9.073	999048032	Propiconazole-II	0.07	0.04	97	5.1		
10.7836	119611006	Fenbuconazole	0.68	0.5	94	7.8		

Fig. 9. DRS report for analysis of peach extract in SIM mode.

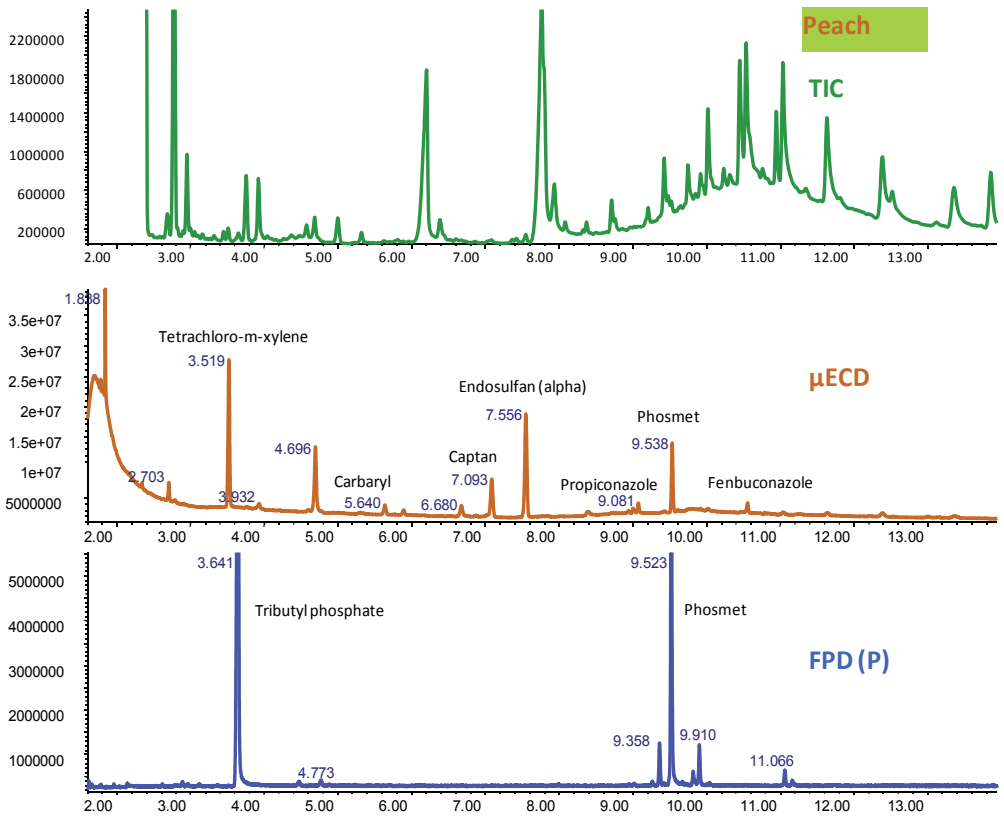


Fig. 10. Simultaneous display of MSD and GC selective detector signals for peach extract.

All of the compounds of interest in Figure 8 were found with AMDIS match factors over 90. Noticeably, the AMDIS quantitation results in Figures 8 (full scan) and 9 (SIM) were very comparable. These quantitation results are actually "semi-quant" results based on one average response factor for all of the 927 compounds in the DRS quantitation method. Figure 10 shows another approach for compound confirmation.
The figure shows simultaneously collected MSD TIC and GC element-selective detector signals [μECD (micro electron capture detector) and FPD (flame photometric detector) in the phosphorus (P) mode] for peach extract. The results were obtained from a single injection with column flow split into three detectors (Meng & Szelewski, 2007). All compounds of interest were shown in the μECD chromatogram at the expected RT. Both SIM and GC element-selective detector analyses are easy and useful approaches to confirm hits found in the DRS screening process.

3.4 Analysis of grape sample data with MFE (Molecular Feature Extractor) and exact mass search (Meng et al, 2009)

Figure 11 shows the raw TIC of grape sample obtained from the LC/Q-TOF.

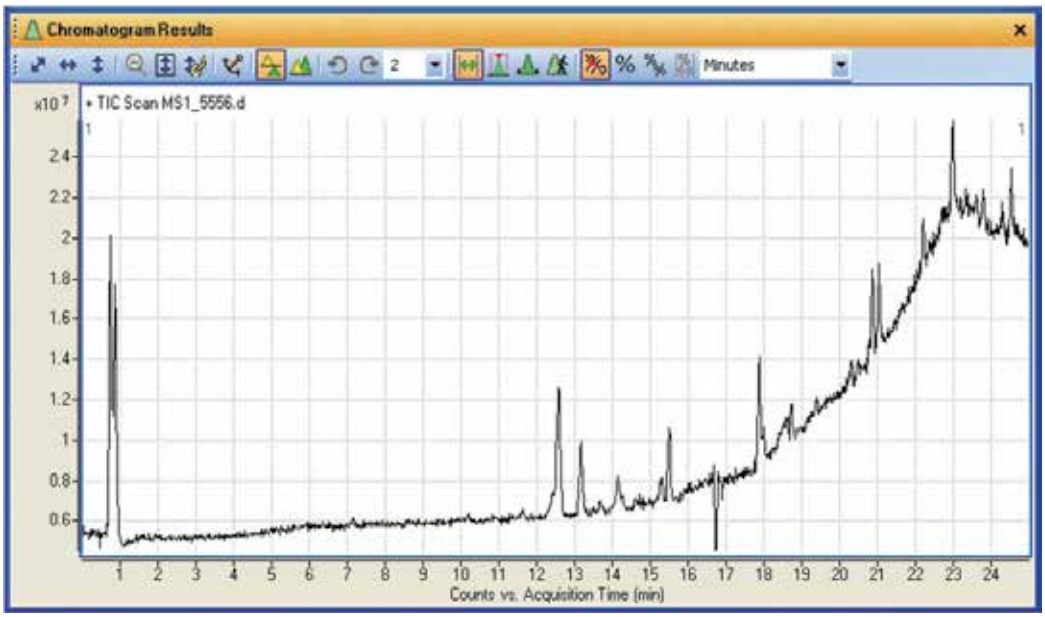


Fig. 11. Raw full spectrum TIC obtained from LC/Q-TOF analysis of grape sample.

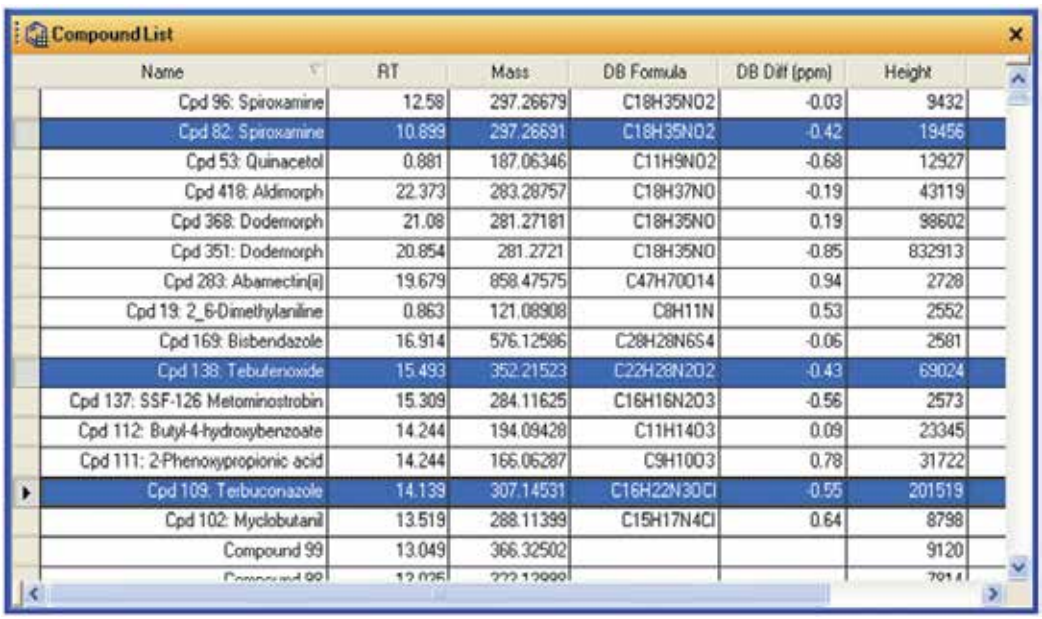
The MFE was used to find the masses of interest within the TIC based on user-defined settings (*see* Table 2 for settings).
The result was 510 potential compounds. The accurate mass measured for each of the 510 compounds was then searched against an Exact Mass Database, using search parameters described in Table 3.
The RT, an optional entry in the database, can be used as a qualifier. Out of the 510 potential compounds, 15 had a match of exact mass with a compound in the database within 3 ppm accuracy. As an example, in the Compound List shown in Figure 12, compound 82 was identified as Spiroxamine.

Extraction	Ion Species	Charge State	Compound Filters	Mass Defect	Results
Peak filter: Use peaks with height ≥ 1000	Positive ions: H, Na, K, NH4	Peak spacing tolerance: 0.0025 m/z, plus 7.0 ppm Limit assigned charge states to a maximum of 1	Relative height ≥ 0.2%	Filtering not used	Delete previous compounds Highlight all compounds

Table 2. Settings for Molecular Feature Extractor (MFE)

Search Criteria	Database	Peak Limits	Positive Ions	Search Results
Match mass only with 3.00 ppm tolerance	Exact Mass Compound database	10	H. Na, K, NH4 Charge state range 1–2 No neutral losses	Limit to the best 5 hits

Table 3. Settings for Database Search



Name	RT	Mass	DB Formula	DB Diff (ppm)	Height
Cpd 96: Spiroxamine	12.58	297.26679	C18H35NO2	-0.03	9432
Cpd 82: Spiroxamine	10.899	297.26691	C18H35NO2	-0.42	19456
Cpd 53: Quinacetol	0.881	187.06346	C11H9NO2	-0.68	12927
Cpd 418: Aldimorph	22.373	283.28757	C18H37NO	-0.19	43119
Cpd 368: Dodemorph	21.08	281.27181	C18H35NO	0.19	98602
Cpd 351: Dodemorph	20.854	281.2721	C18H35NO	-0.85	832913
Cpd 283: Abamectin(a)	19.679	858.47575	C47H70O14	0.94	2728
Cpd 19: 2_6-Dimethylaniline	0.863	121.08908	C8H11N	0.53	2552
Cpd 169: Bisbendazole	16.914	576.12586	C28H28N6S4	-0.06	2581
Cpd 138: Tebufenoxide	15.493	352.21523	C22H28N2O2	-0.43	69024
Cpd 137: SSF-126 Metominostrobin	15.309	284.11625	C16H16N2O3	-0.56	2573
Cpd 112: Butyl-4-hydroxybenzoate	14.244	194.09428	C11H14O3	0.09	23345
Cpd 111: 2-Phenoxypropionic acid	14.244	166.06287	C9H10O3	0.78	31722
Cpd 109: Terbuconazole	14.139	307.14531	C16H22N3OCl	-0.55	201519
Cpd 102: Myclobutanil	13.519	288.11399	C15H17N4Cl	0.64	8798
Compound 99	13.049	366.32502			9120

Fig. 12. A portion of the compound list of grape sample from MFE and Exact Mass Search.

The hits from the database search can easily be confirmed using MS/MS analysis available on the Q-TOF. The three highlighted compounds were selected for targeted-MS/MS analysis. The "DB Diff (ppm)" column in the figure presents the difference in ppm (not amu) between the experimental mass found and the database-listed mass, and shows the excellent mass accuracy of the instrument.
As shown in Figure 13, the grape sample was analyzed again in the LC/Q-TOF-MS/MS mode where both the MS1 full spectrum and the MS/MS full spectrum were acquired.

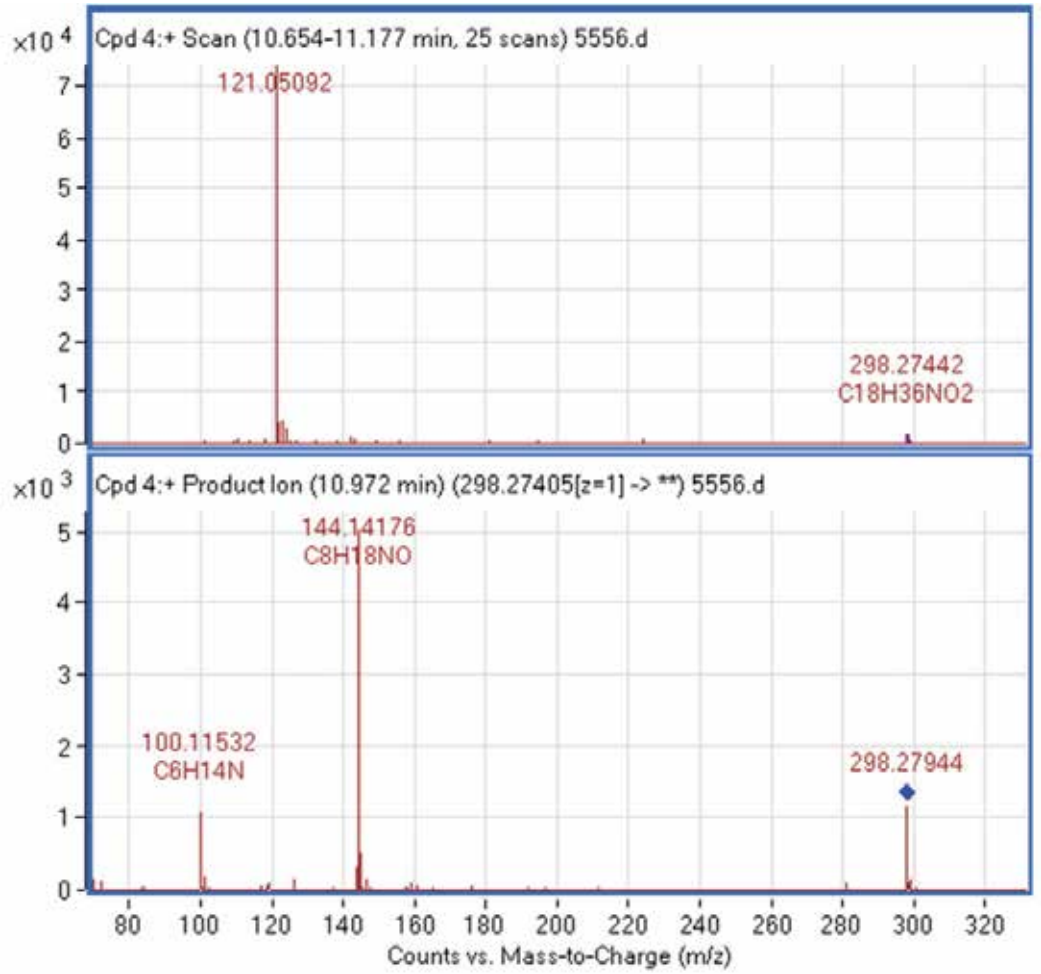


Fig. 13. Analysis of grape sample by LC/Q-TOF full spectrum and MS/MS.

The ion 121.05092 seen in the MS1 mode is the reference ion used for real-time mass axis calibration. The Spiroxamine precursor ion at 298.27442 is found in this scan at RT 10.884 min. The MS/MS full spectrum scan shows the precursor ion 298.27944 and two fragment ions at 100.11532 and 144.14176 as predicted from Spiroxamine's molecular structure. Criteria for finding compounds in the resulting MS/MS chromatogram are listed in Table 4. This additional injection in the targeted MS/MS mode confirmed the hits from the database search.

Integrator	Processing	Cpd TIC Peak Filters	Peak Spectrum	Results
MS/MS Integrator	Maximum chromatogram peak width 0.25 min	Filter on peak area Limit (by height) to the largest 10 peaks	Spectra to include average scans > 10% of peak height Exclude TOF spectra anywhere if above 40.0% of saturation MS/MS peak spectrum background: None	Delete previous compounds Highlight all compounds Extract MS/MS chromatogram Extract MS/MS spectrum

Table 4. Software settings for "Find Compounds by Targeted MS/MS".

3.5 Analysis of strawberry sample data with MFE (Molecular Feature Extractor) and exact mass search (Meng et al, 2009)

A strawberry sample was also analyzed similar to the grape sample above. The MFE produced 822 potential compounds. Figure 14 shows the TIC, the hyperlinked extracted compound chromatogram (ECC), and the mass spectrum for one of these compounds.

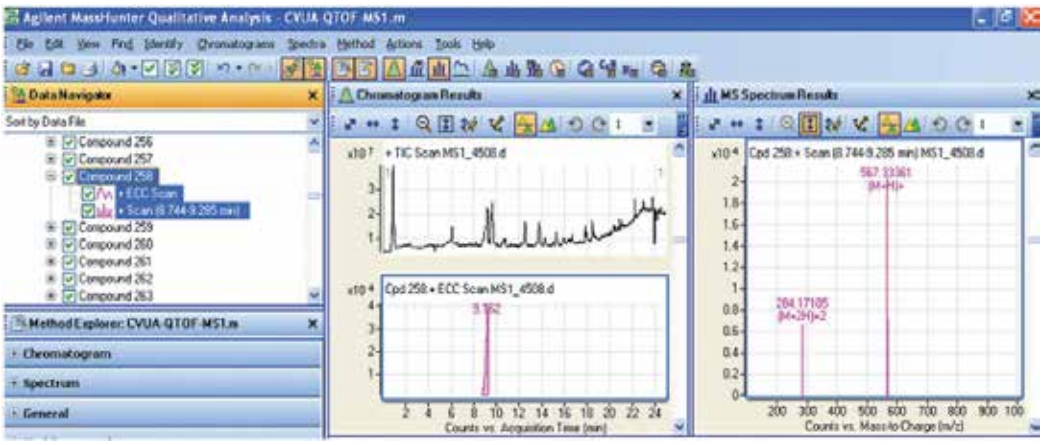


Fig. 14. The ECC and mass spectrum are shown for one of 822 compounds found using the MFE software along with the TIC.

Since ECCs are created when one of the MFE "Find Compounds" algorithms is run, the ECC consists of all the related ions and no chemical noise. The accurate mass of each of these compounds was subsequently searched against a working exact mass database of 1600 pesticides [MassHunter Personal Pesticide Database (G6854AA)]. The criteria used in this search are shown in Table 3. Twenty-six of the 822 compounds had mass matches (3 ppm tolerance) with pesticides in the database. Three plausible exact-mass match compounds, cyprodinil, azoxystrobin, and boscalid were then selected for further confirmation using MS/MS (Q-TOF) analysis with the same instrument. The ECC and mass spectrum for each of the three are provided in Figure 15 along with the database search results showing a difference of less than 1 ppm in experimental and database masses.

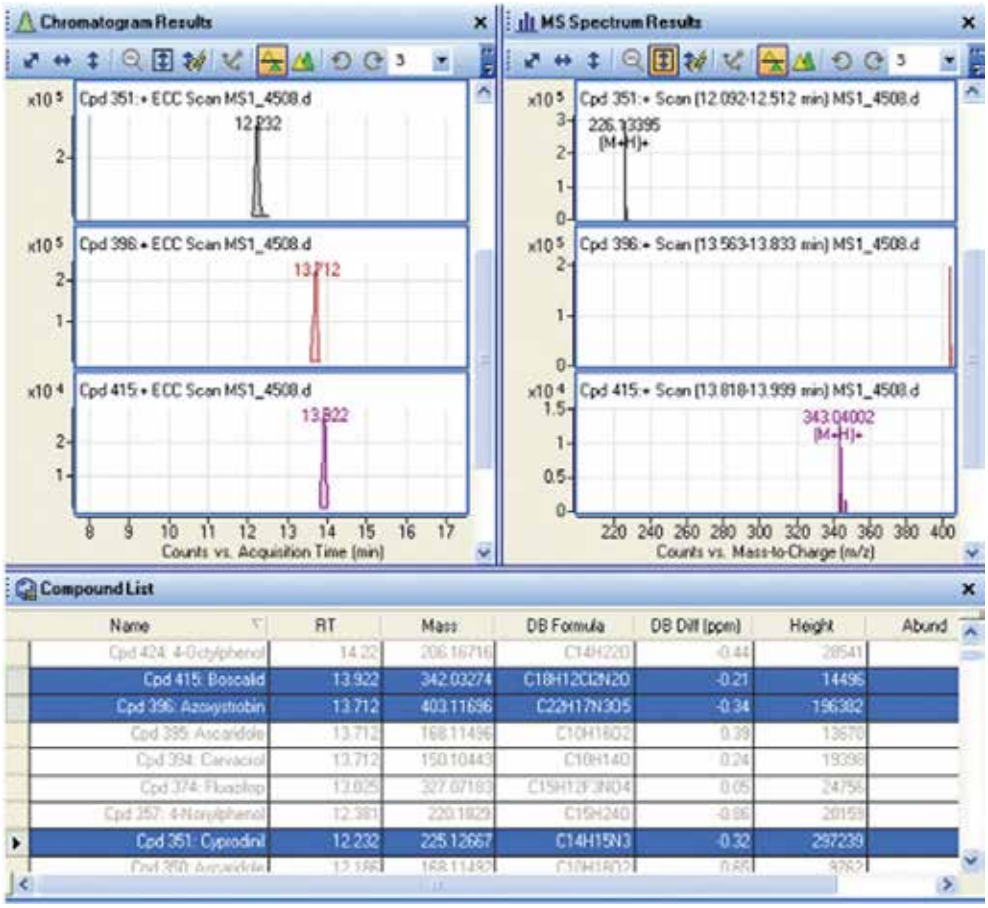


Fig. 15. The hyperlinked ECC and mass spectrum for three positive pesticides, cyprodinil, azoxystrobin, and boscalid, found in the strawberry extract, are shown along with the exact mass database search results (a portion of the results is shown).

The precursor ion (M + H)+ masses chosen for the MS/MS analysis of the strawberry extract were exact masses from the database: 226.13395, 404.12410, and 343.03995 for cyprodinil, azoxystrobin, and boscalid, respectively. Criteria for finding compounds in the resulting MS/MS chromatogram are listed in Table 4. Using the accurate MS/MS masses for the fragment ions, formulas were generated for each compound found in this step.

The confirmation process for azoxystrobin will be further discussed as an example. In Figure 16, the best-fit (with mass accuracy of 0.26 ppm and isotopes) formula generated from the Targeted MS/MS analysis for one of the compounds was $C_{22}H_{17}N_3O_5$, the formula for azoxystrobin.

The two associated fragment masses for this peak had less than 1 ppm difference in mass (0.31 and 0.2 ppm) when compared to the database masses for fragments expected from the $C_{22}H_{17}N_3O_5$ parent formula. In addition, the three isotope masses for the molecular ion all differed by less than 1 ppm. The table outlined in Figure 16 shows that the experimental isotope abundances of the three isotopes match well with the calculated (theoretical) abundances. The boxes in Figure 17 surrounding the isotopes represent the theoretical isotope abundances.

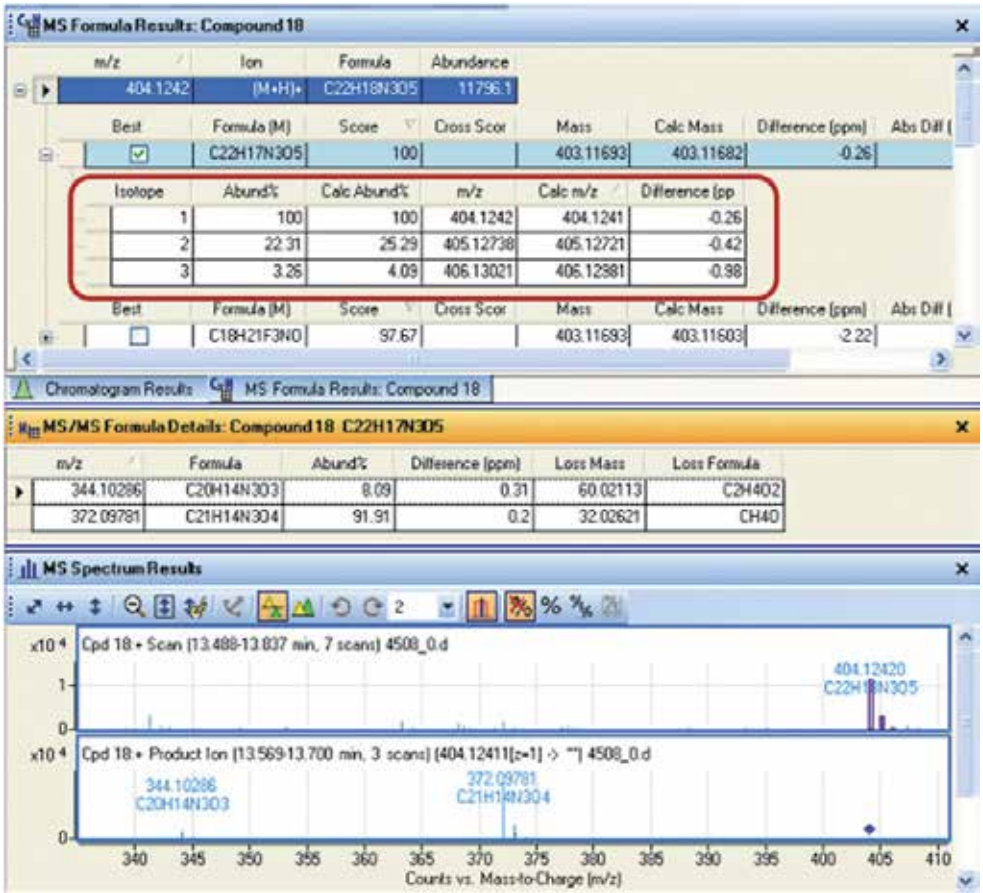


Fig. 16. Results from the formula analysis of MS and MS/MS data of a compound (Azoxystrobin) in the strawberry extract.

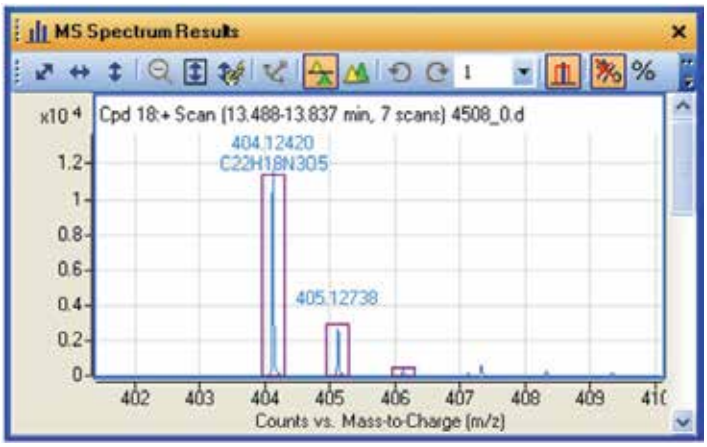


Fig. 17. The experimental isotope abundances of the three isotopes match well with the theoretical abundances (outlined in boxes).

Final confirmation of the structure is obtained by comparing the experimental fragment masses to likely theoretical fragment ion masses. Analysis of the structural formula, as seen in Figure 18, shows two likely fragments, with masses of 344.10351 and 372.09843 that match closely in mass to the two fragment ions found in the MS/MS data: 344.10286 and 372.09781 amu.

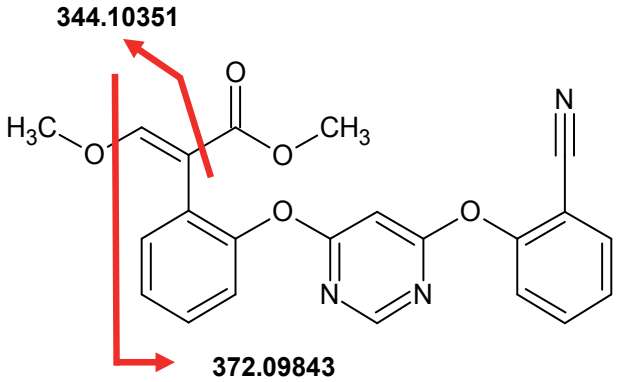


Fig. 18. Structural analysis for Azoxystrobin fragments.

The difference between experimental and calculated is 0.31 and 0.20 ppm, respectively. The accuracy of this comparison, along with the MS data identification and MS/MS formula generation results all strongly suggest the presence of Azoxystrobin in the analyzed strawberry extract.
A diagram depicting this "screen and confirm" workflow is shown in Figure 19.

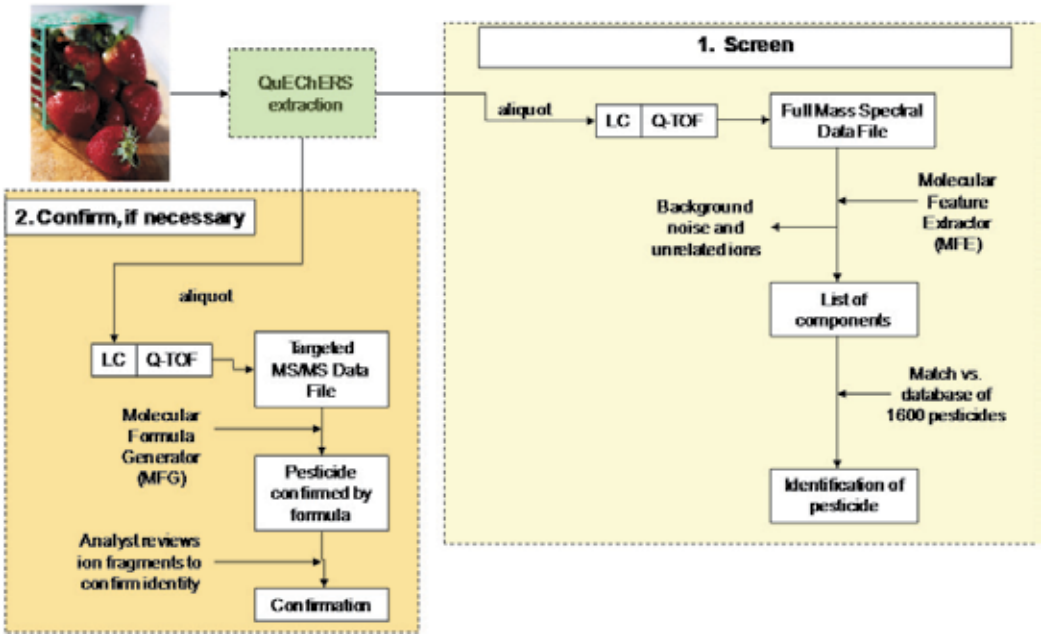


Fig. 19. Screen and Confirm - LC/Q-TOF analysis and software workflow.

