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Pesticides

Strategies for Pesticides Analysis

Edited by Margarita Stoytcheva

PESTICIDES - STRATEGIES FOR PESTICIDES ANALYSIS

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Meet the editor

Margarita Stoytcheva is born in Bulgaria, she graduated from the University of Chemical Technology and Metallurgy of Sofia, Bulgaria, with titles of Chemical Engineer and Master of Electrochemical Technologies. She has Ph.D. and D.Sc. 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 (UABC), Institute of Engineering, Mexicali, as a full time researcher.
She has given courses of general chemistry, analytical chemistry and physical chemistry. Her interests and areas of research are biosensors, electrochemistry and analytical chemistry. Her publications, including articles, books and professional congress proceedings (altogether 150), have been cited more than 300 times in the scientific literature.
She has been a member of the following scientific organizations: New York Academy of Sciences, Association of Scientists (Bulgaria) and Association of Chemists (Bulgaria). Since 2008 she has been a member of the National System of Researchers (SNI) of Mexico.

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Preface

The synthesis, during the 1950s, of organophosphorus, carbamate, organochlorine, and pyrethroid compounds, designed for preventing, destroying, repelling or mitigating any pest, marked the beginning of the contemporary "pesticides era". Users' benefits, because of the successful pesticides application for parasites control of field and fruit crops leading to an increase of the agricultural production, became evident. However, pesticides toxicity and indiscriminate usage caused risks to men and his environment. Therefore, the World Health Organization (WHO), the Food and Agricultural Organization of the United Nations (FAO), the Codex Alimentarius Commission, the EU Commission, and the U. S. Environmental Protection Agency (EPA), among others, enacted the allowable pesticide residue levels in food, drinking water and environmental samples. The European Council Directive 98/83/EC on the quality of water intended for human consumption sets the limit value of the individual pesticides in drinking water at 0.1 ◎g L-1 and of the total pesticides at 0.5 ◎g L-1. According to the U.S. EPA Office of Ground Water and Drinking Water (OGWDW), the health advisory levels for some organophosphorus pesticides in drinking water are: diazinon 3 ◎g L-1, parathion-methyl 2 ◎g L-1, disulfoton 1 ◎g L-1, fenamiphos 2 ◎g L-1, etc. At this time, EPA is reassessing pesticide residue limits in food to ensure that they meet the safety standard established by the Food Quality Protection Act of 1996.

The great public concern and the strict legislation incited the development of reliable, specific, selective and sensitive analytical methods for pesticides monitoring. This book presents some of them.

The first four chapters focus on sample preparation required by the chromatographic pesticides analysis to eliminate interferences and to increase sensitivity. Chapter 1 discusses the current trends in liquid-liquid microextraction for analysis of pesticides residues in food and water. Chapter 2 comments on a number of extraction procedures, as liquid-liquid extraction, solid phase extraction, solid phase microextraction, single drop microextraction, liquid-solid microextraction, microwave assisted solvent extraction, supercritical fluid extraction, dispersive liquid-liquid microextraction and accelerated solvent extraction. Chapter 3 points out on the application of miniaturized ultrasonic extraction for residues analyses of organochlorine pesticides in water and soil samples. Chapter 4 considers factors affecting the accurate quantification of pesticide residues in non-fatty matrices, including effects due to solvent and other materials applied for the residues extraction from the analyzed matrix, molecule polarity, matrix chemical composition, etc.

Chapters 5 to 11 are dedicated to the application of chromatographic techniques in pesticides analysis. Chapter 5 focuses on issues related to the chromatography-mass spectrometry and instrumental approaches to improve selectivity and sensitivity of the determinations. Selectivity enhancement by the negative chemical ionization approach is commented in Chapter 6. Applicability of fast GC for pesticide residues in real-life samples is demonstrated, too. Chapter 7 introduces the principles of the multidimensional chromatography applied in pesticides analyses. In Chapter 8 the authors describe their "rapid and easy" multiresidue methods for determination of pesticide residues in food using gas or liquid chromatography-tandem mass spectrometry. Chapter 9 recommends the continuous human biomonitoring of organochlorine pesticides used in human serum isotope dilution gas chromatography-high-resolution mass spectrometric analysis. Analytical determination of urea pesticides is discussed in Chapter 10. Studying lypophilic properties and bioactivity of pesticides by liquid chromatography is the subject of Chapter 11.