4. Conclusion

GC/MS in the full scan mode combined with deconvolution enables unknown pesticide screening down to the 50 µg/Kg level in various food commodities in a single injection. Both GC/MS and LC/TOF (Q-TOF) screening have the following unique advantages: Crops can be screened for an unlimited number of compounds (depending on the number of compounds in the DRS library or exact mass compound database), and sensitivity is the same regardless of number of compounds screened (unlike QQQ). The hits from the GC/MS screening can be confirmed with an additional injection in the SIM mode or using GC element-selective detectors. LC/Q-TOF also provides the accurate mass MS/MS for confirmation of hits and structure elucidation, thereby frees the analyst from labor-intensive manual comparison of fragmentation patterns. The workflow of "Screen, Confirm, and Quantify" in non-targeted pesticide analysis is explained and illustrated with real samples.

5. Acknowledgement

Authors would like to thank the California Department of Food and Agriculture in Sacramento, CA, for their data files. We also wish to thank Jon Wong of the U.S. Food and Drug Administration (College Park, MD) for providing the peach extract.

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16

Applications of Hadamard Transform-Gas Chromatography/ Mass Spectrometry (HT-GC/MS) to the Detection of Pesticides in Rice

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1. Introduction

The levels of pesticide residues in rice and the standard operating procedures (SOP) for these are often stipulated in many countries (Pareja *et al*., 2011a; Pareja *et al*., 2011b). The conventional analysis of pesticide residues is a labor intensive procedure, since it is necessary to cover a wide range of different chemicals, using a single procedure. These traditional labor intensive extraction methods, such as liquid-liquid extraction, solid-phase extraction and accelerated solvent extraction are still used, whereas the use of the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method is economic and fast (Pareja *et al*., 2011b; Mastovska *et al*., 2010; García-Reyes *et al*., 2009). It has been validated for the extraction of 80 pesticides belonging to various chemical classes from various types of representative commodities with low lipid contents. However, supercritical fluid extraction (SFE) is also a popular method for pesticide extraction from foods (Kim *et al*., 1998; Izquierdo *et al*., 1996; Anastassiades and Schwack, 1998; Eller and Lehotay, 1997; Lehotay, 1997; Bernal *et al*., 2008; France *et al*., 1991; Goli *et al*., 1997). The advantages of SFE are well-known by now, including savings in labor, operational costs, laboratory space, waste minimization and increased selectivity versus traditional methods (Wang and Chang, 1998; Giddings *et al*., 1968; Hawthorne, 1990). When using SFE, in many cases the extracts should be further examined by GC/MS (gas chromatography/mass spectrometry) or HPLC/MS (high-performance liquid chromatography/mass spectrometry) to achieve the necessary selectivity and sensitivity for the different classes of compounds under detection (Hansen *et al*., 1995; Blanch *et al*., 1995; Jacques *et al*., 2007; Seger *et al*., 2004; Stolker *et al*., 2003). In this work, we developed a novel method by combining an on-line sample SFE collection system and the Hadamard transform GC/MS (HT-GC/MS) method for the extraction/detection of pesticide in rice (Fan *et al*., 2010; Johansen *et al*., 1994; Fuoco *et al*., 1997; Aguilera *et al*., 2005; Gmuer *et al*., 1987). The methodology of HT-GC/MS and the design of an on-line SFE sample collection system are demonstrated. Details of the experimental conditions for the determination of pesticides in rice are also reported herein.

2. Experimental

2.1 Reagents

Diazinon, parathion-methyl and chlorpyrifos were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals and gases were of analytical grade and were purchased from commercial sources.

2.2 Apparatus

A gas chromatograph/mass spectrometer (GC 6890 equipped with 5973 mass selective detector; Hewlett-Packard, Avondale, PA, USA) was used in this study. A novel type of Hadamard-injector was prepared in our laboratory; it can be controlled via a personal computer through a PCI 6221 device (National Instruments, USA), according to a series of Hadamard codes. A commercial supercritical fluid extractor (CO_2 pump, PU-1580-CO_2/HPLC pump, PU-2080; JASCO, Japan) was used to the extract the spiked pesticides. The HT-GC chromatograms were calculated using the LabVIEW program.

3. Methodology

3.1 Hadamard transform (HT)

The Hadamard transform (HT) technique has been applied in a variety of fields, including time-of-flight mass spectrometry (Brock *et al*., 1998; Fernández *et al*., 2002; Trapp *et al*., 2004; Fernández *et al*., 2001), Raman spectrometry (Treado *et al*., 1990; DeVerse *et al*., 2000; DeVerse *et al*., 1999), fluorescence imaging (Chen *et al*., 1995; Mei *et al*., 1996; Tang *et al*., 2002; Hassler *et al*., 2005) ion mobility spectrometry (Clowers *et al*., 2006; Szumlas *et al*., 2006), and NMR (Kubo *et al*., 1996; Feliz *et al*., 2006). In addition, the application of the cross correlation technique to chromatographic separation was first proposed by Izawa and coworkers. A Hadamard matrix on the order of n, H_n, is an $n \times n$ of +1's and -1's with the property of the scalar product of any two distinct rows being 0. Thus, H_n must satisfy the following equation,

$$H_n H_n^T = H_n^T H_n = nI_n \quad (1)$$

where H_n^T is the transpose of H_n and I_n is the unit matrix on the order of n. A fundamental equation of the Hadamard transformation is given by

$$[\eta] = [S] \times [C] \quad (2)$$

where η is a series of data, i.e., the observed chromatogram, encoded by a cyclic S-matrix, S, which is the $(n\text{-}1) \times (n\text{-}1)$ matrix consisting of "zero" and "one" elements, and C is a series of data representing a chromatogram. A cyclic S-matrix on the order of $(n\text{-}1)$ is obtained by omitting the first row and column of H_n and then changing +1's to 0's and -1's to 1's. To encode the chromatogram, C, a sample and eluent are introduced into a column according to the PRBS (pseudorandom binary sequence) derived from the cyclic S-matrix. When the elements of the PRBS are "one" and "zero," sample and eluent plugs are introduced into the column, respectively. As a result, the encoded chromatogram, η, is obtained. The encoded chromatogram is decoded to the chromatogram, C, by multiplying an inverse matrix of S, S^{-1}, as follows.

$$[C] = [S]^{-1} \times [\eta] \quad (3)$$

Consequently, the decoded chromatogram shows improvement in the S/N ratio (Fellgett advantage). In both correlation and HT methods, the key technology, based on multiple input techniques according to PRBS, is the injection device, which permits the continuous introduction of a sample. Multiple injection devices for GC have also been developed for correlation GC, in which the solenoid valve (Smit, 1970; Annino and Bullock, 1973), cylindrical slide valve (Kaljurand and Küllik, 1979), and fluidic logic gate (Annino *et al*., 1979) were used. Conversely, in correlation LC, the input signals modulated by PRBS were generated by valve systems (Lub *et al*., 1978; Smit *et al*., 1980; Laeven *et al*., 1983; Mars and Smit, 1990; Kaljurand *et al*., 1992) and by an electrochemical concentration modulator (Engelsma *et al*., 1990).

3.2 Hadamard-injector

A schematic drawing is shown in Figure 1. The developed Hadamard-injector permits a pressurized gas or pressurized liquid to be injected into a separation column according to a pseudorandom binary sequence (PRBS), *i.e.* a Hadamard code. In fact, it was made by

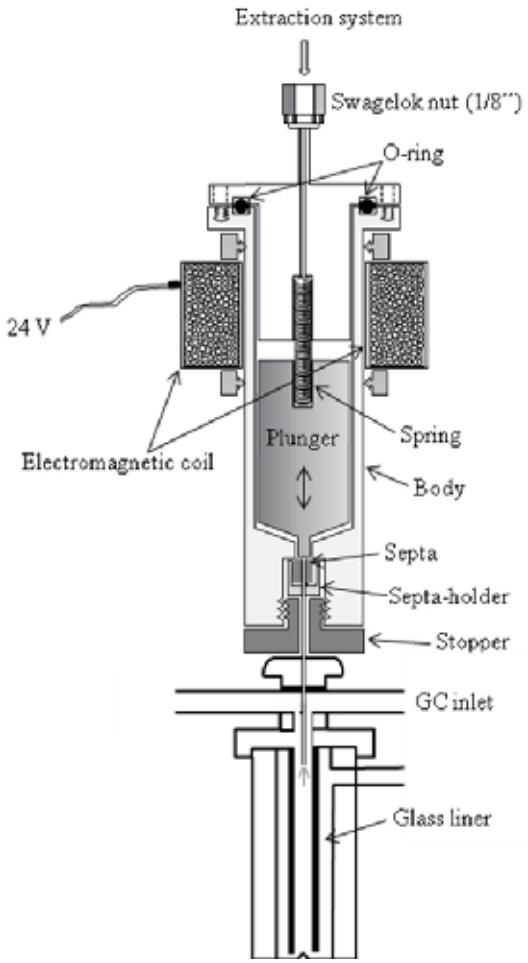

Fig. 1. A schematic drawing of the Hadamard injector used in this study.

modifying a regular pulse nozzle (Lin and Imasaka, 1993). Instead of a pinhole, which is used in general pulse nozzles, a piece of a capillary was used for the introduction of the pressurized sample solution (I.D., 50 μm; 8 cm in length). The body of the Hadamard-injector was made of brass; the plunger had a diameter of 9.5 mm and a length of 34 mm. A 24 V electromagnetic coil and the spring were removed from a solenoid valve (SMC model VX2110: 0~1.5 MPa, Japan), respectively, and used directly. A septa-BTO (Item No. 298735) was inserted into a brass holder, which was used to firmly attach the capillary and prevent gas or liquid leaks, and was sealed with a brass stopper. The Hadamard-injector can be heated and directly inserted into the GC inlet; the injection volume of the sample solution can be adjusted by changing the background pressure (nitrogen gas), the inner diameter of the capillary, the capillary length and the injection time to achieve a micro-controlled injection. During the sample injection process, a personal computer was used to rapidly turn the Hadamard-injector on and off through the PCI 6221 device, according to a series of Hadamard codes, leading to the introduction of the pressurized sample solution through the capillary into the GC column. The injection volume of the pressurized sample solution can be adjusted by changing the background pressure (nitrogen gas), the inner diameter of the capillary, capillary length, and injection time. Figure 2 shows the relationship between the injected volume and injection time based on various background pressures (A, for pressurized gas: 1.3~ 1.8Kg/cm^2; B, for pressurized liquid: 1.5 ~ 3 Kg/cm^2, respectively). Herein, the injected volume was recognized by means of a gas drainage method (Figure 2A) and by weighing the collected liquid (Figure 2B), respectively. As can be seen in Figure 2A (for pressurized gas), when the injection time was adjusted in the range of 1 ~3 s, the injection volume can be controlled from 0.3 to 4.2 μL; in Figure 2B (for a pressurized liquid), when the injection time was adjusted in the range of 0.1 ~1 s, the injection volume can be controlled from 0.04 to 0.3 μL. It should be noted that both show very good linear relationships. The RSD (related standard deviation) values of within-day and between-day were determined to 0.24 ~ 0.38% and 0.27%, respectively, indicating the stability and reproducibility of the procedure. Furthermore, the sample injection time, volume and split-ratio were investigated in detail during GC separation experiments.

4. Results and discussion

Figure 3 shows a schematic diagram of the on-line SFE/HT-GC/MS system. This system consists of a commercial SFE instrument, a commercial GC/MS, a holding tank and the Hadamard-injector. A 2.0 g sample of rice, obtained from a local supermarket, was spiked with three different pesticides (diazinon, chlorpyrifos and parathion-methyl, 30 μg each). The rice sample had first been subjected to a typical GC/MS method to confirm the absence of pesticide contaminants. The extraction liquid was 1.5 mL of acetonitrile. A 0.8 g quantity of glass beads was also placed in the holding tank to suppress bubbling. The CO_2 pressure was set at 20.3 MPa so as to extract the pesticides at a flow rate at 1 mL/min (oven temperature, 50 °C). After adding 15 mL of supercritical CO_2 fluid, the liquid was slowly passed through the spiked rice sample at a constant rate, the extracts were passed through a filter (0.45 μm) and then directly injected into the GC column by a personal computer, which turned the Hadamard-injector on and off, quickly, based on the Hadamard codes. The optimized conditions were a background pressure of 3 Kg/cm^2 and an injection time of 0.2 s. The injection volume was estimated to be ~ 66 nL for a single injection.

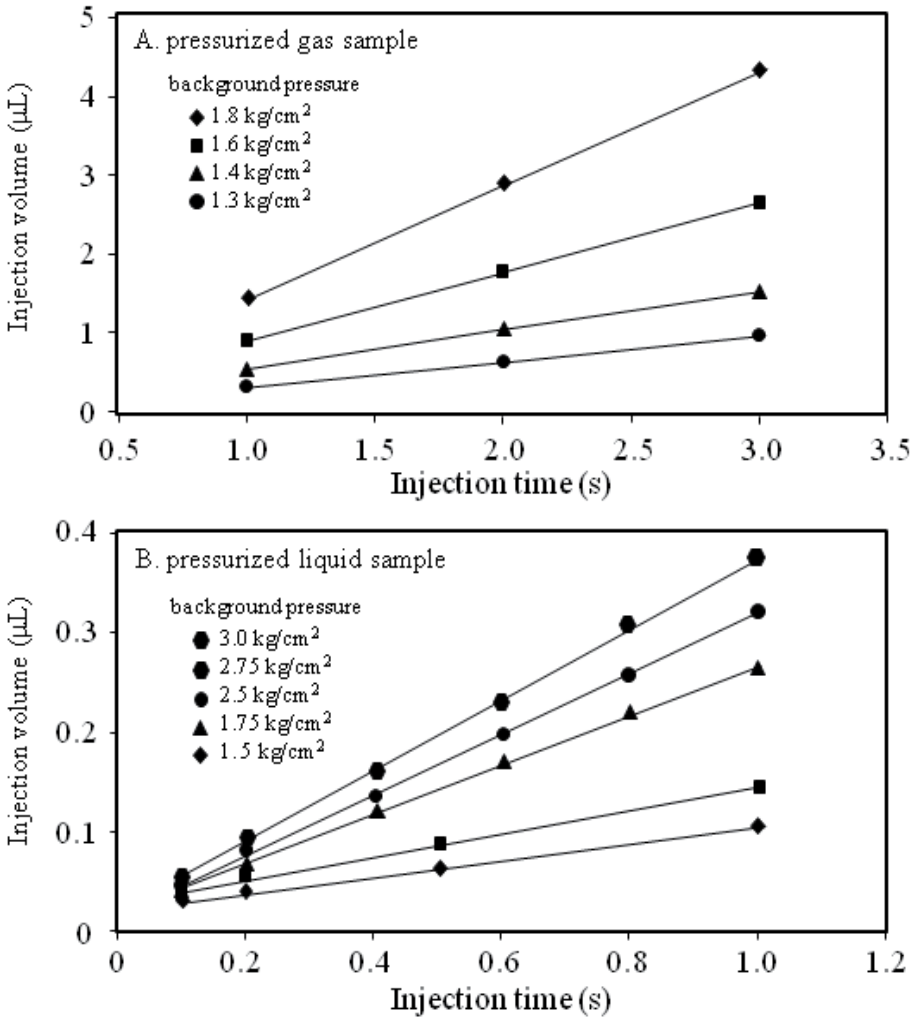

Fig. 2. Relationships between injected volume and injection time based on various background pressures (A, pressurized gas: 1.3 ~ 1.8 Kg/cm²; B, pressurized liquid: 1.5 ~ 3 Kg/cm², respectively).

Figure 4 shows typical GC/MS chromatograms for parathion-methyl standard by means of single injection (frame A) and Hadamard injection (frame B). The concentration level was 20 μg/1 mL acetonitrile. In the case of single injection, injection volume was 66 nL (absolute amount, 1.3 ng). The findings show that the S/N ratio of the chromatogram was relatively poor in frame A. However, under the same experimental conditions, when the Hadamard injection was performed (as shown in frame B; matrix order, n = 255), and each injection volume was still 66 nL, the S/N ratio was dramatically improved. The inset in frame B shows the details of raw data before the inverse Hadamard transformation (chromatogram a, whole chromatogram; chromatogram b, portion chromatogram of n = 127 ~ 238) and the Hadamard code (chromatogram c, n = 127 ~ 238). As can be seen, chromatograms b and c are quite matched, and for this reason, the S/N ratio improved as excepted.

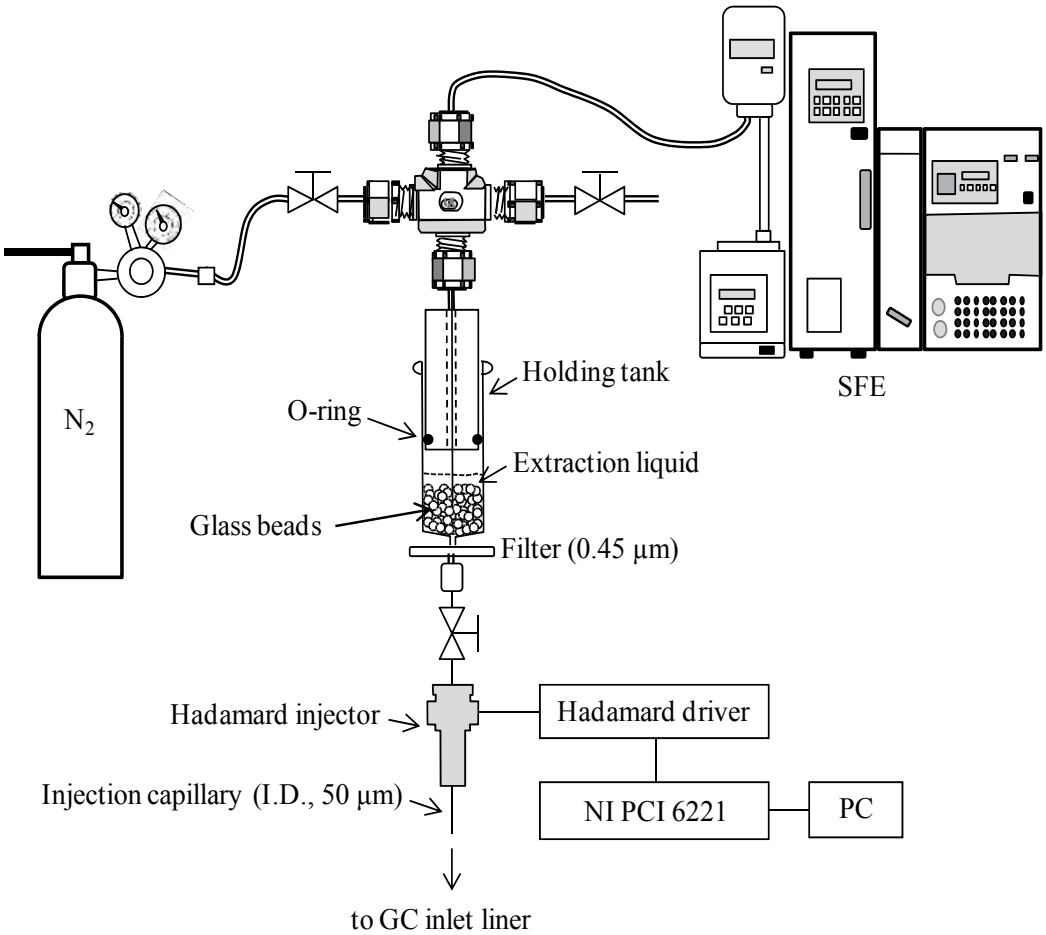

Fig. 3. A schematic diagram of the on-line SFE/HT-GC/MS system.

When analyzing a mixture of different pesticides, the use of Hadamard transform is also helpful to improve the S/N ratio. In this case, three types of pesticides (diazinon, parathion-methyl and chlorpyrifos) were spiked in the sample rice. Figure 5 shows typical HT-GC/MS chromatograms of the on-line SFE extracts from the spiked rice sample. Herein, the SIM mode was used (ion peaks at *m/z* = 125, 137 and 199 were selected for monitoring). Chromatograms a and b show the results obtained for a single injection and a Hadamard injection (matrix order, n = 255), respectively. As can be seen, the S/N ratio is again substantially improved. The detected peaks are also completely consistent with the theoretical prediction. Table 1 shows the relationship between the enhancement in S/N ratios and mass conditions for analysis based on on-line supercritical fluid extraction/HT-GC/MS methods (SIM mode was used; m/z = 125, 137, 199 for diazinon, chlorpyrifos and parathion-methyl, respectively). The results indicate that the on-line SFE/Hadamard-injector described herein also permits precise multiple injections and that it can be used for the determination of actual samples.

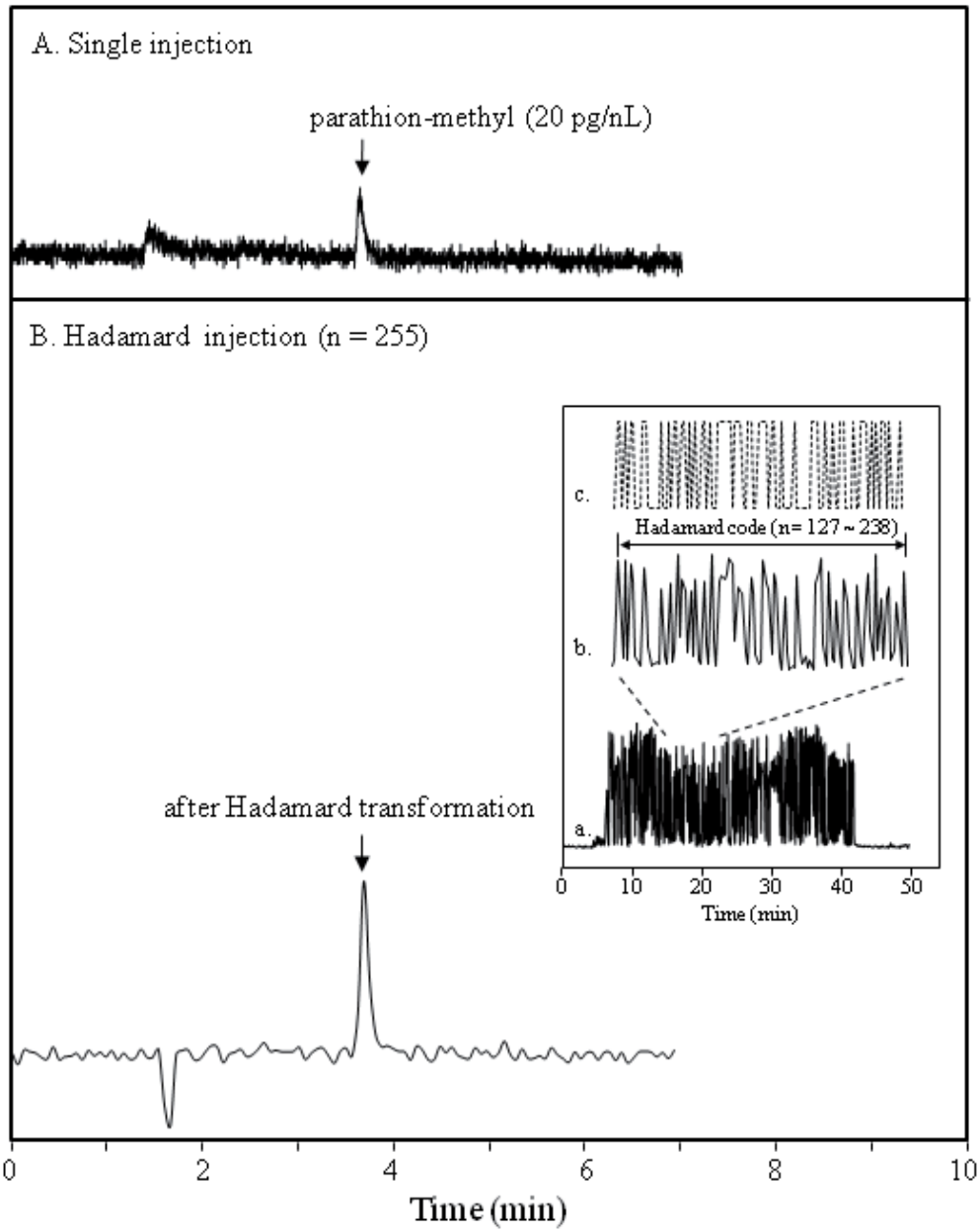

Fig. 4. Typical GC/MS chromatograms for parathion-methyl standard; concentration level, 20 μg/1 mL acetonitrile. In frame A, chromatogram was obtained by single injection (injected volume, 66 nL; absolute amount, 1.3 ng). In frame B, chromatogram was obtained by Hadamard injection (Hadamard matrix, n = 255; each injection volume, 66 nL; total injection volume, 16.8 μL). The inset in frame B shows the details of raw data before the inverse Hadamard transformation (chromatogram a, whole chromatogram; chromatogram b, portion chromatogram of n = 127 ~ 238) and the Hadamard code (chromatogram c, n = 127 ~ 238).

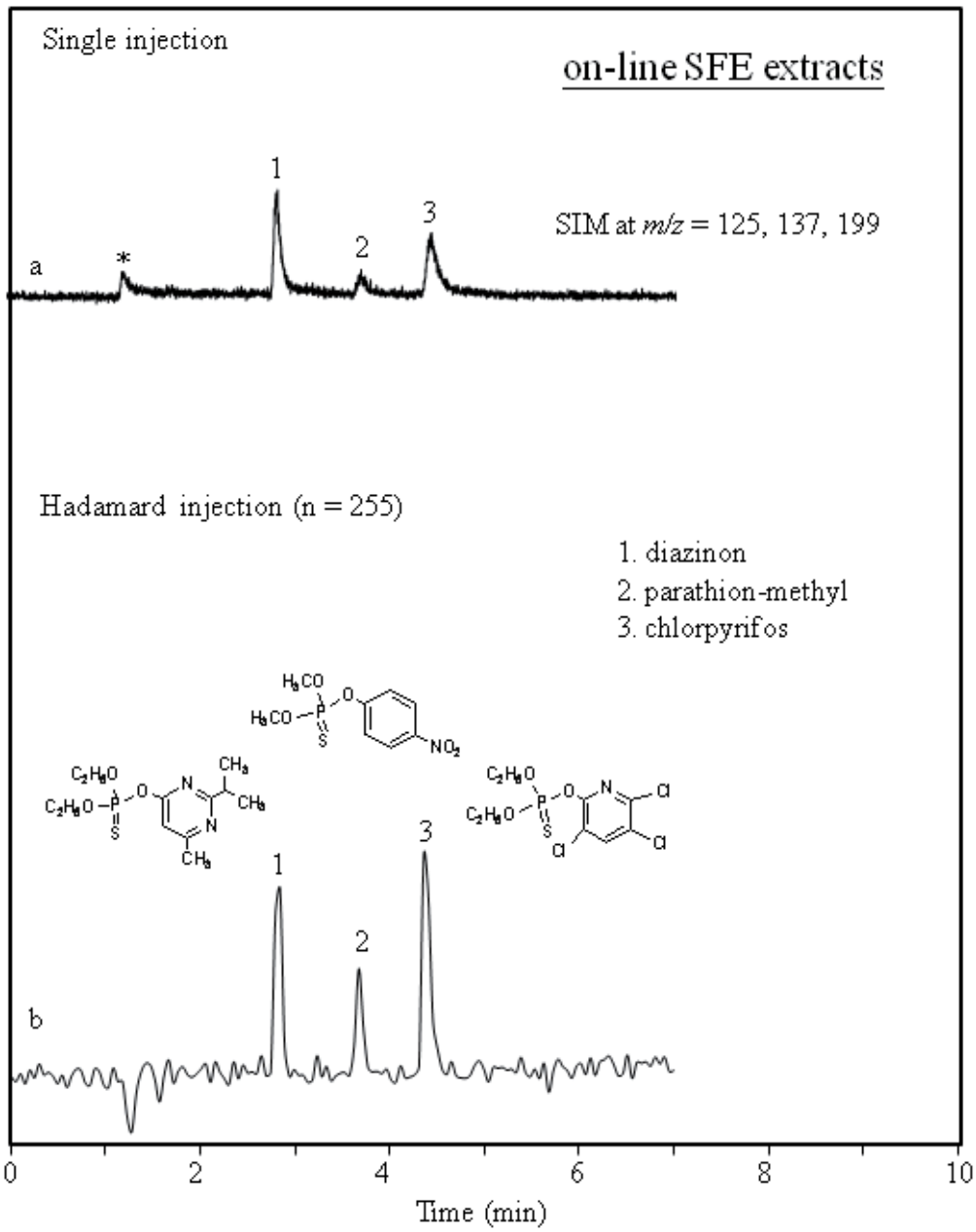

Fig. 5. Typical GC/MS chromatograms of the on-line SFE extracts based on the SIM mode. Chromatograms a and b show single-injection and Hadamard injection (order of matrix, 255), respectively. Diazinon, chlorpyrifos and parathion-methyl (30 μg of each) were spiked to 2.0 g rice. Inset, the raw data shown before inverse Hadamard transformation. The solvent peak is indicated by "*".

Matrix order	Enhancement of S/N ratio
	theoretical
255	8.02
	observed
diazinon	6.8
chlorpyrifos	4.3
parathion-methyl	7.6

The enhancement of the S/N ratio was calculated as the ratio of S/N values obtained in the chromatograms, measured by HT-GC/MS and a single injection method.

Table 1. Relationship between the enhancement in S/N ratios and mass conditions for analysis based on on-line supercritical fluid extraction/HT-GC/MS methods (SIM mode was used; m/z = 125, 137, 199 for diazinon, chlorpyrifos and parathion-methyl, respectively).

5. Conclusion

In this study, we developed novel Hadamard-injectors coupled with sample collection systems. The SFE system was successfully interfaced with the Hadamard-injector and the extracts were successfully collected. The utility of the method was demonstrated using some representative pesticides as model compounds. The device permitted continuous and precise sample injections in HT-GC/MS, resulting in a substantial improvement in S/N ratios through the application of the Hadamard transformation. The enhancement factors for the S/N ratios were matched with the theoretical values. Thus, this method has a variety of applications and could potentially be used in practical trace analysis.

6. Acknowledgment

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17

Highlights of Mass Spectrometric Methodologies in Environmental Pollution

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1. Introduction

1.1 The integrated pest management (IMP)

Phytodrugs or, more commonly, pesticides are chemical devices of both natural and synthetic origin playing an important role in crop protection. International agencies presiding environmental control have recently issued many directives to avoid pollution due, among others, to chemical treatment of fields by agricultural farms. The role of chemical analysis through the many platforms available by applying modern mass spectrometric methods, has become, therefore, of primarily importance either for the control of the health of our planet and for the welfare of many developing and undeveloped countries.

The state-of-the-art of agriculture chains in the world could be represented by a survey published by Botswana scientists on the knowledge and perception of pests, diseases and pest management practices by local vegetable farmers (Obopile et al., 2008). The results showed that pesticides were used and that seven out of twenty-four of them are considered extremely or highly hazardous by the World Health Organisation. Conversely, it is well known that worldwide starving requires a wider distribution of goods, many of them coming from agriculture. Integrated pest management (IPM) represents the only reliable approach helping producers in preserving their crops, therefore training on efficient and safe use of pesticides is needed to minimise their use. Comprehensive studies for each particular problem posed by pest infections are also vital for an environmental friendly approach to the unavoidable use of pesticides (El-Bouhssini et al., 2009; Gennaro et al., 2003).

Mass spectrometry is an affordable chemical method suitable, for its specificity and sensitivity, to deal with identification and assay of molecules in complex mixtures. The method has been implemented in the last years in its sampling, ionizing and analyzing facilities thus allowing molecules from few to million Daltons to be detected. Hyphenation with chromatographic devices helps in the analyses of complex mixtures, whereas parallel and concomitant detection of the separated analytes with other systems such as ultra violet (UV) spectroscopy, pulsed flame photometric (PFPD) (Amiray & Jing, 1998; De Nino et al., 2003) and electrochemical (EC)(Kullman &. Matsumara, 1996; Garrido-Frenich et al., 2001) detectors are determinant in many applications. The evaluation of organophosphates in oranges carried out by GC/MS/PFPD allowed an easy assignment of the analytes in extracted mass chromatogram (A, figure 1).

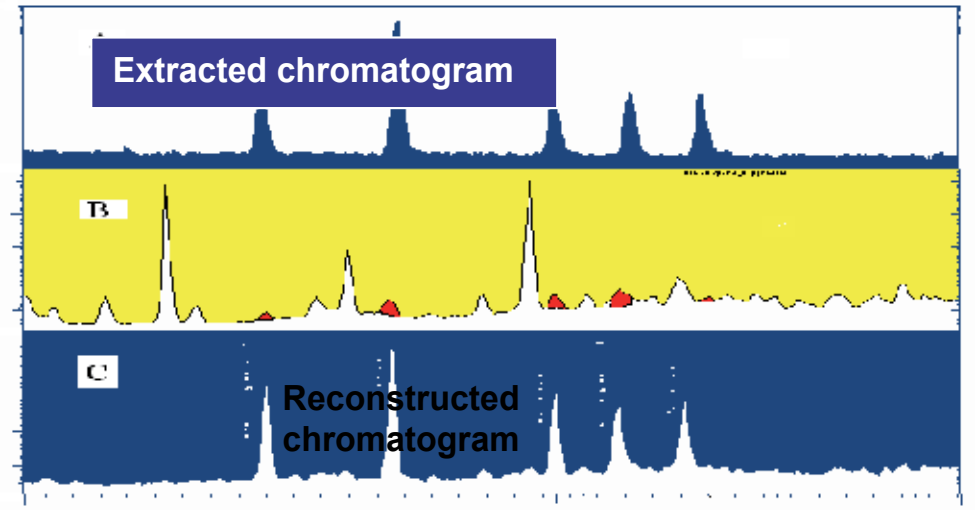

Fig. 1. (A) extracted mass chromatogram typical of the PFPD-identified pesticides (chlorpyrifos-methyl, m/z 125, 286, 288; malathion, m/z 125 and 173; parathion, m/z 109 and 291; chlorpyrifos-ethyl, m/z 314 and 316; and ronnel, m/z 125 and 285); (B) reconstructed mass chromatogram from the EI full-scan spectrum; and (C) PFPD chromatogram obtained for organophosphate standard mixture with –1.85 min offset.

1.2 An overview on the mass spectrometric methodologies

A mass spectrometer is a comprehensive "laboratory" where any class of molecule can be analyzed and fully characterized. A peculiarity of the methodology is represented by the chance of performing quantitative analysis direct from complex mixtures with, or without, a preliminary sample fractionation. Mass spectrometry is based on the identification of ionic species either pre-existent or generated by suitable ionization devices. Electron ionization (EI) has been the first method to be exploited and is still determinant in many analytical laboratory practises. It is based on the action accelerated electrons on gaseous neutral molecules (equation 1)

$$M + e^- \longrightarrow M^{+\cdot} + 2e^- \quad (1)$$

The very simple equation 1 describes how a neutral molecule M can be perturbed by an electron beam causing the formation of the molecular radical species. The minimum amount of energy required for the process is represented by the ionization energy of a given species (Table 1)

Compound	I.E. (eV)	PA (kJ/mol)
Benzene	9.24	750.4
Cyclohexane	9.88	686.9
Cyclohexene	8.95	784.5
1,3-hexadiene;	8.54	769,1

Table 1. value of ionization energy of some typical hydrocarbons

An acceleration energy of 70eV on the average is normally used because it is well above that required by the removal of one electron from a typical organic molecule and it is higher enough to guarantee the efficiency of the process.
The stability of the molecular radical cations thus formed was an important issue of early MS applications since the information on the composition of the analyte could be lost. Chemical ionization (CI) methods were therefore introduced which were based on the proton exchange between the gaseous analyte and an appropriate plasma. The proton affinity (PA, table 1) value provides information on the energetics of ion dissociation. Atmospheric pressure chemical ionization (APCI) source, now available in many commercial instruments, is based on the same principles and has become more popular then CI because it does not require sample evaporation.

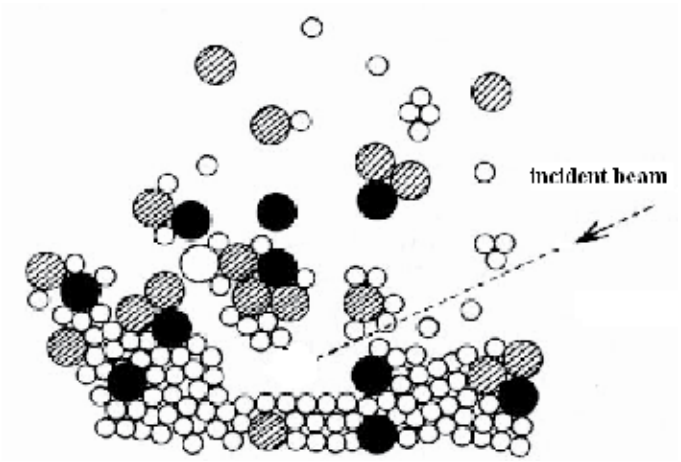

Fig. 2. Schematic of desorption ionization mechanism

A breakthrough in mass spectrometry was represented by the introduction of methods which directly ionize sample solutions after spraying in an electric field (ESI) or deposited on suitable tags and exposed to ion, atom and laser light bombardment (SIMS, FAB, MALDI). Combination of these methods will be addressed in an appropriate chapter. The kinetic energy transferred from the impinging species to the samples, in the desorption ionization methods, is used by the system to drive any kind of chemical reactions, which brings about the formation of protonated/deprotonated species as well ion clusters and electron radical species that are released in the gas phase and analyzed by a suitable mass spectrometer.

2. Liquid chromatography-mass spectrometry (LC-MS)

2.1 Sample preparation

Many environmental samples cannot be analyzed without preliminary sample preparation because they are too dilute or the matrix is too complex. Thus, despite advances in the development of highly efficient analytical instrumentation for their final determination, sample pretreatment remains an important part of obtaining accurate quantitative results. Consequently, the development of an appropriate sample preparation procedure involving extraction, enrichment, and cleanup steps becomes mandatory to obtain a final extract

concentrated on target analytes and as free as possible of matrix compounds. However, the complex sample matrix may contain abundant quantities of chlorophyll, lipids, sterols and other components that can interfere with good sample analysis. The fundamental assumption underlying any methodology for determining residues is that it should guarantee true and precise results at appropriately low limits of detection for a wide spectrum of analytes. Many choices have been proposed for pretreatment and/or extraction of pesticide residues in foods. In most of these the extraction procedure usually involves sample homogenization with an organic solvent, alone or mixed with water or pH-adjusted water, using a homogenizer, blender, or sonicator. In addition to these classical extraction techniques, other more recent approaches, for example QuEChERS, supercritical fluid extraction (SFE), pressurized-liquid extraction (PLE), microwave-assisted extraction (MAE), matrix solid-phase dispersion (MSPD), solid-phase extraction (SPE), solid-phase microextraction (SPME), and stir-bar-sorptive extraction (SBSE), have resulted in new possibilities in sample treatment and advantages such as a substantial reduction of the extraction time and incorporation into on-line flow-analysis systems. Each technique has its advantages and disadvantages and the choice should depend on the analytical problem. All of this extraction and preparation techniques will be discussed in another chapter of the book.

2.2 Sample introduction

LC is complementary to GC, because it permits the analysis of thermally labile, non-volatile, and highly polar compounds. In the past the application of LC in pesticides analysis was usually focused on groups of compounds or single compounds for which no suitable conditions were available for GC analysis. But now LC is particularly adapted to multiresidue analysis of pesticides over a wide range of polarity, including their transformation products, without the need to derivatize any compound. With the trend towards biodegradable pesticides, which are generally more polar than the old ones, LC is becoming the preferred analytical method for most insecticides and their metabolites. The majority of LC-MS is conducted on MS/MS instruments with enhanced selectivity; therefore extensive cleanup is less crucial when compared with analysis by GC-MS and there is a growing trend toward LC-MS analysis without cleanup. Any cleanup of food extracts is still usually performed off-line before LC-MS, whereas the use of online trace enrichment has been successfully used for the determination of pesticide residue in water. SPME, in-tube SPME, and SBSE have all been used as sample introduction devices for LC-MS. These facts together with the ability to cover wide range of physico-chemical properties of pesticides including GC non-amenable analytes predetermine LC the leading approach.

2.3 Chromatography

LC has a variety of separation modes and mobile phases for optimizing a separation. In GC the separation mechanism is based on the interactions between the stationary phase and the solute. In LC the separation mechanisms appear more complicated because of the many primary and secondary interactions between the stationary phase, the mobile phase, and the solute. As mass selectivity does not completely eliminate isobaric interferences and matrix affects that may affect the relative response of analytes. Although separation of all analytes is not considered necessary for detection of pesticide residues due to the high selectivity provided by MS/MS, ion suppression is observed either from coelution with other analytes or, more likely, coeluting matrix components. Also the use of stable isotope dilution assay by inserting labelled analogues of the analyts as intenal standard (Di Donna et al., 2007;

Mazzotti et al., 2009). Stationary phases are usually classified according to the separation mechanisms. To a first approximation these are: (i) adsorption chromatography using silica, alumina, or silica modified by polar groups; (ii) reverse-phase chromatography, using alkyl- or phenyl-bonded silicas, apolar copolymers, or carbonaceous sorbents; (iii) ion-exchange chromatography using sorbents containing ion-exchanger groups at their surface and (iv) size-exclusion chromatography. The most important and widely used separation technique for pesticides is LC on reversed-phase (RP) columns. The mobile phase is important to obtain a good chromatographic separation, but it also affects the analyte ionization and the sensitivity of the mass spectrometer. For example, analyte charge should be suppressed by manipulation of the mobile-phase pH for optimum retention but this can have a detrimental affect on MS response. Contrary to conditions for RP LC retention, for optimized electrospray (ES) ionization, the pH should be adjusted to promote the charged state of the analyte over its neutral species as ionization takes place in the liquid phase. Methanol-water (Leandro et al., 2006; Mickov et al., 2003) and MeCN-water (Di Donna et al., 2007; Mazzotti et al., 2009)mixtures with addition of ammonium acetate (Leandro et al., 2006; Chueng & Wilson, 2006) were mostly utilized as mobile phases at gradient elution conditions. Methanol and/or MeCN are used as organic modifiers, methanol is preferred over MeCN for several reasons.

2.4 Liquid chromatography-mass spectrometry (LC/MS) applications

The different advantages of LC coupled to MS in the field of pesticide residue determination in environmental samples have been addressed (Malik et al., 2010). Moreover, the additional benefits of using MS/MS in terms of increased selectivity and sensitivity have also been discussed. These characteristics, together with the easy compatibility of LC with aqueous samples and the ability to perform most of the analysis without derivatization, make LC-MS/MS the best technique currently available for the determination of polar and ionic pesticides. Although the majority of the methods developed for their application to fruits and vegetables depend usually on the change in the extraction procedures, however in this section the application and the development of the LC-MS methods will be discussed. Quantification of pesticides with LC-MS depends on multiple parameters, such as the choice of the analytical column, mobile phase, flow rate, ionization and acquisition conditions. One of the main problems in the quantitative determination of pesticide residues is that its extraction easily carries away interferences (sugars, cellulose, lipids, etc.) in the final extract. Therefore, quantitative analysis can be severely affected by matrix effects, the most common being the suppression or enhancement of analyte ionization in the mass spectrometer, which lead to unacceptable results if no correction is being made. Signal suppression or enhancement is related to the ionization procedure rather than the analyzer used (Ferrer & Fernàndez-Alba, 2005) and depends on the type of pesticides being analyzed and the type of matrix. This effect is more important when using electrospray interfaces, and the effect is more intense under positive ionization mode. The extent of suppression or enhancement of the signal is typically 0–30% but in some cases, it can be total (Klein & Alder, 2003; Jansson et al., 2004). For this reason, procedures optimized with standards in pure solvent by adjusting MS parameters can lead to wrong conclusions. To evaluate signal suppression, it is a good practice to perform a matrix-matched calibration (standards in identical or similar matrix than sample to be analyzed) or to use appropriate labeled surrogate and internal standards or application of an efficient clean-up step. Also it should be taken into account that the mobile phase is important to obtain a good chromatographic separation, but it also

affects the analyte ionization and the sensitivity of the mass spectrometer (Andreu & Picò , 2005; Medana et al., 2005). Chromatographic separation of ionic compounds can be achieved by different retention mechanisms, mainly, ionexchange and ion-pair reversed-phase liquid chromatography (IP-RPLC). Ion pairing, with the corresponding counter-ions, has been done for acidic and basic analytes (Sancho et al., 2005). For the former, the ammonium cation is the weakest ion-pairing agent, while tri-and di-alkylamines are the stronger ones, and for the latter, perfluorinated organic acids serve as anionic counter-ions (Castro et al., 2001; Sancho et al., 2005). The type and quantity of organic modifier added to the eluent, has to be a compromise between improvement of separation and minimization of suppression of the atmospheric pressure ionization (API) techniques. In the contrary the ion-exchange approach is not very suited to the electrospray interface (ESI) due to the use of buffers with high ionic strength (Castro et al., 2001).

The analysis of pesticides residue may be targeted or non-targeted but always using a multi-residue procedure as generic and simple as possible, reducing to the maximum the clean up steps. The multi-residue methods reported in the literature are very different depending on the food substract. It is not always necessary to extract pesticides from liquid samples, such as juice and wine, with organic solvents because the pesticides are already dissolved in them and can be directly injected in the LC-MS system (Goto et al., 2005). Goto et al., have been developed an analysis method for N-methyl carbamate pesticides in juice and wine that only required sample dilution with ultra-pure water, and LC-ESI-MS/MS determination by direct sample injection into a short column. However, other authors prefer the use of an extraction procedure (Wu et al., 2002), they improved the determination of polar pesticides in water and wine by coupling automated in-tube SPME to LC-ESI-MS. In-tube SPME conditions were optimized by selecting the appropriate extraction parameters, specially the stationary phases used for SPME.

Target analysis is a conventional analysis based on developing a method with standards prior to analysis and monitoring real samples that do not detect compounds not defined in it. The trend within the target analysis is the development of large-scale multiresidue methods (able to determine more than 100 compounds). These methods applied LC–MS/MS, using QqQ, QLT and TOF-MS. LC–MS/MS using QqQ has as a serious limitation the number of compounds that can be simultaneously determined (up to 100–150 depending on the scan speed/dwell time). For example, it has been developed a multi-residue method for the screening, quantification and confirmation of 52 pesticides and metabolites in four fruit and vegetable matrices (Hernández et al., 2006). LC-ToF-MS can be used for the quantitative analysis of pesticides in fruits and vegetables and reports the usefulness of this technique to obtain structural information for unequivocal identification of target compounds provided by elemental composition formula information. A new study were presented by Sage et al. (2002) comparing, the quantitative performance of ToF-MS and LC-MS/MS with MRM for 15 pesticide residues in fruit extracts. This comparative study was concluded that MRM detection is a more sensitive technique than full scan QTOF, allowing lower limits of detection, although LC-ToF-MS could also be used to quantify pesticide residues. This technique provided elevated spectral resolution allowing exact mass measurement and full mass spectral sensitivity for low level analyte detection. The application of TOF or hybrid quadrupole time of flight (QTOF), is increased in the last few years. This increased use is either for metabolite identification studies, for analyte confirmation in positive samples, or for screening methods, based on the high resolution power and accurate mass measurement capabilities of this technique, both in MS or,

preferably, in MS/MS mode in the case of the hybrid QTOF. LC-QToF-MS is more selective because the accurate mass measurement of product ions allows to remove ambiguities. Soler et al. compare four LC-MS systems, equipped with single quadrupole, QqQ, QIT and QToF to evaluate the performance for the analysis of carbofuran and its metabolites. Although quantitative results were best with QqQ, but QToF was the most selective technique because the accurate mass of product ions allowed ambiguities to be removed (Soler et al., 2006). The recently introduced hybrid QLT instrument has also been used to perform MS/MS. This instrument retains classical QqQ modes for quantitative and qualitative analysis (SRM mode and neutral loss scan) and combines them with sensitive ion trap scan modes for the confirmation of analytes or characterization of unknowns, including enhanced product ion mode, time delayed fragmentation, and MS^3 with an ion accumulation capacity higher than a conventional three dimensional ion trap analyzer. The QqLIT analyzer can provide an improved sensitivity in these MS/MS studies and up to 200 compounds can be analyzed in a unique LC–MS/MS run, with 2 SRM transitions (Garcia-Reyes et al., 2007). The working modes (enhanced product ion and MS^3) are useful for the unambiguous confirmation of pesticides with poor fragmentation at low concentration levels, which cannot be easily confirmed by QqQ instruments due to the high SRM ratio between the two transitions (or absence of the second transition) for confirmatory purposes.

Another important issue on pesticide control to ensure food safety, which is still a challenge for the analyst, is the identification of non-target pesticides and metabolites. The non-target analysis offers the possibility of identifying unexpected pesticides, transformation products and/or impurities, or even untargeted compounds that can be toxics. This analysis is more complicated because it requires the identification of unknown compounds. The high resolving power of mass spectrometric techniques, such as TOF-MS, has been applied successfully to this field (Thurman et al., 2005a, 2005b). There are two reports that describe an interesting identification scheme, using a combination of LC-MS/TOF and LC-MS QIT with searching of empirical formulas generated through accurate mass and a ChemIndex or MerckIndex databases. This scheme has been applied to investigate imazalil and prochloraz, the main degradation product of imazalil, and a non-previously reported prochloraz degradation product in citrus fruits extract (Thurman et al., 2005a) and to discover unknown pesticides in tomato skin (Thurman, 2005b). This identification was accomplished basically by combining the information provided by LC–TOF-MS accurate mass analysis with that deduced from the fragmentation pathway of the parent compound and carried out by LC-ion trap MS^n experiments (typically MS/MS or MS^3). To conclude this section, the acquisition of better sensitivity and selectivity of target analytes detection, tandem mass spectrometry (MS/MS) is a generally the preferred option for quantitation purposes. The use of LC–MS/MS with triple quadrupole (QqQ) instruments in multiple reaction monitoring (MRM) mode is so far the more appropriate technique for target analysis. It provides excellent sensitivity and selectivity. This technique is now becoming a well-established approach in the field, as demonstrated by the number of published papers. Also the use of LC–QqTOF-MS is at present one of the most advanced and efficient approaches for screening and identification of non-target pesticides and their metabolites in food.

3. Aplication of spray desorption methods

Recently, a new family of MS techniques has been developed, allows ions to be created under ambient conditions and then collected and analyzed by MS (Cooks et al., 2006).

Ambient mass spectrometry is the ability to record mass spectra on ordinary samples, in their native environment, without sample preparation or preseparation by creating ions outside the instrument. Desorption electrospray ionization (DESI, Figure 3) is an ambient ionization technique that can be used in mass spectrometry for chemical analysis. It is an atmospheric pressure ion source that ionizes gases, liquids and solids in open air under ambient conditions (Takats et al., 2004). DESI is a combination of electrospray (ESI) and desorption ionization (DI) methods. In the DESI process, a spray of charged micro-droplets from a pneumatically-assisted electrospray needle is directed towards the object or analyte of interest in the ambient environment and allowed to impact the surface, giving desorption of the analyte into the gas phase and subsequent ionization.

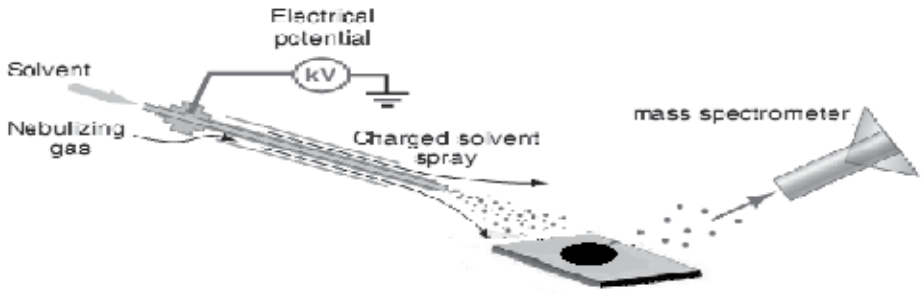

Fig. 3. Schematic configuration of DESI source

DESI approach has been applied in food analysis, in particular 16 different compounds belonging to different classes of agrochemicals (insecticides, herbicides, and fungicides) have been selected as representative of different class of pesticides (García-Reyes et al., 2009). For evaluate the goodness of DESI approach two different approach were performed, direct DESI-MS/MS analysis of fruit peels and DESI analysis of foodstuff extracts, the latter was obtained using the conventional sample treatment protocol used worldwide for pesticide analysis. Identification and confirmation capabilities and sensitivity were examined in detail using different spray solvents, and the optimized method was applied to a wide range of samples obtained by local store. Very low LOD values were obtained under MS/MS experiments, for example,in the case of the ametryn, one of the selected agrochemical choice the LOD is 0.1 μgL^{-1}. Ion suppression due to matrix effects, which are common in electrospray mass spectrometry, were observed but can be reduced with appropriate dilutions, since the sensitivity and LODs obtained (in the micrograms per kilogram range in fruit and vegetable matrixes) were satisfactory even after dilution of the food extracts. With DESI approach, it becoms possible to perform direct analysis of market samples without any further treatment. Therefore the accurate trace quantitation in complex are more attainable using DESI-MS with isotopically labeled standards. Ambient electric discharge provides high density of charged molecules, and metastable neutral species (such as HO •, O •, NO •, etc.), showing attractive attribution for primary ion production.

Low-Temperature Plasma (LTP, Figure 4) is a plasma based technique that operated on the base of the ambient discharge and/or plasma to make the energetic species as the ionization reagents. LTP is obtained by using helium gas as the working medium, and a large number of electrons and positive ions, resulting in the generation of helium atoms at excited energy levels. Besides the metastable helium atoms, the electrons and positive ions can be used as

the carrier of energy and charges for the secondary ionization of the targeted analytes. Because gases (such as moist air, nitrogen gas, helium gas, etc.) can be introduced into the electric field as the discharge medium, the gaseous samples (e.g., air, volatile organic compounds) can be directly analyzed by this technique, showing potential applications for sensitive detecting nonpolar compounds without tedious sample pretreatment. In the LTP stage, gas is supplied continuously between the two electrodes, and plays an important role to maintain discharge, to cool the electrode, and to provide reagent ions.

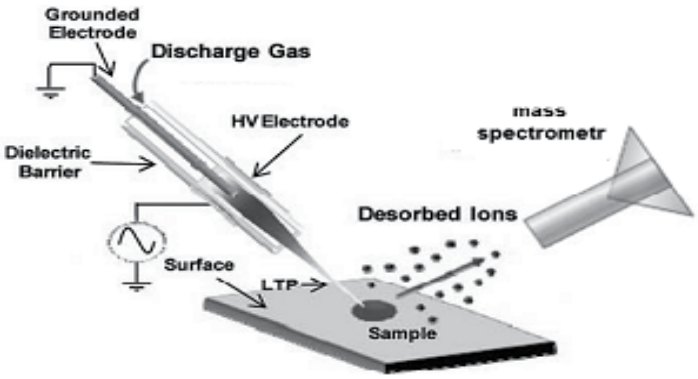

Fig. 4. Schematic configuration of LTP probe

In the LTP probe experiments, the probe is used to directly desorb and ionize the analytes from aqueous solutions. To demonstrate this feature, a glass dish containing 150 mL of deionized water was spiked with atrazine (agricultural herbicide) resulting in a 1 ppm aqueous solution. The solution was placed near the MS inlet, and the LTP probe (He discharge gas) was used to direct the plasma over the liquid surface. The MS spectrum gives a signal at m/z 216 which can be assigned at protonated atrazine, that is confirmed by MS^2 spectrum. The linear dynamic range for atrazine in this case for deionized water varies from 1 ppb to 1 ppm (Harper et al., 2008). LTP-MS has been utilized for the detection of pesticide residues in food. Different multi-class agricultural chemicals were considered (ametryn, amitraz, atrazine, buprofezin, DEET, diphenylamine, ethoxyquin, imazalil, isofenphos-methyl, isoproturon, malathion, parathion-ethyl and terbuthylazine). The experiments were performed directly on fruit peels as well as on fruit/vegetable extracts. Most of the agrochemicals examined displayed remarkable sensitivity in the positive ion mode, giving limits of detection (LOD) for the direct measurement in the low picogram range. The semi-quantitative screening, with LTP-MS, of agrochemicals in foodstuffs were performed using fruit and vegetable extracts (Wiley et al., 2010,). DESI works best with polar analytes that are easy to protonate or deprotonate, although analytes of low polarity are accessible to a certain extent, this was the rationale for developing DAPCI. In order to improve the efficiency of ambient MS in the regime of low-polarity compounds, desorption atmospheric pressure photoionization (DAPPI, Figure 5) has been developed DAPPI represents the adaptation of APPI for the ambient analysis of surfaces analogous to the conversion of APCI to DAPCI. In DAPPI, a heated jet of solvent vapor and nebulizer gas desorbs solid analytes from the surface. A krypton discharge lamp is directed toward the sample so as to irradiate the vapor phase immediately above the surface with UV photons of 10 eV where ionization of the analyte occurs. Like DESI and DAPCI, DAPPI uses a standard API interface to collect the ions (Haapala et al., 2007).

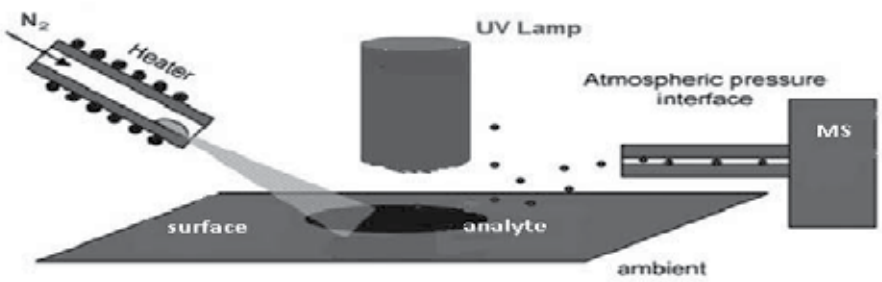

Fig. 5. Schematic configuration of DAPPI source

DAPPI has been used to study a set of harmful compounds typically found in environmental or food samples, DAPPI-MS spectra has reorded in both positive and negative ion modes. The analytes include five polyaromatic hydrocarbons (PAHs) and one N-PAH (acridine), one brominated flame retardant (tetrabromobisphenol A, TBBPA), and nine pesticides. Two authentic samples, orange peel and a spiked soil sample were analyzed by DAPPI-MS in order to demonstrate the suitability of the technique for the analysis of environmental or food samples. The PAHs are neutral non-polar compounds, while the TBBPA and the pesticides are slightly acidic or basic. Limits of detection (LODs, signal-to-noise (S/N) ratio > 3) were determined for all the compounds using a characteristic ion identified from the mass spectrum. If multiple analyte ions were observed in the mass spectrum of a single analyte, the most intense ion was selected as the characteristic ion to be measured. The analysis was carried out in full scan MS mode and the LOD values are averages of four separate sample spots. For the PAH compounds the LODs were determined in positive ion mode and they ranged from 0.1 to 1 ng. The LODs were observed to decrease as the size of the molecule increased. This is expected since the number of delocalized p-electrons increases with increasing molecule size, thus leading to a lower ionization energy. The LODs for the pesticides determined in positive ion mode ranged from 30 to 300 pg. There were no significant differences between the LODs of different pesticides, which is thought to be because of their similar physical and chemical properties, i.e. functional groups containing oxygen and nitrogen (Luosujärvi et al., 2010).

Direct analysis in real time (DART, Figure 6) is another different atmospheric pressure ion source that instantaneously ionizes gases, liquids and solids in open air under ambient conditions. In the DART method, an electrical potential is applied to a gas with a high ionization potential (typically nitrogen or helium) to form a plasma of excited-state atoms and ions, and these desorb low–molecular weight molecules from the surface of a sample (Cody et al., 2005). The cold plasma passes through a second chamber where a second perforated electrode can remove cations from the gas stream that is subsequently heated and passed through a final grid electrode removing oppositely charged species. The ionizing neutral gas may either be directed towards the sampling orifice of an API interface, or analogous to DESI, may hit the sample surface at an angle suitable for its reflection into the entrance of the mass spectrometer.

DART has been applied in several fields, This includes the typical safety-related and forensic usages of ambient MS like detection of explosives, warfare agents, or pharmaceuticals and drugs of abuse. DART is used for the routine rapid analysis of highly

insoluble polycyclic aromatic compounds. Direct analysis of such compounds as solid samples under solvent-free conditions shows that DART is a powerful analytical platform capable of providing high-throughput analysis for these complex samples, and no special sample pre-treatment or instrument setup are required (Domin et al., 2010). Ambient mass spectrometry has been used for the analysis of strobilurin residues in wheat, (DART) ion source coupled with a time-of-flight mass spectrometer (TOF MS), permitted a direct screen of the occurrence of target fungicides in treated grains in less than 1 min. For quantification purpose by DART-TOF MS, an ethyl acetate extract had to be prepared, with the use of a prochloraz as internal standard. The performance characteristics obtained by repeated analyses of extract, spiked at 50 μgkg-1 with six strobilurins (azoxystrobin, picoxystrobin, dimoxystrobin, kresoxim-methyl, pyraclostrobin, and trifloxystrobin), were in the following range: recoveries 78-92%, repeatability (RSD) 8-15%, linearity R^2= 0.9900-0.9978. The analysis of wheat with incurred strobilurin residues demonstrated good trueness of data generated by the DART-TOF MS method; the results were in a good agreement with those obtained by the conventional approach (Schurek et al., 2008).

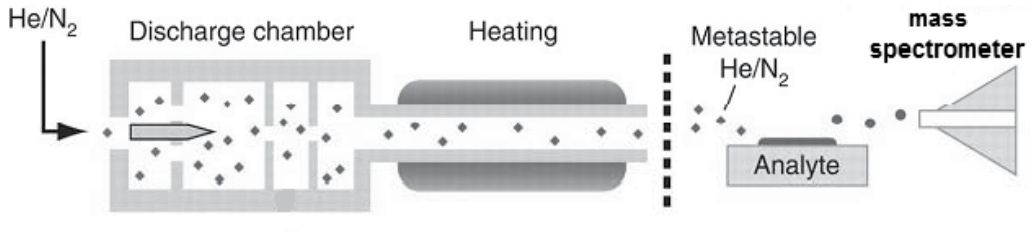

Fig. 6. Schematic configuration of DART source

4. Gas-chromatography mass spectrometry (GC/MS) applications

4.1 Introduction

Considerable efforts have been made to validate analytical methods that allow researchers to obtain reproducible data in the effective sample preparation, separation, identification, and precise quantification of pesticide analytes at the lowest possible level. The trend in the development of new analytical techniques is to simplify the operating procedure, and especially in the improvement and development of new methods by instrumental point of view. This section aims to explore the different gaschromatography-mass spectrometry (GC-MS) methods, both mass spectrometry (MS) and tandem mass spectrometry (MS/MS), developed in recent years for the determination and quantification of pesticides in food, water, soil and air samples. One of the innovations that led in recent years a significant improvement in the analysis of pollutants has been the coupling of fast gaschromatography (fast GC) with different MS devices. In fact, the fast GC-MS allows to process a greater number of analysis while maintaining the same sensitivity and efficiency compared to the GC-MS. Regarding the methods of ionization, the most used are EI and CI while recently is finding a growing use of the application negative chemical ionization (NCI) in MS detection. The most important development in the analysis of pollutants is undoubtedly due to the use of innovative MS analyzers. Indeed, the advent of devices such as ion trap (IT), triple quadrupole (QqQ), time of flight (TOF), and particularly their combinations has attracted the attention of researchers towards the application to the analysis of trace contaminants

and in particular to the multi-residual analysis. The applications of the latest analyzers allow the determination of pollutants in different modes mainly depending on the sensitivity and specificity to be achieved: full-scan, selected ion monitoring (SIM) or selected ion storage (SIS), MS/MS, multiple reaction monitoring (MRM) and selected reaction monitoring (SRM). In the following section it will be discussed the recent applications of GC-MS analysis of the major classes of pesticides: organochlorine, organophosphorus, carbamates, pyrethroids, triazines, and the multi-residual analysis.

4.2 Organochlorine pesticides

A simple and fast analysis of five organochlorine pesticides (OCPs), heptachlor (HTC), aldrin (ALD), dieldrin (DEN), endosulfan-I (EDSI) and endosulfan-II (EDS-II), in aqueous samples was carried out by Tsai et al., (2009) by using the dispersive liquid–liquid microextraction with little solvent consumption (DLLME-LSC) technique for the samples preparation and subsequent gas chromatography/mass spectrometry analysis. The developed procedures were applied to the determination of targeted OCPs in tap water, reservoir water, river water, and sea water. The MS was operated in the selected ion monitoring (SIM) mode for determination of target compounds selecting three ions of each compound. In another approach, the analysis of three chloroacetanilide herbicides, acetochlor, metolachlor and butachlor, in the same matrix was performed with the analytical GC-MS in SIM mode (Xu et al., 2007). The extraction technique developed was the direct immersion solid-phase microextraction (DI-SPME) by means of a PDMS fiber which showed a high affinity for all the three analytes. The SIM technique was also used for determination of nine OCPs in vegetables by single-drop microextraction (SDME) coupled with GC–MS. A solution of p-xylene and acetone (8:2, v/v) was used as organic phase extraction which was subsequently injected for GC-MS analysis (Zhang et al., 2008). The analysis of eighteen organochloride pesticides from complex water samples by ion trap GC–MS in SIM mode was performed with the same single-drop microextraction (SDME) technique (Cortada et al., 2009). The SDME method has been studied by experimental design that allowed the optimization of variables such drop volume, aqueous sample volume, agitation speed, ionic strength and extraction time. A high-level linearity was obtained for the calculated calibration curves for all target analytes. By another method seventeen organichlorine pesticides were analyzed and quantified in air samples by gas chromatography–negative chemical ionization–mass spectrometry (GC–NCI–MS) operating under SIM mode (Yang et al., 2008). The sampling was performed every month within a year from two different urban and suburban sites and extraction was obtained by collecting the atmospheric OCPs on quartz fibre filter (QFF) and polyurethane foam (PUF) using accelerated-solvent extraction (ASE) and subsequent neutral silica solid phase extraction (SPE).

The first application of the triple quadrupole tandem mass spectrometry for analysis of organochlorine pesticides in oils and fats was realized by Patel et al., (2005). GC-MS analysis is preceded by gel permeation chromatography which provides the removal of lipids. For each of nineteen sudied organochlorine compounds, MRM analysis was performed by choosing as the precursor ion the base peak in full-scan spectrum. In some cases were used, in addition to the base peak, even ion masses of lower intensity in order to minimize the matrix effect. Each segment contained a minimum of two and a maximum of four transitions (i.e. one or two analytes per segment). In table 2 are reported the MRM optimization parameters of some studied compounds.

Pesticide	First transition m/z	Second transition m/z	Quantification ions
α-HCH	219 > 147	219 > 183	183
Hexachlorobenzene	284 > 214	284 > 249	249
β–, χ–, δ-HCH	181 > 145	219 > 183	183
Heptachlor	272 > 237	274 > 239	237
Aldrin	263 > 191	293 > 257	191+149
Oxychlordane	187 > 123	185 > 149	123+149
cis-, trans- Chlordane	373 > 264	373 > 266	266
Endosulfan	241 > 206	195 > 125	206
p,p′DDE	246 > 176	318 > 246	176
Dieldrin	263 > 193	277 > 241	193+241
Endrin	263 > 191	281 > 245	191+245
β-Endosulfan	241 > 206	195 > 160	160+206
p,p′-, o,p′-DDT; p,p′-TDE	235 > 165	235 > 199	165

Table 2. MRM optimization parameters in some organochlorine compounds.

The GC–MS/MS triple quadrupole analysis of organochlorine pesticides showed a higher selectivity and allowed the univocal identification and quantification of all the target analytes at low ppb levels in a single analysis. For this reasons a larg number of studies applied the same technique. For example, gas chromatography–triple quadrupole tandem mass spectrometry analysis was recently developed for determination of seventeen organochlorine pesticides in fish feed (Nardelli et al., 2010). The best compromise between sensitivity and selectivity was obtained choosing one precursor and two daughters in multiple reaction monitoring (MRM) mode. The GC-MS/MS analysis showed very low LOD and LOQ values. This method was applied successfully to the monitoring of 37 fish feed samples which showed the natural presence of four pesticides (p,p′-DDE, p,p′-DDD, α-endosulfan and endosulfan sulphate), and HCB was used as internal standard, at a concentration, expressed as sum of isomers, lower than legal limit (50 μg kg^{-1}).
A simple method for determination of nineteen organochlorine pesticides in soil using gas chromatography–tandem mass spectrometry was developed from Rashida et al., (2010). A QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, based on two steps, was used for extraction and clean-up procedure. The MS/MS method included two transitions for each analyte which allowed simultaneous quantification and confirmation of any residues detected.

4.3 Organophosphorus pesticides

Since the prohibition of the first-generation organochlorine pesticides, organophosphorus pesticides have been increasingly important in the global agrochemical industry. The chemical-biological and slow degradation properties together with unscrupulous and extensive uses of this class of pesticides have led to the occurrence of several cases of environmental pollution and food contamination. Basheer et al., (2007) developed a simple binary solvent-based two-phase hollow fiber membrane (HFM)-protected liquid-phase microextraction (BN-LPME) technique followed by analysis under GC/MS selective ion monitoring mode. This method was compared with a classical SPME procedure and both techniques were applied in water samples to determine the presence of six moderately polar

organophosphorus pesticides (OPPs): triethylphosphorothioate, thionazin, sulfotep, phorate, disulfoton, methyl parathion and ethyl parathion. The first sample preparation method was a hollow polyropylene fiber-protected liquid-phase microextraction with a 1:1 mixture of toluene:hexane as extraction solvent over a period of 35 min. The second technique was a direct immersion solid phase micro-extraction (DI-SPME) procedure using a PDMS-DVB fiber and an extraction time of 35 min with stirring. The fiber was desorbed in the injection-port of the GC for 3 minutes at 250 °C. The organophosphorus compounds studied and extracted with both previously cited procedures were quantitatively determined by GC-MS in SIM mode. Linearity, repeatability, relative recoveries and limits of detection (LODs) were investigated under optimized conditions. BN-LPME combined with GC/MS analysis provided repeatability (R.S.D.s ≤ 12%), linearity ($r^2 \leq 0.994$) and LODs in the range of 0.3–11.4 ng L^{-1}. SPME combined with GC/MS analysis provided repeatability (R.S.D.s ≤ 13%), linearity ($r^2 \leq 0.966$) and LODs in the range of 3.1–120.5 ng L^{-1}. The BN-LPME and SPME procedures with GC/MS in SIM mode were applied to the detection of OPPs in domestic wastewater samples and no OPPs were detected in the real samples analyzed. One of the most important pesticide that is used to control the attack of the olive fruit fly, is the phosmet, a non-systemic organophosphorus insecticide. The determination of phosmet residues and its metabolites (phosmet-oxon, phthalimide, N-hydroxymethylphthalimide, and phthalic acid) in olive fruits was developed using matrix solid-phase dispersion (MSPD), and gas chromatography–mass spectrometry (GC/MS). All analytes were determined in selective ion monitoring (SIM). For each compound, at least, a target ion and three qualifying ions were chosen on the basis of their abundance and selectivity. The method showed suitable linearity with correlation coefficients higher than 0.8919 for all the compounds. The LOD values obtained ranged between 0.005 mg kg^{-1} and 0.060 mg kg^{-1}. The limit of quantifications were between 0.070 mg kg^{-1} and 0.15 mg kg^{-1} and resulted below the MRL set by legislation for phosmet. The analysis of real olive samples showed that phosmet concentrations were always lower than the maximum limits set by legislation (Cunha et al., 2007).

The first work was reported using the triple quadrupole for the determination of organophosphorus, has been presented by Qu et al., (2010). This method allowed the determination of twenty organophosphorus pesticides in leek using a modified QuEChERS method for sample preparation. Microwave extraction was used and it offers great reduction of the interference from the sulfur compounds contained in the leeks. The extract was then centrifuged and injected into the GC for GC–QqQ-MS/MS acquiring the data in selected reaction monitoring (SRM) mode. Precursor ion, obtained by the mass spectrum acquired in full scan mode in the range m/z 40-400, was chosen in order to have high m/z ratio (increase in selectivity) and abundance (increase in sensitivity). Selected precursor ion was fragmented by collision-induced (CID) with argon with an energy of 5 to 20 eV, resulting in a spectrum containing the ions produced. In table 3 are reported the data of some organophosphorous studied. This work was clearly demonstrated the selectivity of QqQ analyser which proved to be efficient in the determination of organophosphorous pesticides the vegetables with sulphur-containing compounds.

4.4 Carbamates pesticides

This important class of pesticides have been used extensively since the 1950s, and are widely used since they have high effectiveness and many different biological activities and uses. They are currently used instead of other class of pesticides due to their lower environmental persistence. Since carbamates are thermally unstable, most of the work in the literature were

Pesticide name	Precursor ion (m/z)	Product ion, m/z (collision energy, eV)
Dichlorvos	185	109 (15), 93(15)
Dimethoate	125	47 (15), 93(10)
Fenchlorphos	285	270 (15), 240(15)
Malathion	173	127 (5), 99(15)
Chlorpyrifos	314	258 (12), 286(10)
Parathion	291	109 (15), 137(10)
Trithion	342	157 (10), 199(10)

Table 3. SRM optimization parametersin some organophosphorous studied

performed by liquid chromatography. Nevertheless, several works have been performed using gas chromatography and trying to control as much as possible their termolability. In fact Carabias-Martnez et al., (2005) conducted a chromatographic study of seven carbamates pesticides (aldicarb, carbetamide, propoxur, carbofuran, carbaryl, methiocarb, and pirimicarb) in water by gas chromatography-mass spectrometry (GC-MS) and by both solid-phase extraction (SPE) and solid-phase microextraction (SPME). The same analytical technique was used to determine five carbamate pesticides in water samples using liquid-phase microextraction (LPME) followed by on-column derivatization and gas chromatography–mass spectrometric (GC–MS) in SIM mode analysis (Zhang et al., 2006). For selected-ion monitoring (SIM) mode, one characteristic fragment ion was monitored in addition to the molecular ion of each compound. Single-drop microextraction (SDME) followed by selected ion monitoring gas chromatography–mass spectrometry detection was used for the determination of eight carbamate pesticides (thiofanox, carbofuran, pirimicarb, methiocarb, carbaryl, propoxur, desmedipham and phenmedipham) in water samples (Saraji & Esteki, 2008). In this work were used and compared two different procedures of sample introduction: SDME followed by cool on-column injection without derivatization, and SDME followed by in-microvial derivatization with acetic anhydride and splitless injection. The first method allowed the detection of examined carbamates at concentrations greater than 30 ng L^{-1}, but the main advantages of this method were that it avoids the use of derivatization reagents, reduced analysis times, and reduced the sample manipulation. The SDME followed by in-microvial derivatization with acetic anhydride reached lower values of detection within the range 3–35 ng L^{-1}. However, this technique required longer analysis times and more sample manipulation. Another approche by using ion trap gas chromatography-tandem mass spectrometry (GC-MS/MS) following a low-density extraction solvent-based solvent terminated dispersive liquid–liquid microextraction (ST-DLLME) was developed for the highly sensitive determination of four carbamate pesticides in the water samples (Chena et al., 2010). There is no available at present, studies on the determination of carbamates pesticides using triple quadrupole GC-MS tandem mass spectrometry.

4.5 Other pesticides

Pyrethroids are an important class of pesticides used in many types of agricultural crops as insecticides. Their widespread use is due to high stability and the wide spectrum of action that they present. An efficient methodology has been developed for the determination of pyrethroid insecticide residues in vegetable oils by using combined solid-phases extraction

and gas-chromatography/tandem mass spectrometry (GC-IT-MS/MS) analysis (Esteve-Turrillas et al., 2005). This method has been proposed in order to be used in different arrays as olive, sunflower, corn and soybean oils. Since pyrethroids can also be found in the waters, it was proposed a rapid analytical method for the determination of fourteen pyrethroids in water samples, some of which are critical to water quality (Feo et al., 2010). The method is based on ultrasound-assisted emulsification-extraction (UAEE) with chloroform as extraction solvent followed by gas chromatography-negative ion chemical ionization mass spectrometry (GC-NCI-MS) analysis. Bifenthrin, cyfluthrin, λ-cyhalothrin, cypermethrin, deltamethrin, esfenvalerate, fenvalerate, fenpropathrin, τ-fluvalinate, permethrin, phenothrin, resmethrin, tetramethrin and tralomethrin are the studied compounds. The GC-NCI-MS analysis was achieved in SIM mode using ammonia as reagent gas. Optimization of NCI analysis was performed by developing the parameters of source temperature and system pressure for each compound. It was noted that the NCI mass spectrum of pyrethroids was generally characterized by intensive peaks obtained by loss of the ester substituents that forms stabilized carboxylate ions. Since the information on the diastereosiomer composition of pyrethroid standards was not available, the peaks corresponding to the different diastereoisomers of a single pyrethroid were integrated as sum for the building of the calibration curves. Another family of pesticides that are ubiquitous environmental pollutants are triazines compunds. Triazines, particularly simazine and atrazine, are important herbicides toxic and rather persistent in living systems, soil, and aquatic media. Determination of seven s-triazine herbicides in aquatic media was achieved by immersed solvent microextraction (SME) and gas chromatography–mass spectrometric in SIM mode analysis (Bagheri & Khalilian, 2005). For each compound the MS detection was operated using SIM mode based on the selection of two mass peaks of the highest intensity. For the analysis of triazines in aqueous samples, the same limits of concentration required by the EU and EPA legislation were obtained from Nagaraju & Huang (2007). The proposed method was based on a dispersive liquid–liquid microextraction technique (DLLME) coupled with gas chromatography ion trap mass spectrometric detection. Comparing with solid-phase microextraction (SPME) and hollow fiber protected liquid-phase microextraction (HFP-LPME); the DLLME method developed was a very simple and rapid, requiring less than 3 min. For determination of triazines in the selected-ion storage (SIS) mode, were selected for each compound the most abundant characteristic ions and two characteristic fragment ions, as showed in table 4.

Herbicide	m/z selected for SIS mass detection	Herbicide	m/z selected for SIS mass detection
Simazine (SMZ)	201,186,173 (100, 85, 42)	Bebuthilazine (SBZ)	229, 214, 200 (7.5, 15, 100)
Atrazine (ATZ)	215, 200, 173 (35, 100, 30)	Desmetryn (DMN)	213, 198, 171 (100, 84, 33)
Propazine (PPZ)	229, 214, 172 (30, 100, 58)	Simetryn (SMN)	213,198, 170 (100, 17, 35)
Secbumeton (SBN)	225, 210, 196 (25, 43, 100)	Prometryn (PMN)	241, 226, 184 (74, 65, 100)

Table 4. SIS optimization parameters for the analysis of triazines in aqueous samples.

The method was applied to the analysis of river and tap water samples, but in the real samples studied no triazine was found. A relatively highly solubility in water is shown by acidic herbicide compounds that are often toxic and harmful to health. Their GC monitoring in different environmental matrices cannot be done without any prior derivatization. A derivatization step with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), was needed to carry out the analysis of nine acidic herbicides in water samples by a gas chromatograph coupled to an ion-trap mass spectrometer (Rodrıguez et al., 2005).

4.6 Multiresidue analysis

The possibilities of analysis by GC interfaced to a triple quadrupole or gas chromatography time-of-flight mass spectrometry determination, have made multiresidue analysis, by itself provides a particularly sensitive as the simultaneous determination of numerous different analytes, accurate and extremely advantages in terms of time, and economically from a perspective of accuracy and precision of analytical data. Most of the developed analytical methods using the triple quadrupole focused on food matrices. In a relatively, recent work Garrido-Frenich et al., (2007) developed a new multiresidue method for the simultaneous determination of 100 pesticide residues in olive oil. The analysis was carried out in only 19 minutes by gas chromatography coupled to tandem mass spectrometry using a triple quadrupole mass analyzer. In this work two extraction processes were also tested, and an evaluation of the stability and sensitivity of the chromatographic system has been performed for the tested extraction procedures. The quantitative analysis were performed using caffeine, as internal standard, and in selection reaction monitoring mode, acquiring two or three MS/MS fragmentation reactions for each compound. Two of the most common MS/MS analyzers used in pesticide residue analysis are the ion trap (IT) and the triple quadrupole (QqQ) analyzer. IT and QqQ analyzers are representative of MS/MS in time and MS/MS in space, respectively. Garrido-Frenich et al., (2008) verified the comparison between these two MS/MS approaches, evaluating the obtained performances of GC coupled to the two different analyzers (GC–QqQ–MS/MS and GC–IT–MS/MS) for pesticide residue analysis in food matrices such as cucumber (high water content) and egg (high fat content). The analysis were tested for both systems on 19 pesticides, including organochlorine, organophophorus and pyrethroid pesticides. Two suitable extraction procedures based on the QuEChERS extraction were developed. Both the extracts were submitted to the QqQ mass spectrometric analysis operating in SRM mode and to the MS/MS analysis by ion trap mass analyzer. The QqQ and IT performance was similar in cucumber and egg matrices. However, QqQ provided better sensitivity in egg working in selected reaction monitoring (SRM). At the conclusion of this work MS data and intra-day precision were found similar in QqQ and IT, whereas inter-day precision was found significantly worse in QqQ.

Further evidence of the good obtained results in terms of linearity and sensitivity of gas chromatography-triple quadrupole mass spectrometry method for the determination of pesticide residues in food matrices can be found in another work carried out by Garrido-Frenich et al., (2006a). In this case, were determined some organophosphorus and organochlorine pesticide (thionazin, isofenphos, famfur, p,p′-DDT, mirex, γ-lindane) in meat by GC–MS/MS. In perspective of the importance of determining the presence of trace residues in food and improving the analytical techniques in terms of time and accuracy, the same research group worked to the development of multi-residue methods (MRMs) in fruits and vegetables (Garrido-Frenich et al., 2005; Vidal et al., 2006; Bolaňos et al., 2007; Moreno et

al., 2008). In those methods a screening of an extremely large number of multiclass pesticide residues, such as organochlorine (OCPs), organophosphorus (OPPs), carbamates, pyrethroids, triazoles and dicarboximides, was developed and validated. The methods provided for the use of gas chromatography coupled to a triple quadrupole mass analyzer (GC/ QqQ-MS/MS) using the selected reaction monitoring (SRM) mode. In some cases, the SIM mode was also applied simultaneously. The MS/MS conditions were fixed for each compound, trying to select as precursor ion the highest m/z ratio (greater selectivity) and abundance (greater sensitivity). The same approach was applied to choose the product ions used in the quantitative analysis. According to the validation data and performance characteristics as well as the high sample throughput and low cost, the proposed method is selected for routine application. The same authors proposed the application of the same methodology to the multiresidue determination of organochlorine and organophosphorus pesticides in muscle of chicken, pork and lamb (Garrido-Frenich et al., 2006b). The proposed analytical methodology was applied to the analysis of the pesticides in 10 chicken, 10 pork and 10 lamb samples. Only three OCPs, α-endosulfan, endosulfan sulfate and dichloran were detected in three different lamb samples, while α-endosulfan was detected in only one pork sample, at concentration levels lower than the LOQ values.

In another work carried out by Walorczyk & Gnusowski (2006) a comparison between performances of low-pressure gas chromatography (LPGC– MS) and GC–MS, both with triple quadrupole mass spectrometry, operating in scan, SIM and MS/MS mode was tested. For the screening method step; 78 different target pesticides residues (OPPs, OCPs, pyrethroids, and others) were tested in vegetable matrices and 12 target pesticides in the quantitative method. The study showed that LP-GC–MS technique deeply reduced the analysis time and had a greater ability to the correct identification of pesticides at lower levels since the peaks were improved in both size and shape than conventional GC–MS analysis. It was also found to be superior to the conventional GC with respect to obtained linearity, accuracy and precision parameters. This allowed the presumption that LP-GC based methods, especially those utilizing highly sensitive and specific MS/MS detection mode, might be of practical value in application areas requiring reliable determination at very low concentration levels. Determination of multiclass pesticides in wastewater, surface, and ground water samples (from the Valencia region, Spain) was developed using gas chromatography coupled to mass spectrometry with a triple quadrupole analyzer (Pitarch et al., 2007). The method was applied to the analysis of more than 50 compounds: 19 organochlorine and organophosphorus insecticides, 6 herbicides, 7 polychlorinated biphenyls, 16 polycyclic aromatics hydrocarbons, 2 brominated diphenyl ethers, 3 octyl/nonyl phenols and pentachlorobenzene. They were analyzed in (EI)MS/MS or in negative chemical ionization mode (NCI)MS. The system operated in MS/MS (SRM) mode using argon as collision gas. NCI was tested only for OC pesticides as this ionization mode with the aim to improve the sensitivity in comparison to EI. In negative chemical ionization (NCI), the QqQ system operated in SIR (selected ion recording) mode, using methane as reagent gas. Quantification of samples was carried out by using calibration curves prepared with standards containing five isotopically labeled standards as surrogates. Two MS/MS transitions were acquired for each analyte.

The use of the TOF analyzer has been recently proposed in the complex matrices analysis for its ability to improve the selectivity by narrowing the m/z window, giving better separation of the target compounds from coeluting compounds. The quantification of almost one hundred pesticides in fruit-based baby food, pear and lettuce samples by gas

chromatography–exact mass time-of-flight mass spectrometry (GC–TOF-MS) has been developed by Leandro et al., (2007). The mass accuracy of the TOF instrument improved with increasing concentration of the analytes of interest and was more critical for analytes with low m/z values. A method was developed using programmed temperature vaporiser injection–low-pressure gas chromatography–high-resolution time-of-flight mass spectrometry (PTV–LP-GC–HR-TOF-MS) for the analysis of multiclass pesticide residues in apple-based baby food (Cajka et al., 2008). A comprehensive GC×GC–TOFMS method was optimized for separation of 51 pesticides in grape matrices (Banerjee et al., 2008). The sample preparation was performed using ethyl acetate and subsequent cleaning by DSPE with PSA. A combination of a non-polar and a polar capillary column connected in series allowed chromatographic separation of pesticides studied, thus solving the co-elution problems as observed in full scan one-dimensional GC–MS analysis, and improving the limit of detection by 2–12 times. The same analytical procedure based on comprehensive two-dimensional gas chromatography (GC × GC) coupled with time-of-flight mass spectrometry (TOF-MS) was used for the simultaneous determination of 97 organic contaminants at trace concentration in river water (Matamoros et al., 2010).

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Part 3

Emerging Methods of Pesticides Analysis

18

Chemically Modified Electrodes for Detection of Pesticides

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1. Introduction

Environmental problems and their control have received a great deal of interest and publicity, and the number of pesticides used in agriculture and other applications has been steadily increasing. Environmental scientists are concerned with reliable means for detecting these compounds. Although many advances have been made in this area in recent years, much is yet to be accomplished. Chemistry plays a vital role in environmental protection, and analytical chemistry must meet the challenge of providing qualitative as well as quantitative characterization of pesticides. It has already been warned that some organic and inorganic compounds have harmful effects at substantially lower levels than previously suspected (1). Thus, there is a great necessity for new analytical methods with better precision, accuracy, sensitivity and selectivity. These methods must not be prohibitively expensive and, ideally, could be adapted for measurement in the field.

Existing analytical techniques have limitations when used for environmental monitoring. X-ray and electron diffraction, mass spectrometry, activation analysis, and spectral laser methods are not only sophisticated and expensive, but also require specially trained personnel for proper operation of instrumentation. Although gas-liquid chromatography (GLC or GC) has been used with its variety of sensitive and selective detectors, the technique is limited to volatile compounds (2). Non-volatile compounds are commonly derivatized prior to gas chromatographic detection to increase their volatility. High performance liquid chromatography (HPLC) is useful for non-volatile samples (3), but commercially available detectors are limited in sensitivity and/or selectivity.

A variety of electroanalytical methods have been widely employed for detection of pesticides at bare electrodes (4-7). Drawbacks of bare electrodes have been overcome by the use of electrodes whose surfaces are modified with specific functionalities (chemically modified electrodes or CMEs). This paper summarizes analytical capabilities of electrochemistry for detecting pesticides in the environment. The discussion will be mainly focused on electrochemical detection of pesticides at metalloporphyrin modified electrodes (MPEs). An introduction to CMEs and metalloporphyrin electrochemistry will also be included. Furthermore, the application of modified electrodes for detection of pesticides and the suitability of electrochemistry in conjunction with liquid chromatography (LCEC) for separation of environmental samples followed by detection of pesticides will be discussed.

2. Electrochemical methods

2.1 Electroanalytical chemistry

Electroanalytical techniques are those which show a quantitative relationship between the magnitude of an electrical quantity and the bulk concentration of the analyte. The electrode in solution is, in many respects, a transducer between chemical and electrical entities. The role of the electrode is either to monitor species in solution or to generate new species. Electroanalytical methods are categorized as either static or dynamic. Static methods (e.g.: potentiometry) involve measurements of potential difference at zero current without changing the Nernstian equilibrium at the electrode-solution interface. In dynamic techniques (e.g.: voltammetry, amperometry), the system is disturbed by electrical excitation signals consisting of a variety of potential or current programmes, and the resulting response is measured (8).

Electroanalytical methods have received much attention in the area of environmental chemistry, because they are sensitive and selective. Further, instruments used are relatively simple to operate and potentially portable. Many electroanalytical techniques are used for the analysis of water, atmosphere, solid and food materials. Table I summarizes common electroanalytical techniques and their detection limits (2, 8-10). However, the selection of a particular method and its exact detection limit depend on the nature of the analyte and the sample.

Method	**Concentration/mol dm^{-3}**
Potentiometry	10^{-2} – 10^{-4}
Conductometry	10^{-3} – 10^{-5}
Palarography	10^{-4} – 10^{-6}
Cyclic voltammetry	10^{-4} – 10^{-6}
Rotating disk voltammetry	10^{-4} – 10^{-6}
Steady state amperometry	10^{-5} – 10^{-7}
Amperometry with flow injection analysis	10^{-7} – 10^{-9}
Square-wave and differential pulse voltammetry	10^{-6} – 10^{-8}
Anodic stripping voltammetry with a solid electrode	10^{-8} – 10^{-10}
Anodic stripping voltammetry with a hanging drop electrode	10^{-7} – 10^{-9}
Electrochemistry coupled with liquid chromatography (LCEC)	10^{-9} – 10^{-12}

Table I. Common electroanalytical techniques and their concentration range of detection.

2.2 Electrocatalysis

For a molecule to be directly investigated electrochemically, it must have an electroactive group (electrophore) for reduction or oxidation. In certain cases, the molecule of interest (analyte) shows electroactivity beyond the available potential range of the working electrode, as a result of sluggish kinetics. The rate of electron transfer of such reactions can easily be increased by means of electrocatalysis, a process in which the introduction of a catalyst decreases the reduction (oxidation) potential of the analyte by decreasing the

overpotential, or alternatively, increases the reduction (oxidation) current. Electrocatalysis can also be used to avoid interference from dissolved species (8, 9).
Homogeneous electrocatalysis utilizes the catalyst and the analyte in the liquid phase, while heterogeneous catalysis utilizes the catalyst in the solid phase, usually as a coating on the electrode surface. There are two main types of electrocatalysis, redox and chemical catalysis. Chemical catalysis is a complicated process which involves the electrochemical generation of the active form of the catalyst. Transient formation of an adduct between the active form and the analyte would then take place. The chemical bond thus formed is cleaved yielding product(s) and regenerating the starting form of the catalyst (11).

2.3 Chemically modified electrodes (CMEs)

The concept of CMEs originated in the mid-70's when electrochemists sought to control the chemical nature of electrode surfaces (12, 13). CMEs are different from classical (bare) electrodes in that the electrode surface is altered by immobilizing molecules in a rational fashion so that the electrode thereafter displays new properties. Drawbacks of bare electrodes, such as adsorption of molecules or ions, unpredictable surface reactivity and sluggish kinetics can be overcome by modifying the electrode surface (14). The molecules to be immobilized can be selected on the basis of known and desired properties, for instance, electron transfer mediator catalysts, functionalities which scavenge trace molecules or ions for preconcentration, photosensitizers, corrosion inhibitors and outer sphere electron transfer agents.
The principal routes to the immobilization of substances are, chemisorption, covalent bonding, multimolecular layer deposition and electropolymerization (14). If a catalyst is immobilized on an electrode surface, it is essential to understand the electrochemistry and reactivity of the catalyst in order to utilize it in electrochemical detection. The following section explains the electrochemical behavior of metalloporphyrin catalysts in aqueous and nonaqueous media, and importance of MPEs in the detection of pesticides.

3. Electrochemistry of metalloporphyrins

Metalloporphyrins and their derivatives are well known nucleophiles capable of displacing the halide ion in alkyl and aryl halides yielding the corresponding alkyl or aryl metal-nucleophile complex (15-17). The exact mechanism of the nucleophilic substitution seems to depend on the type of substrate and the metal ion. This process can be catalyzed by supplying electrons via electrochemical, photochemical, or by several solvated ions of alkali metals dissolved in liquid NH_3, or by other electron-transferring agents (18).
Commonly available metalloporphyrins are insoluble in aqueous media, requiring nonaqueous solvents for investigation of porphyrin electrochemistry (19). Metalloporphyrins usually show four electrochemical processes when the last reduction is observed [usually more negative than -1.4 V vs. saturated calomel electrode (SCE)] (20). Cyclic voltammetric studies of cobalt tetraphenylporphyrin [Co(II)TPP] in CH_2Cl_2 shows the expected reduction peaks including the catalytic reductive dehalogenation of the solvent, CH_2Cl_2, a reaction that had not been previously thought of (15).
Although metalloporphyrins have been known to catalyze the reduction of organic molecules containing halogen groups, early studies of catalytic chemistry of organohalides was limited to nonaqueous media at bare electrodes due to solubility problems of many commonly used metalloporphyrins. The behavior of metalloporphyrins in aqueous solution

was later studied by modifying the bare electrode surface with a metalloporphyrin coating, or alternatively, modifying the surface with a foreign matrix (e.g.: polymer) in which metalloporphyrins are immobilized. Electrodes prepared in this manner have shown substantial differences from the solution behavior (15).
Elliot and Merrese introduced the concept of using MPEs for investigation of catalytic properties for the reduction of organohalides (21). They conducted a series of cyclic voltammetric experiments at meso-tetra(p-aminophenyl)porphyrinatoiron(III) modified pyrolytic graphite and glassy carbon electrodes (GCEs) in dimethyl formamide (DMF). The overpotential for the reduction of benzyl bromide, triphenylmethylbromide and hexachloroethane is decreased in the presence of the metalloporphyrin showing that it is an effective catalyst for the reduction of organohalides. During this process, the surface-bound metalloporphyrin is electrochemically reduced to the formal Fe(I) oxidation state when the potential is scanned, followed by nucleophilic substitution. The demonstration of the catalytic electrochemistry of metalloporphyrins at modified electrode surfaces stimulated the development of electrocatalytic detection schemes for organochlorine pesticides. However, this procedure was not employed to develop electrochemical sensors until recently.

4. Chemically modified electrodes for pesticide detection

4.1 General aspects of environmental monitoring

Atomic absorption spectrophotometry is commonly used for monitoring inorganic pollutants, including pesticides which contain heavy metals. In addition, piezoelectric crystals coated with a variety of selective and sensitive substances for the determination of air pollutants including gasses, explosives and pesticides continue to receive attention of environmental chemists (22, 23). Detection procedures for organic and organometallic pollutants, in particular agrochemicals, based on modified electrodes were limited until the late 1980's , although several direct electrochemical determinations had been reported (24). Among them, the use of polarographic methods for the determination of metal containing dithiocarbomate pesticides and organo-tin fungicides demonstrated the potential applicability of electrochemical methods for the detection of pesticides (25).
Differential pulse and square wave modes of polarography have been applied to increase the sensitivity of detection of classical polarography. Nevertheless, the inapplicability of the dropping mercury electrode for steady state amperometric and LCEC measurements has limited its popularity. Mercury coated and amalgamated electrodes have solved this complication, yet maintaining the advantages of the mercury surface. For such investigation, differential pulse mode is commonly used as it is more sensitive than other modes. In this connection, the possibility of using mercury film electrodes for characterization and detection of triphenyltin acetate, an organo-tin mollucicide, had been attempted (26). The detection conducted by differential pulse anodic stripping voltammetry showed that the lower detection limit was 2.5×10^{-9} M. This detection limit was at least an order of magnitude lower than that of other available techniques such as liquid chromatography and differential pulse polorography. However, the use of heavy metal containing environmental pollutants has significantly decreased over the last decade due to environmental and health problems, and consequently development of detection procedures for organometallic pesticides has not been of high priority in recent days.

Application of electrochemical techniques for environmental monitoring, in particular, for the detection of organic pollutants and toxic gases is becoming increasingly popular due to their desirable characteristics (27, 28). The use of modified electrodes with various electrochemical modes has drawn the special attention of environmental scientists because they offer unique advantages (29, 30).

4.2 Detection of pesticides

Brand and Fleet reported the use of mercury coated platinum wires as voltammetric sensors for the detection of dithiocarbomate pesticides (sodium salt) in the late 1960's before extensive research on "modified electrodes" was begun (31). Later, preliminary investigation on the detection of sulphonyl ureas, a new class of herbicides useful for controlling broadleaf weeds, and s-triazine herbicides such as atrazine, prometryne and simazine, with mercury coated GCEs have been reported (32, 33). The detection schemes developed for these two classes of herbicides based on differential pulse polarography at coated GCEs resulted in a lower detection limit of 10^{-8} M at low pH values. It is impossible to use bare electrodes for the above detection because no reduction wave appears even in a solution of 10^{-4} M herbicide (32). In addition to mercury film electrodes, carbon paste electrodes later became attractive due to the fact that they can easily be modified with any material, by mixing with carbon paste (34).

Potentiometric sensors with modified electrode surfaces for environmental analysis include polyvinylchloride (PVC) coated aluminium wires and piezoelectric quartz crystals modified with crown ethers (e.g.: dibenzo-30-crown-10 or DB30C10) for detecting the herbicides, diquat and paraquat (35, 36). The modified piezoelectric crystal demonstrated the use of ion-selective electrodes as sensors for diquat and paraquat in the gas phase while the PVC coated electrode was employed for potentiommetric titrations in solution.

4.3 Pesticide detection with biosensors

A biosensor is an analytical device which converts a biological response into an electrical signal. The term 'biosensor' is often used to cover sensor devices used in order to determine the concentration of substances and other parameters of biological interest even where they do not utilize a biological system directly. Among various kinds of biosensors employed for environmental monitoring, electrodes modified with microorganisms (biofilm electrodes) were first introduced for the analysis of waste water samples. An oxygen electrode whose surface was modified with bacteria or yeast biofilms was used as an amperometric sensor to monitor the oxygen consumption in enzymatic reactions of microorganisms (biochemical oxygen demand or BOD) (37, 38). These BOD probes had a life time of several weeks. After realization of advantages of biosensors, potentiometric and amperometric biosensors with different types of modifiers were designed to detect pesticides in water samples (39-41). There is a vast number of publications devoted to this aspect to date.

5. Metalloprophyrin modified electrodes (MPEs) for detection of pesticides

Although electrodes modified with various reagents had been constructed for detection of pesticides, not much attention had been paid on metalloporphyrins as electrode modifiers. The use of MPEs for detection of clinically important compounds (42) and inorganic ions

(43) as well as the well understood catalytic reduction mechanism of organohalides by metallopophyrins opened a new era of using MPEs for electrochemical sensing of pesticides. Our research group has been expanding the use of electrodes modified with metalloporphyrins and other reagents for the development of novel detection schemes for pesticides, in particular, those which are used on rice cultivation, herbicides and other compounds of environmental significance.

5.1 Electrochemical detection of halogenated, aromatic substances

As indicated earlier, electrochemistry of a metalloporphyrin in nonaqueous medium is different from that in the solid phase in the form of a coating in aqueous or mixed aqueous systems. The notable difference is that the electroactivity of the conjugated system is not evident in aqueous medium, and hence the redox chemistry of the metal center is predominant. Cyclic voltammetry obtained at the tetraphenylcobalt(II) [Co(II)TPP] modified GCE in a mixed aqueous/nonaqueous solvent system (CH_3CN/H_2O; 1:3) shows the reduction of Co(II)TPP to $[Co(I)TPP]^{-1}$ at -0.34 V vs. SCE (15). Similar behavior is observed with Fe(III)TPP modified GCEs as well. GCEs are more suitable for electrochemical detection of substances in aqueous solution because noble metal electrodes such as Pt, Pd and Au in the absence of any analyte show complicated electrochemistry. Hydrogen adsorption, hydrogen desorption, formation of surface oxide and reduction of the oxide are the main complicated processes (9).

Organochlorine herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), are commonly used for post-emergence control of annual and perennial broadleaf weeds in cereals, rice and several other plants. There are several metabolic pathways for 2,4-D and MCPA in the environment including hydroxylation, ring opening and cleavage of the acid side-chain. The toxicity level of these two herbicides for mammals is in the order of 10^{-4} M (several hundred ppm) (44).

The addition of 2,4-D to the electrolyte solution consisting of the Co(II)TPP modified GCE has produced dramatic changes in the voltammogram. Two reduction peaks were observed at -0.15 V and -1.20 V (vs. SCE) during the complete potential scan. The reduction at -1.2 V is 0.15 V more positive than the potential required for the reduction of 2,4-D at bare GCE, demonstrating the catalytic activity of Co(II)TPP. This response is probably associated with the direct reduction of the carboxylic acid group in the herbicide molecule. It would be very difficult to obtain accurate quantitative measurements at such a negative potential at bare electrodes as it is very close to the hydrogen evolution potential. Several interferents, including dissolved oxygen, would be reduced at potentials less negative than -1.2 V, disqualifying the second reduction at the modified electrode for chemical analysis. Thus, the first reduction peak has been explored for quantification of 2,4-D.

Additional electrochemical and controlled experiments conducted at the Co(II)TPP modified GCEs suggested the following mechanism for the first catalytic reduction of 2,4-D at -0.15 V (46).

$$Co(II)TPP + e \rightarrow [Co(I)TPP]^- \qquad \text{Electrochemical step}$$

$$[Co(I)TPP]^- + RX \rightarrow [TPPCo(II)R] + X^- \qquad \text{Chemical step}$$

$$[TPPCo(II)R] + e \rightarrow \text{Products} \qquad \text{Electrochemical step}$$

A similar mechanism is suggested for electrodes modified by other metalloporphyrins, where the metal is reduced from a higher oxidation state to a lower value followed by chemical reaction to form a metal-porphyrin adduct. The last step of the mechanism is the electrochemical reduction of the adduct to form various products. The identification of the products is not necessary for quantitative determinations because the concentration of the analyte depends on the catalytic current generated at the electrode surface.

Both Co(II)TPP and Fe(III)TPPCl modified GCEs operating at a constant potential demonstrated the utility of MPEs for amperometric detection of 2,4-D. The calibration curves obtained at the modified electrode showed a linear dynamic range from 2×10^{-6} M to 1×10^{-5} M (45). Amperometric experiments for 4-chlorophynoxyacetic acid (4-CPA), structurally similar to MCPA, gave a similar linear dynamic range, sensitivity and a lower detection limit. Fe(III)TPPCl modified electrodes were found to be more stable than Co(II)TPP electrodes, but the sensitivity was lower. Successful detection of 2,4-D and 4-CPA by MPEs has extended this methodology for the detection of organochloro pesticides such as propanil, endosulfan and MCPA (46-49). Additionally, it has been shown that MPEs have the ability for selective electrocatalysis of bromo-substituted aromatic substances depending on the position of substitution (50). Another step that has been taken further towards real application in the field of pesticide detection is the use of MPEs to determine the levels of propanil in simulated rice field environment by amperometry (51) and to monitor the activity of propanil by cyclic voltammetry (52). Electrodes modified with free base porphyrins do not produce significant current response in these detections proving the necessity of a metal ion at the center of the porphyrin molecule for electrocatalysis of halogenated aromatic hydrocarbons. Detection limits of steady state amperometric procedures can be further improved by flow injection analysis followed by amperometric detection at least by one order of magnitude (15).

5.2 Electrochemical detection of dalapon

The sensitivity and the limits of detection usually depend on various factors, such as type of the analyte, nature of the modifying agent and method of modification. Co(II)TPP and Fe(III)TPPC1 modified GCEs are found to be more sensitive to aromatic analytes. The detection limit of 2,2-dichloropropionic acid (Dalapon), a general use herbicide, was about two orders of magnitudes lower than that of 2,4-D and 4-CPA (45).

Although metalloporphyrins are good catalysts for reduction of organohalides, thick coatings can prevent the charge (electron) transfer between the electrode surface and the solution as metalloporphyrin molecules can insulate the surface. Thin coatings do not insulate the surface, but they are easily dissociable due to the catalytic reaction with organic compounds dissolved in solution leaving almost a bare electrode. Thus, electrodes modified with thin coatings would not have a long life time, and the coatings of moderate thickness should thus be used as a compromise. Coatings prepared by droplet evaporation technique give a less reproducible response compared to those prepared by dip coating. The thickness of the coating prepared by the dip coating method can be further decreased, if needed, by polishing the modified electrode. This process would eliminate weakly bound porphyrin molecules, and electrodes obtained after polishing have given a higher sensitivity for the analyte.

6. Detection of pesticides by other chemically modified electrodes (CMEs)

Attempts have been taken to explore the possibility of using different types of CMEs for detection of pesticides/agrochemicals. GCEs modified with stearic acid has provided an amperometric sensor for the detection of paraquat, the active ingredient of the herbicide Gramoxone, with a minimum detection limit 6.4×10^{-4} M (53). Another type of modified electrodes is modified carbon paste electrodes where the modifier is incorporated into carbon paste during its preparation. It has been reported that amperometric determination of MCPA at the MnO_2-modified carbon paste electrode results in a very low detection limit of 0.20 ppm. Further, the application of the proposed method for quantitative analysis of MCPA formulations has provided reliable results demonstrating the use of CMEs in real applications (54).

7. Application of chemically modified electrodes in liquid chromatographic detection

In spite of the sensitivity of electroanalytical techniques, they cannot be directly utilized in analyzing real environmental samples, which are usually in a complex matrix of several different components. However, the combination of electrochemistry and liquid chromatography (LCEC) provides very selective and sensitive measurements for environmentally important compounds, for example pesticides, with the aid of chromatographic separation. Consequently, several reports have recently appeared on the application of LCEC techniques for detection of pesticides (55 – 57). Nevertheless, LCEC has yet to fulfill its potential for the analysis of pesticides with CMEs.

8. Conclusion

Monitoring current levels of environmental pollution requires more specific, sensitive and accurate measurements. Existing analytical techniques have several drawbacks, and consequently, electroanalytical techniques attract environmental chemists due to their desirable properties. However, electrochemical detection for environmental monitoring with CMEs is still in its early stage. Polarographic determination of herbicides at mercury film coated electrodes and amperometric detection of organic pollutants with bio-film modified electrodes are among the few established electrochemical techniques. Furthermore, application of MPEs for the detection of pesticides containing halogens has been successful due to the reductive dehalogenation reaction catalyzed by metalloporphyrins.

The use of MPEs as amperometric detectors for pesticides after liquid chromatographic separation of environmental samples (LCEC) would be possible in future. Furthermore, the use of electrodes modified with polymers, biological materials as well as other functionalities for detection of pesticides using LCEC techniques will be a promising research area.

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Voltammetric Analysis of Pesticides

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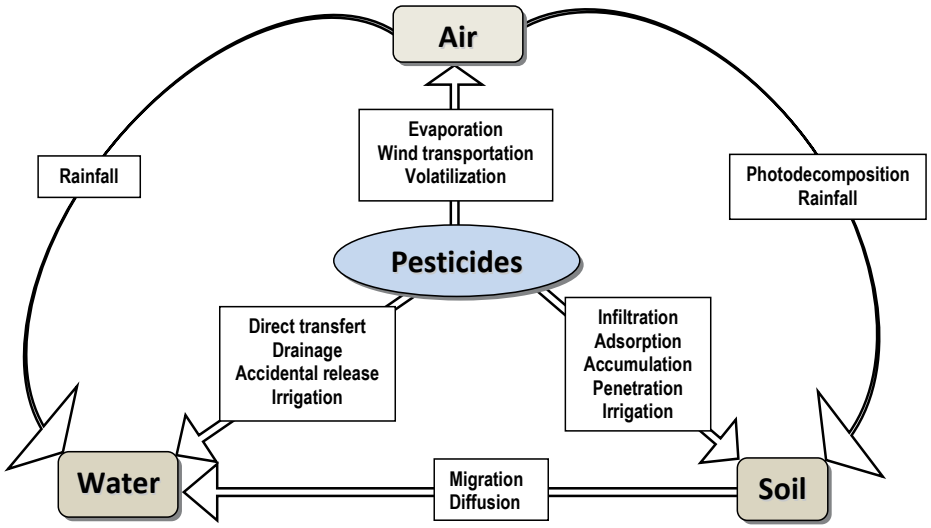
1. Introduction

Pollution due to pesticides is a daily and growing problem, closely related to the intensification of agricultural activities devoted to the satisfaction of human needs in terms of food. In fact, throughout the world, pesticides are intensively used as defoliants or against weed and pests in a large variety of crops (Castanho et al., 2003; De Souza & Machado, 2005a). Also known as persistent organic pollutants due to their low solubility and degradation in the environment, pesticides are thus inherently toxic since their accumulation in living organisms can induce serious diseases.
Several analytical techniques have been used for the analysis of pesticides, which include fluorimetry (Anson & Wade, 1976; Eremin et al., 1996; Coly & Aaron, 1998a,b), capillary electrophoresis (Tomita et al., 1992; Pico et al., 2003), spectrophotometry (Shivhare & Gupta, 1991; Jain et al., 1993), mass spectroscopy (Moyano & Galceran, 1996; Leandro et al., 2006) and mainly gas or liquid chromatography (McGarvey, 1993; Castro et al., 2000; Fillion et al., 2000; Corasaniti & Nistico, 1993). These techniques operate quite well but they present a certain number of disadvantages: they are limited to laboratories, time consuming, expensive, and have to be performed by highly trained technicians (Mulchandani, 2001). During the past decade, scientific works devoted to the implementation of electrochemical devices suitable for the sensing of pesticides have gained growing interest. The main proposal of these studies is the development of convenient, sensitive, selective and low cost tools that could be exploited for a rapid monitoring of pesticides (Tapsoba et al. 2009; Tcheumi et al., 2010). In fact, when pesticides are applied, only a small fraction actually reaches target organisms formed by crop pests and vectors of diseases. Consequently, traces amounts of these chemicals can be detected in surrounding and ground water, in the soil and in the air. Scheme 1 summarizes the most common pathways involved in the dissemination of pesticides in the environment. In another hand, the determination of pesticides and their residues or metabolites in either water or soil solutions is a critical problem for several reasons: (i) the number of chemicals involved is too large, (ii) each of these chemicals displays a specificity in term of reactivity, and (iii) several active ingredients of pesticides are often present in the same matrix. Therefore, the need to design convenient tools for the electroanalysis of pesticides in environmental matrices and in food remains a permanent and challenging question. Following these lines, there is an interest to properly adapt electrochemical methods to build sensors, due to some of their advantageous characteristics such as simple operations, short analysis times, accuracy and high sensitivity (Parham & Rahbar, 2010; Tcheumi et al., 2010).



Scheme 1. Common pathways for the dissemination of pesticides in the three parts of the environment.

In this chapter, the potential contribution of electrochemical methods to the monitoring of pesticides will be exposed. Since chemically modified electrodes have gained great interest for pesticides analysis, a focus will be given to the description of new sensors fabricated in recent years. The future trends in the design of new electrode configurations likely to modernize the electroanalysis of pesticides will be also discussed.

2. Overview of voltammetric methods for the determination of pesticides

2.1 Fundamental concept of voltammetric methods

Voltammetric techniques are undoubtedly the most useful tools in analytical sciences, especially for studies in aqueous media or at solid state of electroactive species. As most chemical and biochemical compounds are either reducible or oxydable, modern research laboratories are usually equipped with electrochemical techniques. Yet, these methods can be used to study the kinetics and thermodynamics of electron and ion transfer processes (Bagotsky, 2005), to investigate adsorption phenomena likely to occur at the electrode surface and to study the reaction mechanisms in organic chemistry or in biochemistry (Bard & Faulkner, 2001). In most voltammetric methods, an electrochemical cell is required which consists of three electrodes: (i) the indicator (or working) electrode at which the electrochemical reaction involving electron transfer takes place, (ii) the reference electrode characterized by a constant potential over time and (iii) the auxiliary electrode at which a counter reaction to that at the working electrode takes place to balance the total charge in the whole system (Gulaboski & Pereira, 2008). From a practical point of view, when a potential E is applied to the working electrode in contact with an electroactive probe, this probe is either oxidized or reduced. Consequently, a change in its concentration occurs at the electrode surface, causing mass transfer towards the electrode. A current then flows through the electrode. By sweeping the potential with time and recording the current (i), a

curve called voltammogram is obtained which is obviously equivalent to record the current as a function of applied potential. The application of voltammetric methods for analytical purposes is due to the fact that the current recorded at the indicator electrode is directly proportional to the analyte concentration (Wang, 2006). There are many voltammetric techniques each characterized by the nature of the potential applied to derive the electron transfer reaction and by the shape of the i *versus* E curve, that is of the voltammogram whose physical parameters are easy to exploit for quantitative analysis.
Concerning the electroanalysis of pesticides most of which are organic compounds bearing electroactive functional groups, cyclic voltammetry and pulse voltammetric techniques (namely differential pulse voltammetry, square wave voltammetry and differential pulse polarography) are frequently used. A brief description of each of these techniques in conjunction with some typical applications will be provided in the forthcoming section.

2.2 Cyclic voltammetry (CV)

Amongst voltammetric methods, CV is quite popular and is generally used to first investigate the electrochemical behaviour of new redox systems (Gulaboski & Pereira, 2008). It offers a rapid location of redox potentials of these systems and a convenient evaluation of the effect of media on the redox process involved (Wang, 2006). CV consists of first applying a linear sweep potential to the working electrode from an initial potential (E_i) to a final one (E_f). After reaching E_f, the sweep is reversed and the potential returns to E_i. During this experiment, a current resulting from the potential applied at a precise scan rate is recorded. The recorded cyclic voltammogram is characterized by five main features: the cathodic and anodic peak potentials (denoted E_c and E_a respectively) to which correspond the cathodic and anodic peak currents (i_c and i_a), and the half-peak potential ($E_{1/2}$). The values of these parameters and the relationship between them provide the basis for classifying cyclic voltammograms as reversible, irreversible or quasi-reversible systems. For adsorption processes and coupled chemical reactions, quantitative data on the kinetics of electron transfer reaction and on the thermodynamics of the redox reaction can be derived, mainly when multiple cycles are subsequently recorded between E_i and E_f. For pesticides analysis, the shape of cyclic voltammogramms can provide informations about the type of working electrode reaction, the number of electrons involved in the electrochemical reaction and about additional process that can eventually occur such as adsorption or coupled chemical reactions (Zoski, 2007). A typical example illustrating the usefulness of cyclic voltammetry in pesticides analysis is presented in Figure 1 where the interfacial behavior of methylparathion was investigated using repetitive cyclic voltammetry in 0.1 M acetate buffer (pH 5), on a glassy carbon electrode covered with a thin film of a gemini surfactant intercalated montmorillonite (Tcheumi et al., 2010). As shown, the electrode response is made of two redox systems: (i) the first one (characterized by Ea_1 = 0.03 V and Ec_1 = 0.00 V) is reversible while the second one formed by one reduction peak (Ec_2 = -0.60 V) is irreversible. One can notice that a global increase of the reversible system's peaks is observed upon continuous cycling, indicating a progressive adsorptive accumulation of methylparathion by the organoclay on the glassy carbon electrode. During the first cyclic scan performed from +0.03 V, the peak of the reversible system did not appear but arises only when the second cathodic peak was formed. This observation showed that the reversible system thus resulted from a species generated by the irreversible system.

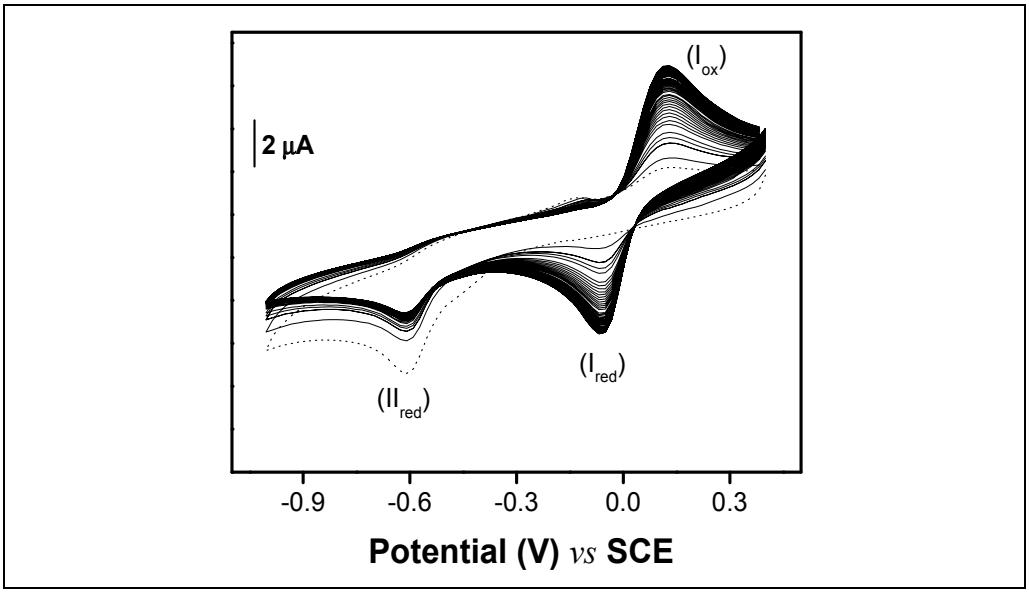
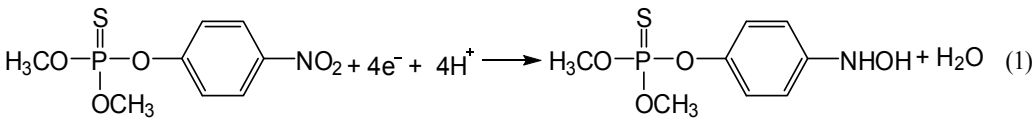
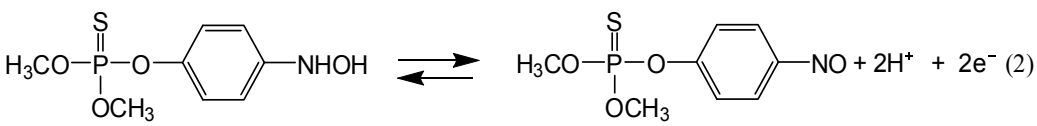


Fig. 1. Multisweep cyclic voltammograms recorded at a scan rate of 100 mV s^{-1} for 41.5 µM methylparathion in 0.1 M acetate buffer (pH 5) on a glassy carbon covered with a gemini surfactant intercalated montmorillonite (From Tcheumi et al, 2010).

Such a behavior was reported for the same pesticide on a nafion coated GCE (Zen et al., 1999), and the irreversible peak was attributed to the reduction of the nitro group of methylparathion to hydroxylamine group (Equation (1)), whereas the reversible system correspond to an electron transfer process indicated in Equation (2) (Zen et al., 1999, Sbai et al., 2007).





2.3 Pulse voltammetric techniques

Pulse voltammetric techniques were introduced in electrochemistry by Barker and Jenkin (1952) to resolve a crucial problem, the lowering of the detection limits of voltammetric measurements which is achieved upon substantially increase the ratio of faradaic to capacitive current (Wang, 2006). In such techniques, the basic principle is the same: a sequence of pulsed potentials is applied to the working electrode, after the potential is stepped, the quite undesirable capacitive current decays exponentially with time to

a negligible value while the faradaic current decays more slowly for the same reaction. This leads to a negligible capacitive current and concomitantly to a significant faradaic current which is related to the electrochemical reaction of interest (Wang, 2006; Gulaboski et al., 2008). There are various pulse voltammetric techniques in modern electrochemistry, classified according to the pulse-wave form and the current sampling mode. The most important instrumental parameters of these techniques are the pulse amplitude (height of the potential pulse), the pulse width (duration of the potential pulse) and the sampling period. The electrode response consists of current peaks the height of which is directly proportional to the concentration of the analyte. Thanks to their great performance, pulse techniques can be exploited via anodic, cathodic or adsorptive stripping modes. Square wave voltammetry, differential pulse voltammetry and differential pulse polarography are the most used pulse techniques for the electroanalysis of pesticides molecules.

2.3.1 Square wave voltammetry (SWV)

SWV is the most advanced and efficient of pulse voltammetric techniques (Osteryoung & O'Dea, 1986; Stojek, 2001). A potential, consisting of symmetrical square-wave pulses superimposed on a staircase-wave form is applied to the working electrode. During each square-wave cycle, the current is sampled twice: once at the end of the forward pulse that gives rise to the oxidative current component, and once at the end of the backward pulse that gives rise to the reduction current component. The difference between the two measurements is plotted versus the base staircase potential and the resulting peak-shaped voltammogram is proportional to the analyte concentration (Kounaves, 1997; Wang, 2006; Gulaboski & Pereira, 2008). Many excellent works dealing with the detection of low amounts of pesticides in food and environmental samples have been published. Figure 2 displays typical square-wave voltammograms obtained on a glassy carbon electrode covered by drop-coating of an organophilic modified clay film. The modified electrode was used to analyze diluted solutions of methylparathion, with concentrations ranging from 4.0×10^{-7} to 8.5×10^{-6} M (Tcheumi et al., 2010). For this particular case, the electrochemical procedure used for the electroanalysis stripping SWV involved two successive steps: an open-circuit accumulation of the pesticide followed by a voltammetric detection in a separate medium. Preconcentration was achieved by dipping the working electrode in a stirred solution of methylparathion at a given concentration, this operation is performed to facilitate the transport followed by the accumulation of the pesticide on the organoclay material. As shown by the inset in Fig. 2, peak currents increase with methylparathion concentration, displaying a linear relationship behavior which was exploited to attain a detection limit of 7×10^{-8} M. SWV has also been shown to be an important technique for the detection of paraquat, a toxic herbicide used to control herbal growth in terrestrial and aquatic environment (El Mhammedi et al., 2007). The working electrode consisted of a carbon paste electrode chemically modified by a synthesized Ca-hydroxyapatite. Many other unique applications of square wave voltammetry include the use of non-modified (Liu & Lin, 2005) and modified (Parham & Rahbar, 2010) carbon paste electrodes for the sensing of organophosphate pesticides, the quantification of pesticides in real water samples using ionomer clay-modified electrode (Zen et al., 1996) and even on non modified electrodes (Garrido et al., 1999, 2001).

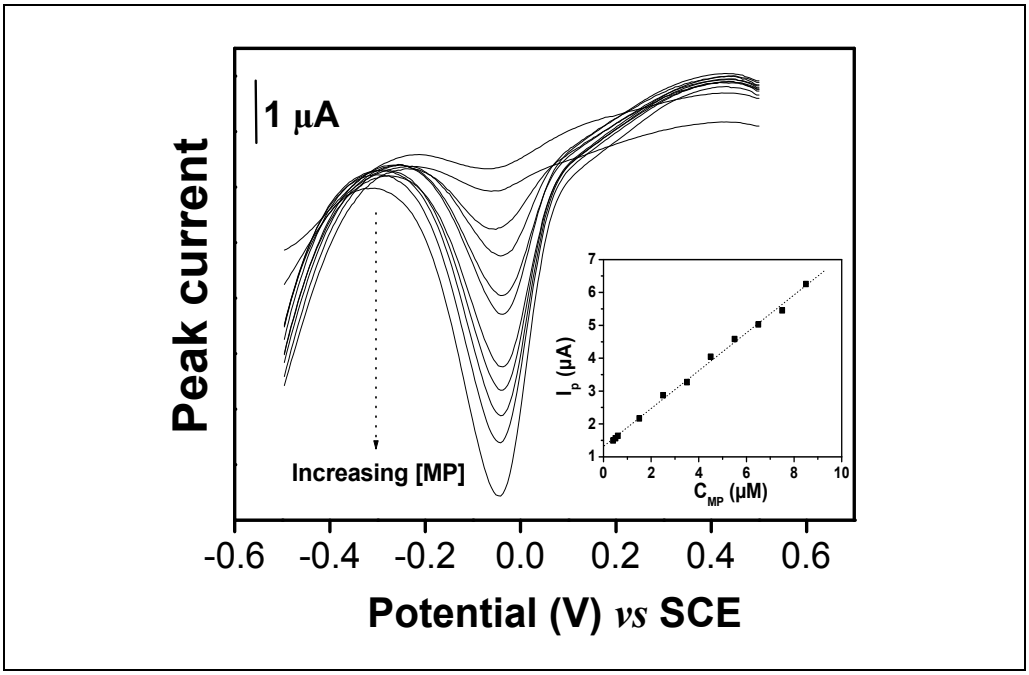


Fig. 2. Square wave voltammograms obtained at a glassy carbon electrode modified by a positively charged gemini surfactant intercalated montmorillonite. The curves were recorded using the "preconcentration at open-circuit/detection sequence". The inset shows a linear relationship between the electrode response and the pesticide concentration ranging from 4 x 10^{-7} to 8.5 x 10^{-6} M (From Tcheumi et al., 2010).

Some recent advances and applications of SWV were described, for which the stripping voltammetry was combined with solid-phase extraction often used in separation science (Gong et al., 2009, 2010). Such a combination was shown to be suitable for decentralized point-of-care or field determination of pesticides in the environment with portable instruments, providing a fast, simple and sensitive electrochemical method for the detection of organophosphate pesticides.

2.3.2 Differential pulse stripping voltammetry (DPV)

Amongst voltammetric methods, DPV is of extreme usefulness as the technique is particularly designed for measuring trace levels of organic and inorganic species (Bagotsky, 2005; Wang, 2006). It consists of small pulses of constant magnitude, superimposed like in SWV on a staircase-waveform. Once more, the current is sampled twice, just before the pulse application and then in the pulse life when the capacitive current has decayed (Skoog et al., 1997; Wang, 2006). The electrode signal, actually the difference between the currents measured for each single pulse consists of peaks the height of which is directly proportional to the concentration of the corresponding analyte. The widespread application of DPV is also due the peak-shaped of voltammograms which results in improved resolution between two species with similar redox potentials as peaks separated by 50 mV may be measured (Wang, 2006). Some recent applications of the technique for the fabrication of sensors include the use of carbon paste electrodes for the detection of either dinitrophenolic

herbicides (Sreedhar et al., 2003) or neonicotinoid insecticides (Papp et al., 2010). Differential pulse voltammetric determination of herbicides at a clay-modified electrode has been also reported by Manisankar et al. (2004a,b; 2005a).

2.3.3 Differential pulse polarography (DPP)

DPP is a subclass of voltammetry in which the operational principles of DPV are applied using either a dropping mercury electrode or a static mercury drop electrode as indicator electrode. Although mercury is highly toxic and thus less present in electrochemistry laboratories, DPP remains nowadays a useful analytical technique for pesticides analysis, for two main reasons: (i) various functional groups (i.e. carbonyl, nitro, disulfide, azo, quinone) present in pesticide molecules are reducible at mercury electrodes which display wide cathodic ranges (surtension), (ii) many organic and inorganic compounds tend to be adsorbed at mercury electrode; this fact can be favourably exploited for their accumulation prior to their detection. Thus, Sreedhar et al. (1997) reported the utilization of DPP for voltammetric sensing of three fungicides; namely folfet, phosmet and dialifos that contain carbonyl group. In this study, both standard addition and calibration methods were used to determine folfet and phosmet at nanomolar level. Castanho et al. (2003) developed a differential pulse polarographic method to detect methylparathion in water and soil suspensions. Their method allowed the recording of the sorption isotherms of the pesticides on three Brazilian soils. Another recent relevant work was reported by Balaji et al. (2010a) who described a method to study the voltammetric behaviour of fluazinam, a dinitropesticide. This method was successfully employed for the detection of the pesticide in various environmental matrices. These authors also developed a differential pulse polarographic method to determine fluochloralin in formulations, grains, soils and spiked water samples (Balaji et al., 2010b).

These studies reveal that pulse voltammetric techniques are particularly designed for measuring very low levels of pesticides in the environment, mainly when they are used in connection with modified electrodes as we will also discuss in this chapter. Taken together, SW and DP voltammetries are commonly exploited via adsorptive stripping mode where traces of pollutants are accumulated by adsorption. This approach offers improvement in selectivity and sensitivity. For this to be achieved, the active surface of solid electrode substrate (glassy carbon, platinum, SnO_2 for example) is modified by a compound or a material exhibiting some chemical affinity towards the target analyte (Walcarius et al., 2003; Tonle et al., 2005). These modified electrodes, engineered to respond selectivity and sensitivity to pesticides are focussed on electrochemical sensors and biosensors.

3. Electrochemical sensors based on chemically modified electrodes

During the past years, analytical systems based on modified electrodes have been developed, aiming at providing researchers with efficient amperometric sensors of diverse nature. These electrodes which combine the individual and specific properties of each of their components can be classified on the basis of their physical and chemical configurations. Hereafter will be described the major chemically modified electrodes used for the detection of pesticides. The most described chemically modified electrodes in the field of pesticide control include carbon nanotubes modified electrodes, carbon paste electrodes, clay film-modified electrodes and immobilized enzyme electrodes.

3.1 Carbon nanotubes modified electrodes

During the last years, several research teams have exploited either single-wall carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) to build amperometric sensors by means of modified electrodes. Yet, CNTs are of special interest due to their inherent electronic, metallic and structural properties (Ajayan, 1999), and can be directly used as electrode modifier, or in combination with another component dotted with attractive properties. MWCNTs served as excellent electrode material on a chitosan matrix for the sensitive detection of triazophos pesticide (Du et al., 2007). Some other works have been reported in which MWCNTs were combined with conductive polymers to form composite structures which then associated the properties of both components: the well-known electrocatalytic and adsorption characteristics of MWCNTs, and the bound active surface functionalities of conductive polymers. Following the same line, an efficient sensor was obtained by covering a MWCNT modified glassy carbon electrode with polypyrrole and polyaniline, and used to electrochemically reduce isoproturon, voltage and difocol (Manisankar et al., 2008). More recently, a viable nanocomposite platform formed by a basal plane pyrolytic carbon electrode nanostructured with Ni(II)-phtalocyanine and MWCNTs has been shown to exhibit sensitive electrocatalytic properties towards the detection of asulam pesticide (Siswana et al., 2010). Two years before, these authors described the electrocatalytic detection of amitrole on MWCNTs using the basal plane pyrolytic graphite electrode modified by a Fe(II) tetra-aminophtalocyanine (Siswana et al., 2008). The results show that CNTs can lead to a widespread range of electrochemical sensors for the detection of pesticide contaminates.

3.2 Carbon paste electrodes (CPEs)

CPEs, invented by Adams (1958) are formed by a mixture of carbon (graphite) powder, a pasting liquid (binder) and usually a third component (in solid state) whose behaviour is to be studied or which displays favourable interactions towards a target analyte. Recently, a well documented review was published on the electrochemistry and electroanalytical applications of CPEs, on the occasion of the half-of-century anniversary of their discovery (Svancara *et al*., 2009). Undoubtedly, CPE remains a powerful and useful tool with a wide diversity of applications, especially for the building of low-cost and sensitive sensors for the detection of extremely low concentration of electroactive species (the lowest limits of detection achieved at CPEs lie between 10^{-11} and 10^{-16} M, as summarized in the review by Svancara et al. (2009)). These applications are closely related to some of their typical properties such as low background currents, long-time stability, high polarization limits (both in anodic and cathodic directions), easy fabrication and comfortable renewal of the paste (Svegl & Ogorevc, 2000; Svancara et al., 2009).

During the past decade, CPEs have been shown as attractive tools for the detection of pesticides. A mixed-ion fluorohectorite heterostructure incorporated in a CPE was used as an electrochemical sensor for the detection of the herbicide 2,4-dichlorophenoxyacetic acid, reaching concentrations up to 20 μM (Ozkan et al., 2002). A highly sensitive amperometric method based on a ferophtalocyanine chemically modified CPE coupled with enzymes co-immobilized onto the surface of a dialysis membrane was developed for the detection of organophosphorous pesticides (Anton et al., 2003). This work opened the way to the construction of an amperometric biosensor for paraoxon and carbofuran pesticides detected at concentrations up to 10^{-10} M. Using a bare CPE and a sepiolite (clay material) modified CPE, Sreedhar et al. (2003) described a sensitive adsorptive stripping voltammetric method

for the determination of dinoseb and dinoterb herbicides. More recently, Papp et al. (2010) performed the voltammetric investigation of thiamethoxam (a neonicotinoid insecticide) using a tricresylphosphate-based CPE. The so-developed method allowed a direct determination of the insecticide in a river water samples. One could also cite a relevant work by Parham & Rahbar (2010) who quantitatively detected methylparathion in water samples by the means of a ZrO_2-nanoparticles modified CPE.
As demonstrated by these examples, CPE remain actually a very useful tool for the electroanalytical detection of pesticides. Thus, new electrochemical sensors could be favourably tailored for a specific analytical task, depending on the nature and the chemical reactivity towards the pesticide, of the compound added to the matrix formed by carbon particles and the binder.

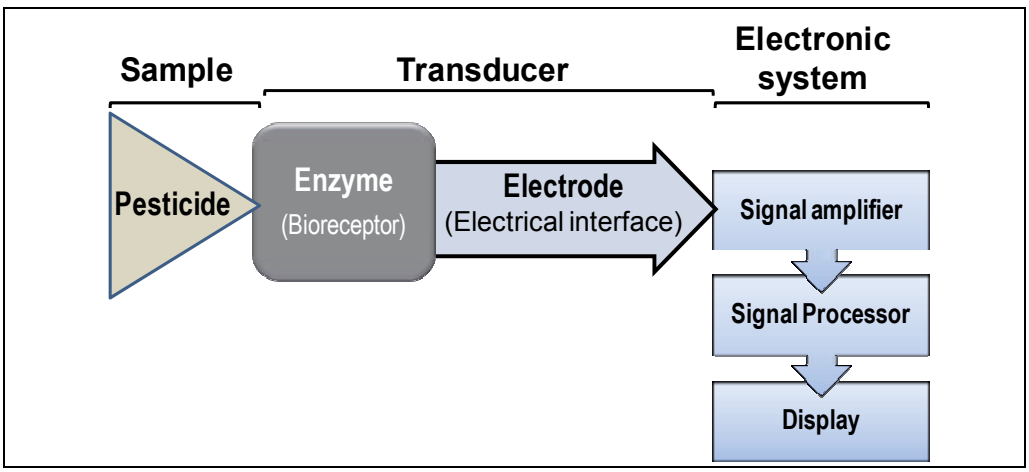
3.3 Clay film-modified electrodes (CFMEs)

CFMEs are obtained by covering the surface of conductive solid substrates (glassy carbon, Au, Pt, SnO_2 or pyrolitic graphite) by a thin membrane of clay. They were first introduced by Ghosh and Bard (1983) to elucidate charge phenomena in the film, and the effect of the film structure on the mobility of the electrochemical probe to the electrode surface (Naegeli et al., 1986; Subramanian & Fitch, 1992; Fitch et al., 1996). This particular type of electrodes has been actively investigated for its potential applications in electrocatalysis (Premkumar & Ramaraj, 1997; Moronta, 2004; Paramasivam & Anandhan, 2005), in the electroanalysis of organic and inorganic compounds (Ballarin et al, 2000; Jieumboue et al., 2009, Tonle et al., 2009) and in biochemical analysis (Coche-Guerente et al. 1998; Mbouguen et al. 2007). It has been shown that the diffusion of an electroactive species within the film at a CFME depends on several factors such as the pore structure of the film, the size of the probe, its concentration, the ionic strength and the composition of the supporting electrolyte (Naegeli et al., 1986; Fitch et al. 1996). Two main approaches are usually used to build CFMEs: (i) the spin-coating of a colloidal suspension enriched with clay particles, (ii) the drop-coating on the active surface of the electrode of the clay suspension, both followed by drying either in air or in an oven at a given temperature. As stated by Navratilova & Kula (2003), the way of drying, the type of clay and the size of clay particles are crucial factors for obtaining a film with required stability, uniformity and thickness.
The high adsorption power of clays and clay minerals, combined with their particular colloidal properties allowed their exploitation as suitable modifiers for the elaboration of clay film-modified electrodes used to analyze pesticides. In such electrodes, pesticide molecules containing functional polar groups are directly bound to negatively charged smectite-type clay particles either by electrostatic interactions or by an ion exchange process. Yet, smectites are characterized by internal surfaces accessible by polar organic molecules and by flexible adsorptive properties (Villemure & Bard, 1990). As smectites can act as carrier of organic contaminants, sensitive voltammetric methods were developed by several authors. Manisankar et al. (2004b) studied the redox behaviour of endosulfan and orthochlorophenol using a sodium montmorillonite clay-modified glassy carbon electrode (GCE), and demonstrated the suitability of the modified electrode for the determination of these pesticides by means of differential pulse stripping voltammetry. The same research team reported the development of an electrochemical trace determination method for isoproturon and carbendazim in soil and water spiked samples, using once more a sodium montmorillonite clay-modified GCE, in the presence and in the absence of cetyltrimethylammonium bromide (CTAB) surfactant (Manisankar et al., 2005b). Following

these lines, they showed that the presence of CTAB at a heteropolyacid montmorillonite clay-modified GCE enhanced the electrode response and hence the stripping voltammetric determination of isoproturon (ISO), carbendazim (CAR) and methylparathion (MP), leading to low limits of detection (1, 10 and 20 ng mL^{-1} for ISO, CAR and MP respectively; Manisankar et al., 2006). Significant advances have also been noticed in the field of CFMEs via the use as electrode modifiers of organoclays issued from the intercalation of cationic surfactants in the interlayer space of montmorillonite. In fact, the presence of surfactant within the clay gallery changes the inherent properties of the latter from hydrophilic to organophilic, leading thereby to a hybrid material useful for the uptake of organic pollutants. Thus, Tcheumi et al. (2010) exploited a gemini surfactant intercalated montmorillonite complex to efficiently accumulate methylparathion pesticide. By increasing the concentration of the dimeric surfactant in the clay, they observed the enhancement of the electrode response due to the hydrophobic character of the organoclay and to favourable hydrophobic-type interactions towards MP. This modified electrode allowed the stripping determination of methylparathion with a detection limit of 7×10^{-8} M.

3.4 Immobilized enzyme electrodes

Electrochemical biosensors have attracted much attention over the last two decades for environmental chemistry. A biosensor can be defined as an analytical device which tightly converts a biological response to a quantifiable and processable signal (Grieshaber et al., 2008). The basic principles and architectures of electrochemical biosensors have been reviewed by several authors (Pohanka et al., 2001; Mulchandani et al., 2001; Mostafa, 2010). A typical biosensor for pesticides detection is formed by three main components: the bioreceptor (enzyme) coupled to an electrochemical interface (electrode), and an electronic system designed to convert the biological response from the electrode into a quantifiable and processable signal (Scheme 2). The steps involved in the analysis process, described in detail by Davis et al. (2007) are summarized as follows: once the electrode is exposed to a pesticide solution, an interaction occurs between the pesticide and the enzyme, diminishing or destroying completely its activity.



Scheme 2. Major components of a typical biosensor for pesticide detection

This inhibition can then be easily quantified by further exposure to the initial substrate concentration and comparison with the response prior to pesticide exposure.
Numerous works have been undertaken during the last couple of decades on the exploitation of electrochemical biosensors for pesticides monitoring, and some of the best limits of detection (LOD) achieved are summarized in Table 1.

Pesticide	*Bioreceptor	Technique	Limit of detection	Reference
Parathion	PH	Amperometry	1 ng L^{-1}	Sacks et al., 2000
Parathion Paroxon	PH	Amperometry	15 nM 20 nM	Chough et al., 2002
Parathion Carbaryl	AChE	Cyclic voltammetry	**9.0– 0.3 µg L^{-1}	Pedrosa et al., 2008
Carbaryl	AChE	Amperometry	5 x 10^{-5} M	Josiane & Sergio, 2008
Paraoxon Parathion	OPH	Potentiometry	2 x 10^{-6} M	Schöning et al., 2003
Paraoxon Methylparathion	OPH	Amperometry	9 x 10^{-8} M 7 x 10^{-8} M	Mulchandani et al., 2001
Carbofuran Paraoxon	AChE BuChE	Amperometry	0.2 nM 0.6 nM	Albareda et al., 2001
Carbofuran Dichlorvos	ChO ChE	Amperometry	0.01 ppb 1.3 x 10^{-3} ppb	Snejdarkova et al., 2003
Carbofuran	AChE	Amperometry	1 nM	Olga & Jon, 2007
Carbaryl Carbofuran	AChE ChO	Amperometry	2 µg mL^{-1}	Palchetti et al., 1997
Methylparathion Diazinon Carbofuran Carboy	Tyr	Amperometry	6 ppb 19 ppb 5 ppb 10 ppb	Yaico et al., 2007
Diazinon Dichlorvos	Tyr	Amperometry	5 µM 75 µM	Everett & Reichnitz, 1998
Ziram Diram	Tyr	Amperometry	0.074 µM 1.3 µM	Perez-Pita et al., 1997
Ethylparaoxon	AsO	Amperometry	1 ppm	Rekha et al., 2000

*See section 6 for abbreviations - **The detection range is for both pesticides

Table 1. Description of some enzymatic biosensors used for direct detection of pesticides

The detection of pesticides at low levels can't easily be achieved by direct detection of the pesticide itself, but rather by the detection of its inhibitory effects on enzyme reaction (Davis et al., 2007). However, Schöning et al. (2003) realized a miniaturized enzyme biosensor for the direct determination of organophosphorus pesticides using an organophosphorus hydrolase modified semiconductor. This sensor showed certain selectivity and stability as it allowed the discrimination between organophosphorus and other pesticides, requiring a single-step and showing a long period of stability. A wide range of immobilization techniques were investigated on mutant acetylcholinesterase enzymes, giving rise to

sensitive and selective sensors which were used to measure concentrations of several enzymes down to levels hitherto undetectable (Davis et al., 2007).
One could also mention a quite simple method proposed by Pedrosa et al. (2008) for the detection of parathion and carbaryl in natural waters and in food samples using a self-assembled monolayer (SAM) acethycholinesterase electrochemical biosensor. From this investigation, the usefulness of the SAM for the fabrication of the biosensor was demonstrated as its deposition on the gold electrode used as electrical interface did not inhibit the electron transfer process between this gold electrode and the electrolyte. Despite these relevant works, the lack of selectivity in most cases is to be mentioned. In fact, immobilized enzymes are often inhibited by many chemicals such as heavy metals and other electroactive species likely to coexist in the matrix to be analysed. Additionally, multi-step protocols are required for analysis with inhibition based sensors, limiting thereby their application for on-site monitoring (Schöning et al., 2003). At present, there are several types of electrochemical biosensors exploited for the recognition followed by the quantification of pesticides. The works exposed in this section have shown that the construction of new and efficient biosensors could be expected by different immobilization strategies taking into consideration the nature of the transducer material, the long-term stability and the selectivity of the final device.

4. Practical considerations and validation of voltammetric methods

The first thing to be considered for an electrochemical experiment is the availability of equipments. The required basic instruments includes an electrochemical cell (in a three electrode configuration in most cases), the voltamperometric analyser formed by a potentiostat (main component) coupled to a computer in modern tools. Some other specific components such as a faradaic cage or a deoxygenating gas tube could be added when required, depending on the exigencies of the experiment.
Nowadays, a large number of voltammetric analysers are commercially supplied at relatively modest prices, so that from 5000 € (in 2011) one can intend to perform experiments on a voltammetric system interfaced to a computer. For more information on modern and miniaturised instrumentation, one can consult the current suppliers websites presented in Table 2.
After the instrumentation, some practical criteria to be taken into consideration for a voltammetric method designed for environmental monitoring are closely related to selectivity, sensitivity and accuracy. For sensors, the stability, the reproducibility and the cost of the global sensing procedure are some key parameters to be properly evaluated.
Table 3 summarizes some low detection limits achieved at carbon paste and film electrodes chemically modified within the past two decades. For practical guidance, the electrode configuration and the nature of the supporting electrolyte are provided.
The characteristics of sensors are hereafter summarized according to definitions formulated by the Analytical Chemistry Division of the International Union of Pure and Applied Chemistry (Kutner et al., 1998).
Concerning the selectivity and sensitivity, the modifier agent for biosensors and modified electrodes should be conveniently chosen on the basis of selective and favourable interactions towards the analyte, improving thereby the device selectivity. Moreover, the detection limit should be sufficiently low for the purpose envisaged.

Supplier name	Website
Radiometer analytical (France)	www.radiometer-analytical.com
BASi (USA)	www.BASInc.com
EG&G PAR (USA)	www.egg.inc.com/par
BioLogic (USA)	www.bio-logic.us
Eco Chemie (The Netherlands)	www.brinkmann.com
Solartron (USA)	www.solartron.com
Palm Instruments (The Netherlands)	www. Palmsens.com
CH-Instruments (USA)	www.chinstruments.com
Dropsens (Spain)	www.dropsens.com

Table 2. Some common electrochemical instrumentation suppliers

*Electrode configuration	*Supporting electrolyte	Analyte	LOD	Reference
CPEs modified with a ferophtalocyanine and immobilized enzyme	50 mM PB (pH 7.5)	Paraoxon Carbofuran	10^{-10} M	Anton et al., 2003
CPE modified with tricresylphosphate	BRB (pH 7.0)	Thiamethoxam	7.29 μg mL^{-1}	Papp et al., 2010
CPE modified with sepiolite (clay)	BRB (pH 4.0)	Dinoseb Dinoterb	10^{-10} M 5.4 x 10^{-10} M	Sreedhar et al., 2003
Blank CPE	0.2 M AB (pH 5.2)	Methylparathion	0.05 μM L^{-1}	Liu & Lin, 2005
CPE modified with ZrO_2 nanoparticles	AB (pH 5.25)	Methylparathion	2 ng mL^{-1}	Parham & Rahbar, 2010
CPE modified by amberlite XAD-2 resin	0.1 M AB (pH 6)	Paraquat	0.1 mg L^{-1}	Alvarez et al., 1992
CPE covered by a enzymatic membrane	PB (pH 7.3)	Heptenophos	0.3 mg L^{-1}	Skladal, 1992
GCE modified by PEDOT*	C_2H_5OH/H_2O (50%, v/v) of 0.1 M H_2SO_4/0.1 M KOH or BRB	Dicofol Cypermethrin Monocrotophos Chlorpyrifos Phosalone	11.20 mg L^{-1} 2.50 μg L^{-1} 2.23 μg L^{-1} 305 μg L^{-1} 1.80 μg L^{-1}	Manisankar et al., 2005a
GCE covered by a thin film of sodium MMT	0.1 M H_2SO_4 in C_2H_5OH/H_2O (50%, v/v) /0.1 M KOH / 0.1 M KCl or BRB	Carbendazim Isoproturon	5 ng mL^{-1} 1 ng mL^{-1}	Manisankar et al., 2005b
GCE covered by a film of heteropolyacid MMT	0.1 M H_2SO_4 in C_2H_5OH/H_2O (50%, v/v)	Isoproturon Carbendazim Methylparathion	1 ng mL^{-1} 10 ng mL^{-1} 20 ng mL^{-1}	Manisankar et al., 2006
MWCNT-GCE PAN-MWCNT-GCE PPY-MWCNT-GCE	C_2H_5OH/H_2O (50%, v/v) of 0.1 M H_2SO_4/0.1 M NaOH or BRB	Isoproturon Voltage (RS) Dicofol	0.1 μg L^{-1} 0.01 μg L^{-1} 0.05 μg L^{-1}	Manisankar et al., 2008
GCE modified by a dimeric surfactant intercalated MMT	0.1 M AB (pH 5)	Methylparathion	7 x 10^{-8} M	Tcheumi et al., 2010
Fe-TAP-MWCNTP-PGE	0.1 M PB (pH 12) + 0.05 M Na_2SO_4	Amitrole	0.5 nM	Siswana et al., 2008
Graphite covered by PTFE	PB (pH 7.4)	Thiram	12.9 μg L^{-1}	Fernandez et al., 1995
pNITSPc/Nafion-CFME	0.2 M AB (pH 5.2)	Methylparathion	0.1 mg mL^{-1}	Sbai et al., 2007
pNITSPc/p-PPD-CFME	0.2 M AB (pH 5.2)	Methylparathion	40 μg L^{-1}	Tapsoba et al., 2009

*See section 6 for abbreviations.

Table 3. Some best detection limits achieved within the two past decades on carbon paste and film coated electrodes for pesticide analysis.

The linear concentration range should be as large as possible, and the calibration highly reproducible. The modification procedure leading to the chemically modified electrode or to the biosensor should be easy to perform; for biosensor especially, the immobilization of the bioreagent should be permanent whilst the enzyme activity is kept free from deterioration and devaluation. The measurements should be sufficiently reliable and repeatable.

5. Future trends in pesticides control by voltammetric methods

In this chapter, the role voltammetric methods play for the monitoring of pesticides both in environment and food has been examined. This was done by reviewing momentary various methods and their possible combinations leading to efficient tools for the electroanalysis of pesticide molecules. Key learnings on the electrochemical sensors were discussed, and the summarizing of the recent advances demonstrates that the sensors up-to-date described offer high sensitivity and fast analysis, and can be used under field conditions. As proven by the number of applications reviewed, it appears that voltammetric methods are extensively applied for the detection of pesticides in various matrices. Moreover, voltammetry constitutes an alternative to chromatographic and spectroscopic methods usually used for the determination of pesticides.

Concerning the monitoring of pesticides, future trends will be the development of innovative technology for miniaturised and disposable tools combining the following features: stability, high selectivity, highest accuracy and low detection limits. These tools may also meet the requirements for on field detection and real-time analysis by non specialised operators. Thus, some key areas offering great promise in the elaboration of new electroanalytical strategies may be properly developed, which include new electrode configurations, miniaturized devices and the preparation of new nanocomposite materials.

5.1 New electrode configurations

In order to improve the sensing ability of voltammetric techniques, significant efforts are being made nowadays. The miniaturization of working electrodes has led to the emergence of microelectrodes that are electrodes with at least one dimension not greater than 25 μm (Wang, 2006). In comparison with conventional working electrodes, such electrodes present several advantages: their small size allows their use in the study of reactions and electrodic processes in lower conductivity solvents and in the absence of the supporting electrolyte, thereby minimizing the cost of sample manipulations. Moreover, microelectrodes can be used to explore microscopic domains, to measure local concentration profiles (in vivo analysis) whilst offering lowered limits of detection in microflow systems.

A wide operability of microelectrodes was demonstrated by de Souza et al. who reported the use of gold microelectrode and square wave voltammetry for the analytical determination of low concentration of dichlorvos pesticide in pure and natural water samples (2005a) in one hand, and trace amounts of paraquat in lemon and orange juice samples (2005b), in natural water, food and beverages (2006) in the other hand. Experiments were performed without any pre-purification or preparation step of the samples.

Sbai at al. (2007) reported for the first time the use of a carbon fiber microelectrode modified with combinations of nickel(II) tetrasulfonated phtalocyanine and nafion films for a rapid detection of methylparathion pesticide. The advantage of nickel complexes-based associated to nafion coating as new electrode material was thus demonstrated. That work was further extended by Tapsoba et al. (2009) who exploited the same nickel(II) complex but now

electroformed, in combination with a para-phenylenediamine electropolymerized coating to detect methylparathion for concentrations up to 40 μg/L. Taking in to advantage the practical applicability of wall-jet electrode in stripping analysis, that is maintaining under well-developed convection-diffusion conditions the flux of electroactive analyte to the working electrode; Manisankar et al. (2005a) studied the electrochemical behaviour of some pesticides on a poly 3,4-ethylenedioxythiophene modified wall-jet electrode. These few examples reveal the possibility to exploit unmodified or chemically modified microelectrodes for the construction of sensitive devices for pesticide monitoring.

5.2 Miniaturized devices

The development of miniaturized and disposable voltammetric sensors is undoubtedly a challenge for coming research work devoted to environmental and food control. Although the size of a sensor is not so important for environmental monitoring, the growing tendency towards miniaturization is observed nowadays for several reasons: (i) research laboratories need to reduce the amount of used chemicals which have an impact not only on the environment (secondary pollution) but also on the economy (Suziki, 2000; Rodriguez-Mozaz et al., 2004); (ii) inexpensive, easy-to-handle and small size sensors are suitable for on-field screening applications; (iii) the need to minimize sample pre-treatment in order to reduce analysis time and allow probing of natural speciation (Brett, 2001).

Biosensing is extremely important for pesticides control; furtunately, electrochemical biosensors do not require heavy and sophisticated equipment for the signal processing (Pijanowska & Torbicz, 2005). In addition, biosensors are easy to calibrate. Hence, miniaturized biosensors able to provide results in real time are regarded as cost-effective and portable analytical tools. In this sense, disposable carbon paste electrodes with embedded enzyme, screen-printed electrodes covered by a pesticide recognition compound are also potential options for the elaboration of sensitive working electrodes. Screen-printed electrodes based on thick-film hybrid technology offer a number of advantages: they are convenient for works on microvolumes and for decentralized assays. The feasibility of this particular aspect has been proven by Suprun et al. (2005) who reported on the use of a screen-printed carbon electrode for the detection of aldicarb, paraoxon and methylparathion

5.3 New electrode materials for biosensors

The preparation of nanomaterials-modified electrochemical transducers could be extended, based for example on the conjugation of adsorptive carbon nanotubes and electroactive polymers on which enzymes as organophosphatase should be immobilized for the detection of organophosphorus pesticides. Such a combination may improve the electrochemical properties of transducers: fast electron transfer or high signal to noise ratio along with sensitivity and selectivity. Some key reviews on the nanotechnology immobilization of enzymes for the elaboration of biosensors have been published (Merkoci & Alegret, 2005; Davis et al., 2007; Kumar, 2007; Merkoci, 2009; Stoytcheva et al., 2011) that demonstrate the interest of several nanomaterial-enzyme configurations.

6. List of abbreviations

AB: Acetate buffer
AChE: Acetylcholine esterase
ADH: Aldehyde deshydrogenase

AsO: Ascorbate oxydase
BRB: Britton Robinson buffer
BuChE: butyrylthiocholine esterase
CFME: Carbon fiber microelectrode
ChE: Choline esterase
ChO: Choline oxydase
CPE: Carbon paste electrode
Fe-TAP-MWCNTP-PGE: Iron(II) tetra-aminophtalocyanine electropolymerized onto a MWCNT-modified basal plane pyrolytic graphite electrode
GCE: Glassy carbon electrode
MMT: Montmorillonite
MWCNT: Multiwalled carbon nanotubes
MWCNT-GCE: MWCNT film coated GCE
OPH: Organophosphorus hydrolase
PAN: Polyaniline
PAN-MWCNT-GCE: PAN electrodeposited on MWCNT-GCE
PB: Phosphate buffer
PEDOT: Poly 3,4-ethylenedioxythiophene
PH: Parathion hydrolase
pNITSPc/Nafion-CFME: Polytetrasulfonated phtalocyanine/nafion film coated on a CFME
pNITSPc/p-PPD-CFME: Polytetrasulfonated phtalocyanine/*para*-phenylenediamine film coated on a CFME
PPY: Polypyrrole
PPY-MWCNT-GCE: PPY electrodeposited on MWCNT-GCE
PTFE: Polytetrafluoroethylene
Tyr: Tyrosinase

7. Conclusion

A description of the state of art and a global overview of voltammetric methods currently developed and applied for the determination of pesticides residues in real environmental matrices and food were provided. By a comparative approach, the usefulness of voltammetry and its advantages towards classical methods of pesticides analysis were also demonstrated. A focus has been given to practical considerations, as pesticides form a wide range of compounds, the electrochemical behavior of each of them being strongly related to its physicochemical properties. The constitution, the preparation methods and the specific features of chemically modified electrodes that have found numerous important applications for the detection of pesticides during the past two decades were also presented, and a brief discussion on future trends in the electroanalysis of pesticides using non conventional electrodes discussed.

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The Potential of Flow-Based Optosensing Devices for Pesticide Assessment

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1. Introduction

The rise of intensive agriculture in the last decades has originated the massive use of pesticides (herbicides, fungicides or insecticides), which has become a serious environmental problem and a potential risk for human health. Pesticides residues can be often found in soils, natural waters, atmosphere and agricultural products and cause adverse effects on humans, plants, animals and ecosystems, even at low concentration levels. In order to guarantee consumer safety and regulate international trade, Maximum Residue Limits (MRLs) for pesticides in foodstuffs have been established by several Government agencies and European Union Commission (European Union, 2005).

Therefore, nowadays the development of new analytical methodologies capable of determining trace levels of pesticides in the environment is one of the most important tasks in analytical science. The determination of pesticide residues in food matrices is a formidable challenge mainly because of the small quantities of analytes and large amounts of interfering substances which can be co-extracted with them and, in most cases, adversely affect the results of an analysis (Wilkowska & Biziuk, 2011). Pesticide analysis in food samples has been usually carried out by means of multi-residue methods that use gas chromatography (GC) as the preferred technique because many of these compounds are low polar, volatile and thermally stable (Guan et al., 2010; Hunter et al., 2010). Nevertheless, new pesticides, which show a more specific mode of action and have a higher polarity and lower persistence than old ones, have been developed in the last years. Most of these novel compounds can be conveniently separated by high-performance liquid chromatography (HPLC) (Fu et al., 2009; Soler et al., 2008; Wu et al., 2002). Currently, pesticides comprise more than 1200 active ingredients, which are formulated in thousand of different commercial products (Ahmed, 2001). Since they present very different physic-chemical characteristics and large differences in volatility, polarity and persistence, both GC and HPLC coupled to mass spectrometry detection (GC-MS, HPLC-MS) are used as complementary techniques in pesticide analysis (Garrido et al., 2005; Pang et al., 2006).

Nevertheless, although pesticide residue analysis methodologies in different matrices by GC and/or HPLC are well established, there is still a need for sensitive, faster, easy-to-use and cost effective procedures as real and practical alternatives to the robust and efficient chromatographic methods. These procedures would allow the rapid detection of pesticides, being used for a preliminary screening in laboratories where a large number of samples have to be processed in a short time. In response to this need, several spectroscopic

methods, based on the use of a solid phase, have been developed in the last years, due to their inherent features as selectivity, sensitivity and low cost of analysis. This methodology, called solid-phase spectrometry (SPS) (Matsuoka & Yoshimura, 2010), can be performed in batch mode (M.L. Fernández et al., 1998; Richter et al., 2002) or in automatic mode (López et al., 2007a; Traviesa et al., 2004). In both of them, the species of interest (analyte or a derivate) is retained on an appropriate solid support and the direct measurement of the light absorption or emission of this latter is carried out. Sensitivity and selectivity are the two most remarkable analytical features of this methodology. This is due to the separation of the species of interest from the sample matrix and its preconcentration on the solid support, that is, in the zone itself where it will be measured with a non-destructive molecular spectroscopic detector.

The combination of SPS with flow-injection analysis (FIA), the so called flow-through optosensors (Ruzicka & Hansen, 1985), combines the advantages of both methodologies, simplicity, rapidity, sensitivity, selectivity and low cost. In this kind of systems the separation and retention of the species of interest on the solid phase take place in the detection area itself and simultaneously with it. Therefore, these systems integrate, in the space and time, several analytical processes, separation, preconcentration and detection. It is necessary to clarify that flow-through optosensors combine flow-injection techniques with detection on an optically active surface packed in a flow-cell and, consequently, the analytical signal is measured directly on the solid support. Nevertheless, flow systems using a solid support which is not packed into a flow-cell have also been reported for the determination of pesticides. They are usually based on the use of a biological recognition element which is immobilized on a solid support placed into an immunoreactor (biosensors), and the measurement is carried out in solution after the elution of the monitored species from the solid surface. Sometimes, they are also called flow-through optosensors in literature although, according to the first definition of these systems (Ruzicka & Hansen, 1985), this denomination is not appropriate. They will be also reviewed here, but in a minor depth than flow-through optosensors.

2. Flow methodologies in optosensors

The basic components of any flow system include: propelling unit, device/s for the introduction of solutions into the system, flow-through cell and detector. To date, four different flow methodologies have been used in the development of optosensors for the determination of pesticides, which will be here described (Table 1).

2.1 Flow-injection analysis (FIA)

A FIA system includes a peristaltic pump to propel the solutions, a series of plastic tubes to transport them, injection valves to introduce constant volumes of the solutions in the system, a flow-cell and the detector. The valves usually employed are six-way rotary valves, which are manually-controlled and allow the insertion of a defined volume of solution in the system. The main advantages of a conventional FIA system are high throughput, repeatability, versatility and automation. This was the first flow methodology used in the development of optosensors for the analysis of pesticides (Agudo et al., 1993) and the most frequently used one in flow-based biosensors (Roda et al., 1994; Varsamis et al., 2008) and chemical optosensors (Badía & Díaz, 1999; Piccirilli & Escandar, 2009).

2.2 Sequential-injection analysis (SIA)

SIA was introduced as a following generation in the development of FIA, with the aim of improving automation and versatility of FIA systems. The key components of a SIA manifold are a multi-position valve and a bi-directional syringe pump (both automatically controlled by a computer). Sample and reagents are introduced in a holding coil before being impelled by the piston of the pump through a mixing coil to the detector. The multi-position valve avoids the use of additional valves, such as in conventional FIA systems, in which several valves are needed. The great advantages of this technique are an important saving in samples and reagents, robustness and versatility, whereas a lower analysis rate and complicated software are the main drawbacks. To date, SIA has been applied only to the development of immunosensors in pesticide assessment (González et al., 2005; Herranz et al., 2008).

2.3 Multicommutation flow-injection analysis (MCFIA)

In MCFIA the sample injector used in FIA is replaced by three-way solenoid commutation valves. Each solenoid valve acts as an independent switch and the whole system is automatically controlled by a computer. These valves are similar to an electronic circuit, with a variable number of active nodes, and present two different positions: "ON" and "OFF", allowing the effective control of sample and reagents dispersion and widening the scope of applications in flow analysis. Compared to conventional FIA, the injected volume can also be adjusted by controlling the commutation timing via software. Hence, the high repeatability in multicommutation systems is associated with the precision in time measurement. In addition, as sample and reagents are injected into the flow system only when necessary, the consumption rate and waste generation can be minimized. Recently, several MCFIA fluorescent chemical optosensors have been proposed (Llorent et al., 2007; López et al., 2007a) for determination of pesticides.

2.4 Multi-syringe flow-injection analysis (MSFIA)

This technique is based on the use of multiple syringes which are used as liquid-propelling devices. The variety of available syringes with very different capacities offers a broad selection of flow rates. The implementation of three-way solenoid valves at the head of every syringe allows the injection of precise and well-defined volumes of sample and reagents. MSFIA was introduced as an alternative to its predecessor techniques, combining manifold operation and high sample throughput of FIA together with the robustness and versatility of SIA. Peristaltic pumps are still the most common liquid-propelling drives in FIA and MCFIA. In these systems, the flexible Tygon tubing of the pumps needs to be replaced periodically. Nevertheless, the pump tubing lifetime can be eliminated in MSFIA. In addition, reagent consumption is reduced by more than 10 times in comparison with common flow injection procedures. To date, only a flow-based optosensor using MSFIA has been proposed. The system allowed the determination of one of the main degradation products of pesticides derived from naphthalene acid, 1-naphthylamine (Guzmán et al., 2006).

The introduction of SIA, MCFIA and MSFIA has provided an additional grade of automation, when comparing to FIA, due to a complete absence of human intervention during each measurement. This is due to the automatic control of the flow of solutions. In addition, it has allowed the reduction of the reagents and sample consumption, which makes these methodologies to be more suitable for routine analysis.

Flow methodology	Propulsion unit	Injection unit	Other elements
FIA	Peristaltic pump	Six-way rotary valves	-
SIA	Bidirectional syringe pump	Multi-position valves	Holding coil
MCFIA	Peristaltic pump	Computer controlled three-way solenoid valves	-
MSFIA	Multisyringe module	Computer controlled three-way solenoid valves	Holding coil. Reaction coil

Table 1. Flow methodologies used in optosensors for determination of pesticides

3. Chemical flow-based optosensors

A large number of optical chemical sensors based on light absorption or emission detection has been presented over the past 20 years for pesticide assessment. Next, the main characteristics and applications of these systems are reviewed.

3.1 Solid supports

The purpose of the solid support in flow-based optosensors is the retention of the analyte or a product generated from this latter and, consequently, its preconcentration and separation of the sample solution on a very little amount of it. Therefore, its selection mainly depends on the nature of the target species. For the selection of an appropriate solid support, it is necessary to take into account that it has to satisfy some requirements such as: (a) to be compatible with the detection system used, that is, to provide an appropriate background signal; (b) the particle size has to be large enough to avoid overpressure in the system; (c) to guarantee reproducibility in the response of the sensor; and (d) the retention/elution process of the species of interest has to be quick enough. The solid support is usually packed into a commercial flow-cell that is placed in the detection area. Sometimes, in the case of the simultaneous determination of two or three analytes or the removal of a potential interfering species, solid supports are packed into little minicolumns inserted on-line in the flow injection system.

The most frequently used solid support in flow-based optosensors for pesticides analysis has been C_{18} bonded silica gel, an adsorptive hydrophobic material, which is very appropriate for neutral species that show low solubility in water (Llorent et al., 2007; López et al., 2009a). Nevertheless, this sensing material presents an important drawback to take into account, its low selectivity because of the adsorptive nature of the retention process. Although usually this solid support has been used for filling a commercial flow-cell, a recent application proposed its use in form of extraction disks (Guzmán et al., 2006). The C_{18} extraction disks present some advantages over conventional resins, such as higher flow rates, low overpressures and better reproducibility. When using these extraction disks the detection is carried out by measuring the intensity of reflected incident light at the surface of the disk with a bifurcated optical fiber.

Polystyrene-type and gel-type ion exchangers have also been used for the retention of charged species. The first ones, constituted by a hydrophobic aromatic matrix, are usually discarded when working in UV region because of their very high background but they can

be used for Visible measurements. As an example, the use of a Dowex 50W-X8-200 cation-exchange resin for the retention of paraquat and its posterior reaction with a chromogenic reagent (Agudo et al., 1993) can be cited. With respect to gel-type exchangers, dextran polymers such as Sephadex resins have been the most widely used. Their ionic character allows the exclusion of a lot of compounds accompanying to analyte in samples. It is worth of mentioning the use of Sephadex QAE A-25 for the analysis of azoxystrobin in grapes, wine and must (López et al., 2007a). The use of this anionic sensing material allowed a high tolerance of the proposed method to the presence of other fluorescent pesticides such as carbofuran, imazalil, carbaryl and bendiocarb.

Non-ionic resins, known like macroporous polymers, have also been used sometimes, although their high background in the UV and Visible regions make them useful only for luminescence measurements. This is the case of Amberlite XAD 7 resin which was used for the phophorimetric determination of naptalam (Salinas et al., 2004).

On the other hand, a fluorescent optosensor has been proposed for the determination of warfarin based on the use of a β-cyclodextrin bonded (Cyclobond I) as sensing support (Badía & Díaz, 1999). Cyclodextrins are macrocyclic glucose oligomers in which the oligosaccharide ring forms a torus, whose outer surface has a hydrophilic character; on the contrary, the inner cavity is hydrophobic. The interest of these materials comes from their ability to bind a variety of guest molecules inside the non-polar cavity. Warfarin could be determined in waters without any previous treatment with a detection limit of 2 $\mu g\ L^{-1}$.

Recently, the use of nylon powder as a new sensing material has been investigated for the determination of thiabendazole (Piccirilli & Escandar, 2007, 2009). This material is obtained by scratching 6,6-nylon probes and sieving the resulting powder through a stainless steel strainer. Although nylon particles have heterogeneous shapes, they allow reproducible analytical signals and show good mechanical and chemical resistance. The application of this new material to the fluorescent analysis of thiabendazole (Piccirilli & Escandar, 2007) did not show any advantages when comparing to the use of other adsorbents such as C_{18} silica gel. Nevertheless, the immobilization of this fungicide on nylon powder was sufficiently efficient as to allow its phosphorescence emission and develop for the first time a phosphorimetric flow-based optosensor (Piccirilli & Escandar, 2009). A significant improvement in selectivity of thiabendazole determination was obtained, avoiding the interference originated by others fluorescent pesticides such as carbaryl, carbendazim or 1-naphthylacetic acid.

A very recent way to achieve the tailored selectivity of analytes is the use of molecularly imprinted polymers (MIPs). Molecular imprinting is usually a process of copolymerization of functional and cross-linking monomers in the presence of a template molecule. The removal of the template molecules leaves a predetermined arrangement of ligands and a tailored binding pocket (Alexander et al., 2006). Such imprinted polymers show an affinity for the template molecules over other structurally related compounds. MIPs are successful to enhance the selectivity of a luminescence optosensing system and they are considered as one of the simplest, most straightforward and cost-effective methods for developing artificial receptors for toxic organic species. The implementation of MIPs in a flow-based optosensor for analysis of pesticides was described for the first time for the sensing of two monoamine naphthalene compounds (MA-NCs), 1-naphthylamine (1-NA) and 2-naphtylamine (2-NA), considered as priority contaminants. The measurement of the room-temperature fluorescence emission of every one of them, at their respective optimal excitation and emission wavelengths and at an isoemissive point, allowed their individual and total (NA) determination, respectively (Valero

et al., 2009a), in drinking waters. Nevertheless, the interference of 1-naphthalenemethylamine (1-NMA), which was also adsorbed on these MIPs, was serious and could not be avoided. On the other hand, there was no interference of 1-naphtol and 2-naphtol, both considered as the most important potential interfering species. Later, the same authors developed a very similar MIP fluorescence optosensor for the simultaneous determination of 1-NA and 2-NA in the presence of 1-NMA without sample pre-treatment. The determination was possible by processing fluorescence data with multivariate calibration (Valero et al., 2009b).
The use of MIPs as recognition elements in chemiluminescence sensors is another promising approach for the improvement of both sensitivity and selectivity. In addition, the template molecules adsorbed can be destroyed through the chemiluminescence reaction, and the templates that have been reacted can be easily washed off using water as eluent, so avoiding the use of buffer solutions or organic solvents for MIPs regeneration purposes (Fang et al., 2009).

3.2 Flow-cells

Two aims have to be pursued in the choice of an appropriate flow-cell for the development of an optosensor. On the one hand, the target species has to be retained in an area of the solid support as small as possible, in order to obtain its highest preconcentration in the detection area and, consequently, the highest sensitivity. On the other hand, the light beam of the detector must be focused on this area without loss of light to the surrounding zones. In addition, it is necessary to take into account that the light path can not be too long since it would originate a high background signal of the solid support, which would be incompatible with the measurement.
The most appropriate commercial flow-cells for the development of flow-based optosensors are Hellma 138-OS (Fernández et al., 1991) and Hellma 176.052-QS (López et al., 2007b; Piccirilli & Escandar, 2009), for absorption and emission measurements, respectively (Figure 1). The cells are blocked in the outlet with glass wool to prevent the particle displacement by the carrier stream. Then, the solid support, as a slurry suspension, is loaded with the aid of a syringe and the inlet is kept free. The packing level of solid support in the flow cell is a key variable. It has to be filled just up to a height which enables the light beam to pass completely through the solid layer. If the solid support does not reach the optical path, the

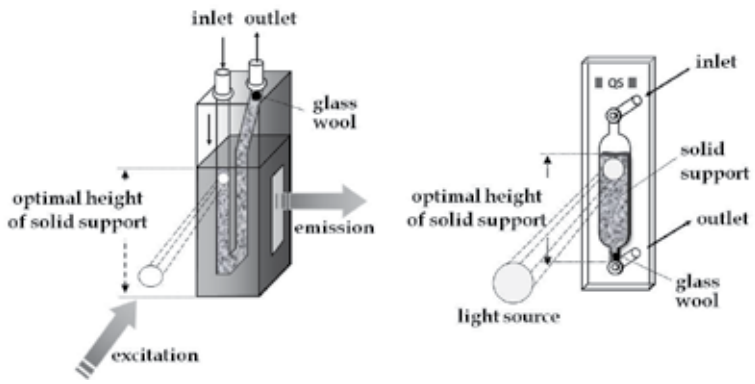

Fig. 1. Flow-cells used in chemical optosensors. (a) fluorescence and phosphorescence measurements: Hellma 176.052-QS, 25 μL inner volume, 1,5 mm optical path; (b) absorbance measurements: Hellma 138-OS, 50 μL inner volume, 1 mm optical path.

light beam passes through the solution and, consequently, a decrease in the signal is obtained. If the packing top is far above the light beam, the support area with the highest concentration of the retained species of interest would fall outside the irradiated area and so, a lower and wider signal would be obtained. Therefore, the top of the support has to be kept as close as possible to the light beam, so resulting in higher sensitivity.
A piece of V-shape colourless glass tube (5 mm x 5 cm i.d. length) has been also used for packing the solid support when using chemiluminescence detection (Fang et al., 2009). After stuffing both ends of the tube with glass wool, it was connected to the flow system and placed just in front of the photomultiplier.
In the case of using optical fiber reflectance for detection, the solid support is placed just at the end of the fiber by using home-made devices (Guzmán et al., 2006).

3.3 Regeneration of solid support

A very important and key requirement of flow-based optosensors is the successive reutilization of the solid support placed into the flow-cell for a large number of measurements, which makes them reusable. It involves the regeneration of the solid support after every injection of sample solution and analytical measurement, which can be achieved in two different ways (Figure 2): (a) the carrier solution itself acts as eluting solution, so desorbing the species monitored from the solid support after every injection of sample. This is the simplest procedure to regenerate the solid support since it is not necessary the use of an additional eluting solution. Although this procedure allows a high throughput, the transitory retention of the target species in the detection area also originates a decrease in the analytical signal obtained; (b) after every injection of sample, an eluting solution is passed through the sensing zone in order to remove the species retained on it. This is necessary when the species monitored is permanently retained on the solid support or its elution from this latter by the carrier solution itself is very slow. Sensitivity achieved is higher than in the previous case, but the use of the eluting solution provides a lower throughput and shortens the lifetime of the solid support, due to the successive compression and swelling of this latter. The introduction of the eluting solution in the flow-system can be carried out by inserting a defined volume of that or passing it through the solid support for a period of time.

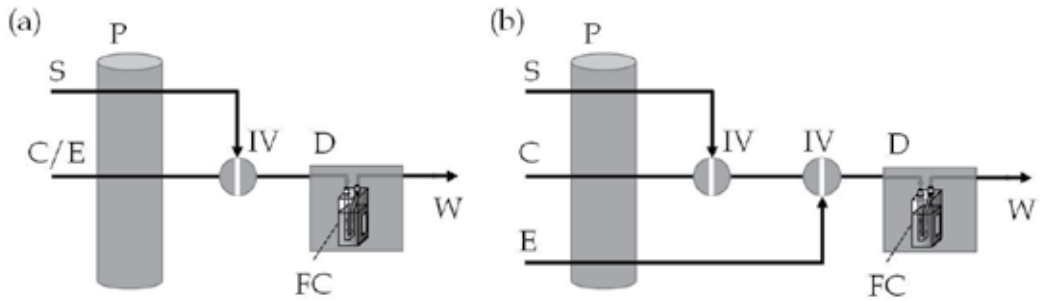

Fig. 2. Manifold configurations for regeneration of the solid support. (a) Without eluting solution; (b) With eluting solution. S, sample solution; C, carrier solution; E, eluting solution; P, peristaltic pump; IV, injection valve; D, detector; FC, flow-cell filled with solid support; W, waste

3.4 Mono-sensing optosensors

In this type of flow-based optosensors, the solid support responds only to an analyte in the sample (Table 2). The determination of this latter can be carried out with or without the previous on-line generation of a derivative product.

The direct measurement of a property of the analyte itself is the simplest approach used in the development of flow-based optosensors and it constitutes an interesting contribution to green analytical chemistry since it does not need solutions besides the carrier and (sometimes) eluting solutions. Fluorescence is the most frequently used detection technique in this type of mono-sensing optosensors, although very recently has been proposed the phosphorescence determination of thiabendazole in waters (Piccirilli & Escandar, 2009). An example of these systems is the optosensor developed for the screening of bitertanol in banana samples, in which the native fluorescence of the pesticide was monitored at 261/326 nm after its retention on C_{18} silica gel (Llorent et al., 2007). The pre-treatment of the sample was accomplished by QuEChERS methodology (Anastassiades et al., 2003) consisting of an acetonitrile extraction/partitioning and dispersive solid phase extraction (SPE) clean-up with primary secondary amine (PSA). The sensor showed good tolerance to the presence of other common pesticides such as simazine, imazalil, dimethoate or aldicarb. Nevertheless, the interference of carbofuran and carbaryl was very serious and the authors proposed two changes in the procedure in order to their elimination. The proposed method fulfilled the MRL established for bitertanol in banana by European Union, 3 mg kg^{-1}.

As fluorescence native is not a common characteristic of pesticides, different strategies have been used for the determination of non-fluorescent pesticides, such as chemical derivation or UV-irradiation. The chemical derivation of the analyte has been carried out by means of the formation of a chromogenic product which is retained on the solid support (Agudo et al., 1993; Guzmán et al., 2006) or using a chemiluminescent reaction (Fang et al., 2009). In this latter application, maleic hydrazide (MH) was selectively immobilized on a MIP filling the flow-cell. Then, luminol and potassium periodate solutions were delivered to the cell and originated strong chemiluminescence. This approach did not provide a high throughput since the measurement involved: (a) immobilization of the analyte on the MIP, (b) washing of the MIP to remove the resting sample solution, (c) delivering of chemiluminescent reagents to the flow-cell, and (d) washing of the MIP for its regeneration. Nevertheless, the MIP used as recognition material of MH allowed to detect this latter in vegetable samples without purification step.

Other interesting strategy for derivation of pesticides is photochemically-induced fluorescence (PIF) technique. It consists of the on-line generation of fluorophores from non-fluorescent or weakly-fluorescent analytes by means of an UV lamp and presents inherent advantages over ordinary chemical reactions such as quicker reaction rate, fewer chemicals involved and smaller dilution factor. The typical manifold used for this strategy is shown in Figure 3. As can be seen, the photodegradation of the pesticide is carried out by inserting a photoreactor just before the detector. This latter is constructed by coiling a PTFE tubing (0.8 mm i.d.) around a low-pressure mercury lamp (8 – 15 W, 254 nm), which is placed into an aluminium box to permit the maximum reflectance of UV light and heat dissipation. An important aspect to take into account in this type of optosensors is that the regenerating solution (carrier itself or eluting solution) has to be able to elute not only the species monitored but other products of the UV irradiation of the analyte, which possibly can remain retained on the solid support and interfere the next determination. This strategy was applied for the first time to the fluorimetric determination of the neonicotinoid insecticide

Pesticide	Principle	Solid phase / flow system	Detector	LOD (μg L^{-1})	Reference
Paraquat	reaction with dithionate	Dowex 50W-X8 / FIA	AB	0.11	Agudo & Valcárcel, 1993
1-naphthylamine	Griess reaction	C_{18} disk / MSFIA	RF	1.1	Guzmán et al., 2006
Warfarin	inclusion complex	Cyclobond I / FIA	FL	2	Badía & Díaz, 1999
Warfarin	direct measurement	Sephadex QAE A-25 / FIA	FL	4.1	Ruedas et al., 2001
Thiabendazole	direct measurement	nylon powder / FIA	FL	2.8	Piccirilli & Escandar, 2007
Carbaryl	direct measurement	MIP / FIA	FL	0.27	Sánchez et al., 2007
Bitertanol	direct measurement	C_{18} / MCFIA	FL	14[a]	Llorent et al., 2007
Diphenylamine	direct measurement	C_{18} / MCFIA	FL	60	J.F. García et al., 2005
Thiabendazole	direct measurement	C_{18} / MCFIA	FL	90[a]	J.F. García et al., 2006
Azoxystrobin	UV irradiation	Sephadex QAE A-25 / MCFIA	FL (PIF)	2.4[b] 5.4[c] 6[d]	López et al., 2007a
Imidacloprid	UV irradiation	C_{18} / FIA	FL (PIF)	1.8	López et al., 2007b
Linuron	UV-irradiation; micellar medium	C_{18} / FIA	FL (PIF)	220 130	Piccirilli et al., 2008
Metsulfuron methyl	UV-irradiation; micellar medium	C_{18} / FIA	FL (PIF)	0.14	López et al., 2009a
Thiabendazole	direct measurement	nylon powder / FIA	PH	4.5	Piccirilli & Escandar, 2009
Maleic hydrazide	reaction with luminol-KIO_4	MIP / FIA	CH	60	Fang et al., 2009

[a] μg kg^{-1}; [b] wine; [c] must; [d] grapes (μg kg^{-1}); AB: absorptiometry; RF: reflectometry; FL: fluorescence; PIF; photochemically induced fluorescence; PH: phosphorescence; CH: chemiluminescence; MCFIA: multicommutation flow-injection analysis; FIA: flow-injection analysis; MSFIA: multisyringe flow-injection analysis

Table 2. Mono-sensing flow-based chemical optosensors for pesticide assessment

imidacloprid in vegetables (López et al., 2007b). The on-line photochemical conversion of the insecticide was combined with the measurement of the generated fluorescent photoproduct when retained on C_{18} silica gel. The method was applied to the analysis of imidacloprid in peppers, river, well and irrigation waters. The detection limit obtained was comparable to those obtained using conventional chromatographic methods.

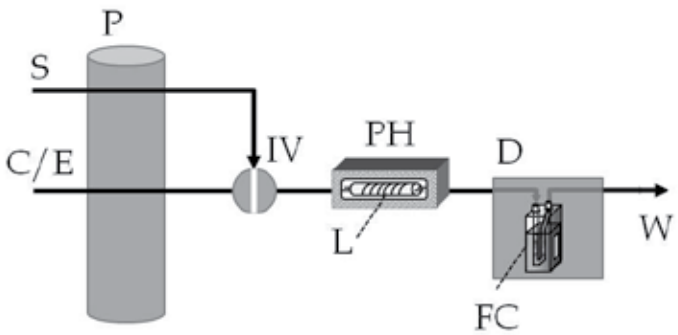

Fig. 3. Manifold configuration for photochemically-induced fluorescence (PIF) detection. S, sample solution; C/E, carrier/eluting solution; P, peristaltic pump; IV, injection valve; PH, photoreactor; L, UV lamp; D, detector; FC, flow-cell filled with solid support; W, waste

3.5 Multi-sensing optosensors

When the sensing zone of an optosensor is able to respond to more than one analyte from only an injection of sample, it is called multiparameter or multi-sensing optosensor (Table 3). These systems are more difficult to develop, as more complex series of requirements have to be accomplished for this purpose. Three different strategies have been used in the development of this kind of flow-based optosensors, which have allowed the simultaneous determination of two or three analytes.

3.5.1 Separation in a minicolumn

One of the most frequently used strategies for the development of multi-sensing systems has been the introduction in the flow-system of an on-line minicolumn filled with an appropriate solid support (Figure 4) (Llorent et al. 2005; Ruedas et al., 2002). Its aim is to discriminate in time the arrival of the analytes to the sensing zone. These minicolumns are home-made, in glass, and they are placed just before the flow-cell (Figure 4a). The length of the material packed is a key variable since it has to be sufficient as to allow a complete separation of the analytes in the minimum possible time. Appropriate working conditions have to be established as to allow the selective retention of one (bi-parameter sensor) or two (three-parameter sensor) of the analytes on the solid support packed into the minicolumn, while the other one(s) reach(es) the detection area. This requires a carrier/eluting solution allowing different retention-elution kinetics of the analytes in the minicolumn. After the measurement of the first analyte, the use of additional eluting solutions allows the desorption of the analyte(s) retained in the minicolumn and their transport to the sensing zone. Figure 4b shows the recording signals obtained for the simultaneous determination of three analytes when varying the length (amount) of the solid support packing in the minicolumn. As can be seen, the separation is only possible for a length of "d" mm.

Pesticide	Separation strategy	Solid phase / Flow system	Detector	LOD (μg L^{-1})	Reference
Carbofuran propoxur carbaryl	measurement at nine λ	C_{18} /FIA	AB	15 15 15	B. Fernández et al., 1991
Naptalam 1-naphthylamine	measurement at two λ	Amberlite XAD 7 / FIA	PH	8.1 11.2	Salinas et al., 2004
Thiabendazole metsulfuron methyl	separation in minicolumn	C_{18} / FIA	FL (PIF)	2.5 3.3	López et al., 2009b
Fuberidazole carbaryl benomyl	separation in minicolumn	C_{18} / FIA	FL	0.09 6 9	J.F. García et al., 2004a
Thiabendazole warfarin	separation in minicolumn	C_{18} / FIA	FL	2.35 0.54	Ruedas et al., 2002
Benomyl carbendazim carbofuran	separation in minicolumn	C_{18} /FIA	FL	35 15 68	Llorent et al., 2005
Naphtylamine (1-*plus* 2-isomers)	isosbestic point	MIP / FIA	FL	45 45	Valero et al., 2009a
1-Naphtylamine 2-naphtylamine	PLS-1, N-PLS, U-PLS	MIP /FIA	FL	15 33	Valero et al., 2009b
α-Naphtol o-phenylphenol thiabendazole	PLS	C_{18} / FIA	FL	- - -	Domínguez et al., 2007
Fuberidazole o-phenylphenol	separation in the flow-cell	C_{18} /MCFIA	FL	0.18 6.1	Llorent et al., 2006
Benomyl carbendazim	separation in the flow-cell	C_{18} / FIA	FL	7.5 3.0	J.F. García et al., 2003
Benomyl thiabendazole	separation in the flow-cell	C_{18} / FIA	FL	3.6 0.06	J.F. García et al., 2004b

AB: absorptiometry; FL: fluorescence; PIF; photochemically induced fluorescence; PH: phosphorescence; FIA: flow-injection analysis; MCFIA: multicommutation flow-injection analysis

Table 3. Multi-sensing flow-based chemical optosensors for pesticide assessment

Thiabendazole and metsulfuron methyl were simultaneously determined in water samples by placing in the flow system a minicolumn packed with C_{18} silica gel, the same solid support used as sensing zone (López et al., 2009b). Firstly, the sample was injected into a 15% methanol solution, which originated a weak retention of thiabendazole in the minicolumn and its elution by the carrier solution itself, being monitored by measuring its native fluorescence. After this, metsulfuron methyl was eluted from the minicolumn with a 60% methanol solution and monitored after its UV-irradiation and generation of a strongly fluorescent photoproduct.

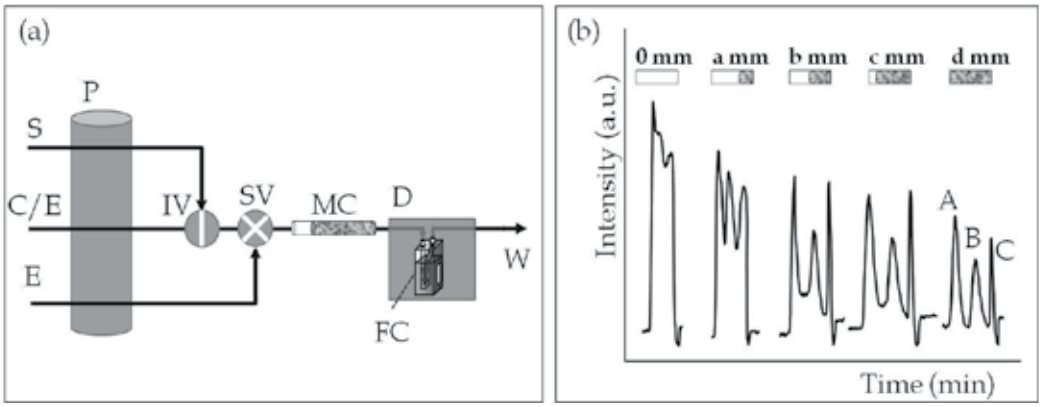

Fig. 4. Simultaneous determination of pesticides using a minicolumn inserted before the flow-cell. (a) Manifold configuration for determination of two species: S, sample solution; C/E, carrier/eluting solution; E, eluting solution; P, peristaltic pump; IV, injection valve; SV, selection valve; MC, minicolumn filled with solid support; D, detector; FC, flow-cell filled with solid support; W, waste. (b) Recording signals obtained for determination of three species (A, B and C) using different amounts (or lengths) of solid support in the minicolumn: d: optimal amount of solid support.

3.5.2 Separation in the flow-cell

A very simple design for the on-line separation of analytes, without involving the use of additional devices in the manifold, is the integration of the minicolumn into the same flow-cell. The minicolumn is replaced by the introduction in the flow-cell of an additional amount of solid support and, therefore, the level of support in that is higher than the usual one. The separation of the analytes takes place in the zone of the support above the detection area and allows a sequential arrival of them to this latter. This strategy involves some advantages like: (a) simplicity in manifold and procedure, (b) higher sensitivity, and (c) higher throughput.

As an example, the simultaneous determination of two pesticides, A and B, is shown in Figure 5. The signals recorded for A and B at two different excitation/emission wavelengths ($\lambda_{ex}/\lambda_{em}$) are shown for different additional amounts of solid support in the flow-cell. As can be seen, both analytes show a significant spectral overlapping and their simultaneous determination is not possible without a previous separation.

This strategy has been only used for the simultaneous determination of two pesticides (J.F. García et al., 2003, 2004b; Llorent et al., 2006) by measuring their native fluorescence. In one of the applications developed, multicommutation principles were also applied (Llorent et al. 2006). Fuberidazole and o-phenylphenol could be simultaneously determined, despite their severe spectral overlapping, by using an additional amount of solid support (C_{18}) in the flow-cell and two different carrier/eluting solutions. The use of a 30% methanol:water (v/v) solution originated a very strong retention of o-phenylphenol on the upper part of the support, in a zone situated above the irradiated one, while fuberidazole was transitorily retained and monitored. Then, a 60% methanol:water (v/v) solution allowed the elution of o-phenylphenol and its monitoring.

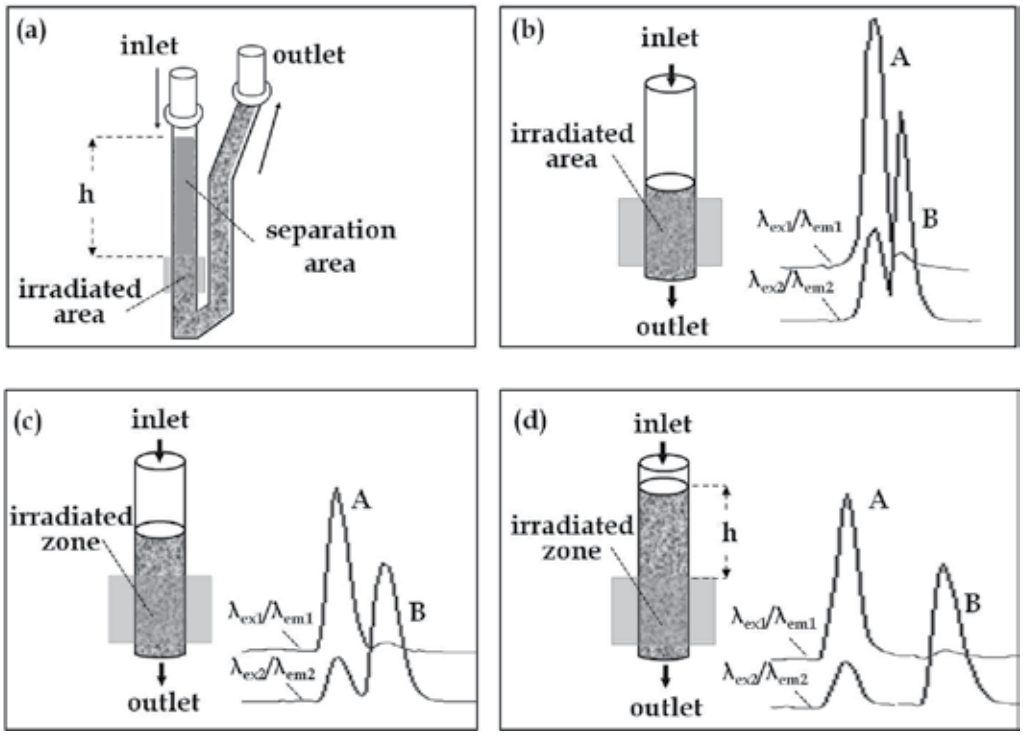

Fig. 5. Simultaneous determination of two analytes (A and B) in the flow-cell itself: (a) detail of the flow-cell filled with an additional amount of solid support; (b) - (d) recording signals of A and B for different amounts of solid support. h: optimal height of solid support above the detection area

3.5.3 Separation with mathematical treatment

The simultaneous determination of two or three analytes can be also carried out without their previous on-line separation, but making use of a mathematical treatment of their analytical signals. Several chemometrics algorithms such as first-order multivariate calibration partial least-squares (PLS) algorithm and second-order algorithms such as multiway PLS (N-PLS) and unfolded PLS (U-PLS) have been applied for this purpose. These approaches can resolve overlapping signals and reduce interference problems as well as background noise (Picón et al., 2000). In this way, the resolution of three pesticides, α-naphtol, thiabendazole and o-phenylphenol, at μg L^{-1} level was possible by developing a FIA-based system with fluorimetric detection, using C_{18} silica gel as active sorbent substrate in the flow-cell (Domínguez et al., 2007).

4. Flow-based biosensors

Biosensors have been defined as analytical devices which tightly combine biorecognition elements with physical transducers for detection of the target compounds (Guilbault et al., 2004). The majority of biosensors developed to date have used an antibody or enzyme (purified or in the form of whole cells) as the biocomponent, being fluorescence and chemiluminescence the most frequently used detection techniques. The biological

recognition element is usually immobilized in an immunoreactor placed before the flow-cell. As stated in *Introduction*, these systems cannot be considered strictly as flow-through optosensors, since the analytical measurement is carried out in solution. Nevertheless, they will be also reviewed here. To the best of our knowledge, to date only two flow-through biosensors, in which the biorecognition element is placed into the flow-cell, have been proposed (Pulido et al., 2000, 2006).

4.1 Enzyme inhibition-based biosensors

Many biosensors which are used for pesticide detection are based on the inhibition reaction of several enzymes in the presence of pesticides. In these biosensors, the biological element is an enzyme which reacts selectively with its substrate. Given that pesticides selectively inhibit the activity of certain enzymes, their activity and the resulting product concentration is affected. This inhibition is analytically useful and has been used advantageously in the development of many biosensing devices. The development of biosensors based on immobilized enzymes came about to solve several problems such as loss of enzyme (especially if expensive), maintenance of enzyme stability and shelf life of the biosensor, and additionally to reduce the time of the enzymatic response (Amine et al., 2006). Enzymes immobilized on suitable supports and packed into a reactor column, in combination with a flow injection system, have been widely used as biological recognition elements of biosensors for on-line monitoring of pesticides. The inhibition of the enzyme activity can be monitored by different optical techniques, being chemiluminescence the most frequently used technique. The chemical immobilization of the enzyme involves the formation of a covalent bond between some groups of the enzyme and a water-insoluble support (M. García et al., 1986).

Biosensing analytical devices, based on the acetylcholinesterase (AChE) inhibition test, using the monitoring of hydrogen peroxide generated as a result of the oxidation of choline produced from acetylcholine hydrolysis in the presence of choline oxidase have been reported for the quantification of carbamate and organophosphorus pesticides. In one of these approaches a column, placed inside a luminometer, was packed with cholineoxidase and peroxidase immobilized on methacrylate beads and AChE was added to the samples or immobilized in another column packed with the same solid support but placed outside the luminometer (Roda et al., 1994). The use of soluble AChE provided the best results when comparing with immobilized enzyme. The developed system allowed the determination of paraoxon and aldicarb with detection limits of 0.75 and 4 μg L^{-1}, respectively. It was applied to the analysis of paraoxon in water, soil and vegetables.

In other work, cholinesterase (ChE) was immobilized in a gel obtained by hydrolysis and condensation of tetramethyl orthosilicate (TMOS) (Navas & Ramos, 1997). A non-fluorescent synthetic substrate (indoxyl-acetate) was hydrolyzed by ChE to yield a highly fluorescent product (indoxyl), whose rate of formation was lowered in the presence of organophosphorus pesticides due to the inhibition of the enzyme.

Some authors have used a pH-sensitive transducer in the development of optical AChE-based biosensors. In these systems, the pH change generated by the release of acetic acid during the enzymatic reaction between acetyl choline and AChE is measured. The monitoring of the pH change can be carried out by using a colorimetric or fluorimetric indicator such as fluorescein isothiocyanate (FITC) (Rogers et al., 1991), thymol blue (Andres & Narayanaswamy, 1997) or chlorophenol red (Xavier et al., 2000).

4.2 Photosystem II (PSII)-based biosensors

These biosensors are reported to be able to detect herbicides such as triazine, diazines, phenolic and urea herbicides in the environment (Moreland & Novitzky, 1987; Vedrine et al., 2003). These substances inhibit photosynthetic electron flow by blocking the PSII quinone binding site and thus modify chlorophyll fluorescence. Some of these biosensing systems use isolated chloroplasts or intact cells of algae. Based on this principle a multi-strain algal biosensor was constructed for the rapid detection of five water-soluble herbicides. Nine microalgal strains were immobilized on an array biochip using permeable membranes and chlorophyll fluorescence was monitored (Podola & Melkonian, 2005). This inhibition capacity has also been used for the development of a chemiluminescent biosensor to detect low levels of atrazine and diuron (Varsamis et al., 2008). Thylakoid membranes, isolated from fresh spinach leaves, and horseradish peroxidase (HRP) enzyme were immobilized on magnetic beads and chemiluminescence of the photosynthetically produced H_2O_2 was measured, which was disrupted by both herbicides. The integration of the two-step reaction was achieved by designing a micro-system consisting of two Perspex (polymethylmethacrylate) blocks sandwiching a short fluidic channel made from silicon elastomer. The system contained two active zones: (i) immobilized PSII and (II) immobilized HRP to catalyze luminol/hydrogen peroxide chemiluminescence. The limits of detection obtained were 0.14 and 0.043 ng L^{-1} for atrazine and diuron, respectively.

4.3 Immunosensors

Immunosensors are biosensors that use antibodies (Ab) or antigens (Ag) as the specific sensing element and provide concentration-dependent signals. They consist of two processes, a molecular recognition process, for sensing the specific Ag–Ab binding reaction at the surface of receptor, and a signal-transfer process, for responding to changes in a parameter of the receptor caused by the specific binding. They have also shown to be useful for the determination of pesticides in water, food and soil samples. Among the advantages of these methodologies we can point out the high sensitivity and selectivity of antibodies which, in many cases, allows the direct detection of the target analytes in buffered samples without further treatment. Immunosensors can be divided into two classes, depending on if labels are used or not: labeled type and label-free type (Jiang et al., 2008).

4.3.1 Labeled-type

In labeled immunosensors, a label is used to quantify the amount of Ab or analyte bound during an incubation step. For this purpose, enzymes such as HRP, glucose oxidase and fluorescent or electrochemiluminescent probes have been widely used. The amount of target analyte can be inferred from the amount of labels that binds to the solid support. Usually, two different approaches are used: *sandwich* and *competitive* assays (Figure 6).

The *sandwich assay* consists of two recognition steps. In the first step, the Ab is immobilized on a transducer surface, which allows capturing the analyte of interest. In the second step, a labeled secondary Ab is added to bind with the previously captured analyte. The immunocomplexes (immobilized Ab-analyte-labeled Ab) are formed and the signals from labels are proportional to analyte concentration.

In *competitive assays*, the analyte and a constant amount of labeled analyte compete for a limited number of Ab binding sites. As the analyte concentration increases, a minor amount

of labeled analyte is retained on the transducer, originating a decrease in the signal if Ab-bound labeled analyte is detected (Herranz et al., 2008).

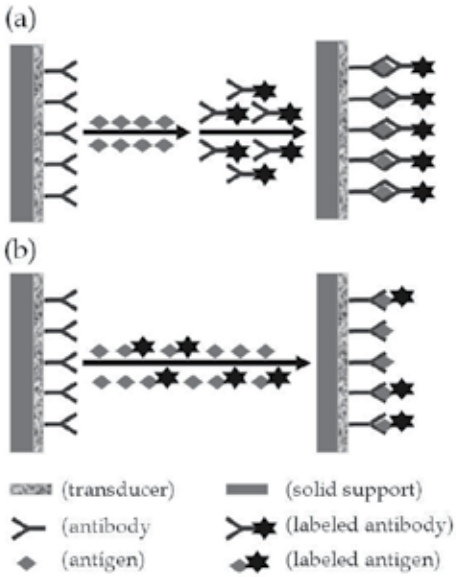

Fig. 6. Diagram for labeled immunosensors. (a) *sandwich* assay; (b) *competitive* assay

Two different strategies are used for the determination of the target species: (i) the solid support is placed into an immunoreactor column (reactor) and the amount of labeled analyte bound to the Ab is monitored; (ii) the solid support is placed into a flow-cell in the detector and the analytical signal of the labeled analyte-Ab complex is measured. The first strategy has been the most frequently used in the development of immunosensors for the determination of pesticides (Figure 7), implementing SIA methodology. However, these systems cannot be called flow-through optosensors, since the measurement is carried out in solution.

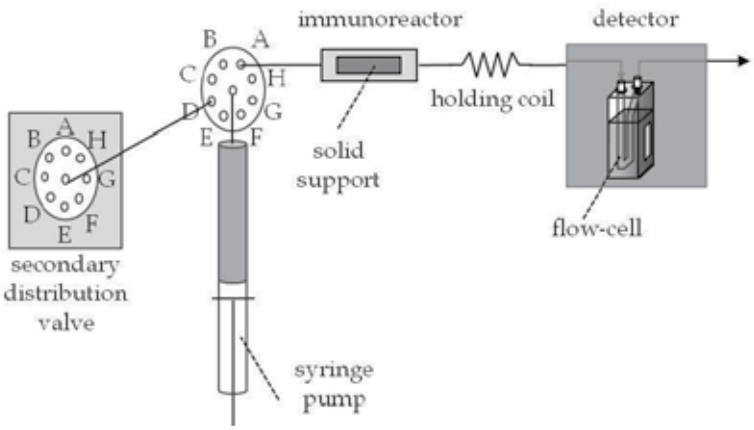

Fig. 7. SIA manifold used in labeled immunosensors, with immobilization of antibody in an immunoreactor

An example of biosensors including an immunoreactor is the derivatization-assisted enzyme immunosensor developed for the determination of glyphosate in water and soil samples (González et al., 2005). Enzyme substrates 3-(p-Hydroxyphenyl)-propanoic acid (HPPA) and H_2O_2 were used and the product of the enzymatic reaction was transferred from the reactor to the flow cell and the fluorescence peak was registered.
On the other hand, it is worth of mentioning the immunosensor proposed for determination of isoproturon in drinking water (Pulido et al., 2006). The solid support used, consisting of a sol–gel glass doped with anti-isoproturon monoclonal antibody, was placed in this case into the flow-cell of the spectrofluorimeter. Free isoproturon in solution competed with a fluorescent conjugated isoproturon and reduced the support bonded fluorescence in a concentration-dependent manner. A detection limit of 9.7 ng L^{-1} was obtained and recoveries higher than 90% for tap and well water. A similar fluoroimmunosensor for isoproturon, using two different solid supports (CPG and CPG-Protein A) and placing the immobilizated Ab directly into a flow-cell located in the detection area, was also described (Pulido et al., 2000).
Antibody immobilization on solid-phase has been carried out by using several coupling strategies, such as immobilization via controlled pore glass (CPG) (González et al., 1997), controlled pore glass bound to protein A (CPGA) (Herranz et al., 2008) or Protein A/G (González et al., 2005). Although the labeled immunosensors are usually more sensitive, they usually are not capable of real-time monitoring of the Ab–analyte reaction and increase both operation and development costs compared to label-free immunosensors.

4.3.2 Label-free type

Two different strategies, both based on competitive formats, have been used for the development of label-free immunosensors: *direct* and *indirect* determination. In the first type, the analytical signal measured is directly proportional to the concentration of analyte, being its main advantage simplicity of the system. The second type, based on an inhibition test, has been proven to be an effective method for the determination of pesticides. Firstly, a protein conjugate of the pesticide is immobilized onto the surface of a transducer, and then analyte–Ab mixtures are preincubated in solution. After being injected on the sensor surface, a competition is established between the analyte present in solution and pesticide protein-conjugate on solid support. Consequently, Ab binding to the immobilized conjugate is inhibited by the presence of the target pesticide. Surface plasmon resonance (SPR) technology is the most usually used detection system for this kind of immunosensors. SPR immunosensing involves the immobilization of Ab (or analyte) on a thin gold surface deposited on the reflecting surface of a glass prism. Interaction of both analyte and Ab on the surface originates a change in the refractive index of the solution due to shifts in mass occurring after biomolecule binding (Dutra & Kubot, 2007). This change is directly related to the concentration of analyte in the surface layer. Some of the main advantages of SPR technology are its versatility and capability of monitoring binding interactions without the need for labeling of the target compounds and its outstanding attributes of miniaturization and portable instrumentation.
A rapid and simple immunosensor for indirect determination of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was developed based on surface plasmon resonance (SPR) technology (Gobi et al., 2007). The sensor device was fabricated by simple physical adsorption of an ovalbumin conjugate of 2,4-D on an SPR thin-film gold chip. The selective

binding of a monoclonal Ab against 2,4-D (2,4-D-Ab) is followed by an increase in SPR angle. The biosensor allowed a detection limit of 0.1 μg L^{-1} and showed a high tolerance to the presence of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).
It is also worth mentioning the performance of a SPR immunosensor system for the simultaneous detection of DDT, chlorpyrifos and carbaryl (Mauriz et al., 2007). The multi-assay was carried out by using a two-channeled configuration for multiple immobilizations of derivatives of the three pesticides. Different immobilization formats were used. The immobilization of a single derivative allowed the simultaneous monitoring of antibody interactions occurring separately on each individual flow cell. In contrast, multisurface sensor with several immobilized derivatives involved the use of one independent flow cell for the analysis of two or three analytes.
Tables 4 and 5 show some of the flow-based biosensors developed for determination of pesticides.

Pesticide	**Principle**	**Solid Phase/ Flow system**	**Detector**	**LOD (μg L^{-1})**	**Reference**
Simazine	antibody (labeled type)	CPGA / SIA	FL	0.0013	Herranz et al., 2008
Carbaryl	antibody (labeled type)	CPG / SIA	FL	0.029	González et al., 1997
Glyphosate	antibody (labeled type)	Protein A/G / SIA	FL	0.021	González et al., 2005
1-naphtol	antibody (labeled type)	Protein A/G / SIA	FL	12	Penalva et al., 2000
Isoproturon	antibody (labeled type)	CPGA CPG / FIA	FL	3.0-4.5 3.0	Pulido et al., 2000
Isoproturon	antibody (labeled type)	sol-gel glass / FIA	FL	0.0097	Pulido et al., 2006
2,4-D	antibody (label-free type)	gold film / FIA	SPR	0.1	Gobi et al., 2007
Atrazine	antibody (label-free type)	gold film / FIA	SPR	0.020	Farré et al., 2007
DDT carbaryl chlorpyrifos	antibody (label-free type)	gold film / FIA	SPR	0.018 0.050 0.052	Mauriz et al., 2007

FL: fluorescence; SPR: surface plasmon resonance; CPG: controlled pore glass; CPGA: controlled pore glass bound to protein A

Table 4. Applications of flow-based immunosensors to pesticide assessment

Pesticide	Principle	Solid Phase/ Flow system	Detector	LOD ($\mu g\ L^{-1}$)	Reference
Paraoxon aldicarb	AChE inhibition	Eupergit C / FIA	CH	0.75 4	Roda et al., 1994
Methidation naled mecarbam fenitrothion azinphos-ethyl	ChE inhibition	sol-gel crystal/ FIA	FL	57600 360 13900 17800 17900	Navas & Ramos,1997
Bendiocarb methomyl echothiophate paraoxon	AChE inhibition	quartz fibers / FIA	FL	- - - -	Rogers et al., 1991
Carbofuran paraoxon	AChE inhibition	ITC glass / FIA	RF	3.1 24.7	Andres & Narayanas-wamy, 1997
Propoxur carbaryl	AChE inhibition	CPG / FIA	RF	8 500	Xavier et al., 2000
Atrazine simazine isoproturon diuron DNOC	inhibition of PSII in algae	quartz microfibre filter / FIA	FL	0.25 0.5 0.025 0.025 5	Vedrine et al., 2003
Atrazine diuron paraquat simazine isoproturon	inhibition of PSII in algae	array biochip membrane / FIA	FL	0.5 - 10	Podola & Melkonian, 2005
Atrazine diuron	inhibition of PSII in thylakoid membranes	magnetic beads / FIA	CH	1.4E-04 4.3E-05	Varsamis et al., 2008

AChE: acetylcholinesterase; ChE: cholinesterase; PSII: photosystem II; RF: reflectometry; FL: fluorescence; CH: chemiluminescence; CPG: controlled pore glass; ITC: isothiocyanate; FIA: flow-injection analysis

Table 5. Applications of flow enzyme- and photosystem-II-based biosensors to pesticide assessment

5. Conclusions

Flow-based optosensors constitute a simple and inexpensive tool for routine analysis of pesticides. They offer advantages such as high sensitivity, high selectivity, high throughput, low cost of equipment and they can be considered as an interesting alternative to the well established chromatographic methods (HPLC and GC). The recent introduction of new flow methodologies such as SIA, MCFIA and MSFIA has allowed remarkable improvements in

flow systems such as higher repeatability, minor consumption of sample and reagents, complete automation, robustness, versatility and minimization of waste generation. All these methodologies have proven their applicability to the analysis of pesticides in complex matrices, with detection limits comparable to those obtained with chromatographic methods. To date, the proposed flow-based optosensors have shown to be very useful for the monitoring of one, two or three pesticides. Therefore, they are not appropriate for multiresidue analysis, although they are very useful for a preliminary screening in laboratories where a large number of samples have to be processed in a short time. In the future, the implementation of other flow methodologies such as multipumping or lab-on-valve could contribute to enhance miniaturization and versatility of systems in the design of automatic methods for routine analysis.

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Edited by Margarita Stoytcheva

The book offers a professional look on the recent achievements and emerging trends in pesticides analysis, including pesticides identification and characterization. The 20 chapters are organized in three sections. The first book section addresses issues associated with pesticides classification, pesticides properties and environmental risks, and pesticides safe management, and provides a general overview on the advanced chromatographic and sensors- and biosensors-based methods for pesticides determination. The second book section is specially devoted to the chromatographic pesticides quantification, including sample preparation. The basic principles of the modern extraction techniques, such as: accelerated solvent extraction, supercritical fluid extraction, microwave assisted extraction, solid phase extraction, solid phase microextraction, matrix solid phase dispersion extraction, cloud point extraction, and QuEChERS are comprehensively described and critically evaluated. The third book section describes some alternative analytical approaches to the conventional methods of pesticides determination. These include voltammetric techniques making use of electrochemical sensors and biosensors, and solid-phase spectrometry combined with flow-injection analysis applying flow-based optosensors.

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