Chapters 12 and 13 address the immunoassay- and biosensors-based techniques for pesticides quantification. Chapter 14 is centered on the development of electrochemical sensors based on chemical or biological recognition processes and the advantages provided by nanomaterials electrode modification. Chapter 15 reviews the principles of the electrochemical biosensors-based methods for organophosphorus pesticides determination as methods of choice for "in situ" and "on line" application. The recent trends in the development of electrochemical biosensors, including nanomaterials transducer modification and genetic engineering of the biological recognition element are revised. Chapter 16 reports the results obtained by applying a gold-mercaptobenzothiazole-polyaniline-acetylcholinesterase-polyvinylacetate thick film amperometric biosensor for the detection of selected organophosphorus and carbamates pesticide in the nanomolar concentration range. Chapter 17 describes a miniaturized and portable new conductometer coupled with haloalkane dehalogenase, an enzyme able to cleave chlorinated chemicals, to detect pesticides applying a bipolar pulse technique.

The book contains up-to-date publications of leading experts. It addresses the key problems in pesticide analysis related to the sample preparation techniques and the application of the current chromatographic and alternative biosensors-based methods. The references at the end of each chapter provide a starting point to acquire a deeper knowledge on the state of the art. The edition is intended to furnish valuable recent information to the professionals involved in pesticides analysis.

Finally, it is my pleasant duty to acknowledge each of the authors for contributing their chapters to this volume.

Margarita Stoytcheva
Mexicali, Baja California
Mexico

1

Current Trends in Liquid-Liquid Microextraction for Analysis of Pesticide Residues in Food and Water

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1. Introduction

Since the middle of last century, the use of organic synthetic pesticides became a widespread practice, in order to better prevent, control and destroy pests. Despite their usefulness in the increment of food production, the extensive use of pesticides during production, processing, storage, transport or marketing of agricultural commodities can led to environmental contamination and to the presence of residues in food. Real and perceived concerns about pesticide toxicity have promoted their strict regulation in order to protect consumers, environment and also the users of pesticides. Thus, reliable and accurate analytical methods are essential to protect human health and to support the compliance and enforcement of laws and regulations pertaining to food safety.

The first analytical methods for pesticide analysis were developed in the years 1960s, employing an initial extraction with acetone, followed by a partitioning step upon addition of a non-polar solvent and salt; these methods involved complex and solvent-intensive cleanup steps. Moreover, the instruments available for analysis of the target compounds had a relative low selectivity and sensitivity. The development of technology and robotic in the 1990s allied to the aim to reduce manual interference and to allow sample preparation during non-working time, has boosted the development of automated sample preparation techniques such as supercritical fluid extraction and pressure liquid extraction. Though initially very promising, these techniques have not succeeded in the field of pesticides analysis for various reasons, namely high price and low reliability of the instruments, and inability to extract different pesticide classes in foods with the same efficiency, often requiring separate optimization for different analytes. Later, a successful simplification of "traditional" solvent sample preparation, QuEChERS (quick, easy, cheap, effective, rugged, and safe) was presented by Lehotay and collaborators (Anastassiades et al., 2003). This procedure, involving a simple extraction/partition using acetonitrile and salts followed by a simple dispersive cleanup, has been adopted for the analysis of many pesticide residues in food (Cunha et al, 2010). Two similar QuEChERS methods achieved the status of Official Method of the AOAC International (Lehotay, 2007) and European Committee for Standardization (CEN) standard method EN 15662 (Standard Method EN 15662). Unfortunately, the analysis of QuEChERS extracts in acetonitrile by GC-MS is not totally straightforward. Several facts can occur: degradation of the GC column by the polar solvent,

vapor overload of the insert liner due to the high thermal expansion coefficient, contamination of the system by co-extractives (Hetmanski et al., 2010), and reduced enrichment factors.
Recently, the development of new analytical equipment, namely tandem mass spectrometers coupled to LC and GC systems, allowed improvements in the sensitivity, selectivity, and speed of analysis. Although the prohibitive costs of such equipments make them unattainable to many groups working in this field. Such improvements in sensitivity and selectivity could also be accomplished by innovative sample preparation techniques recently introduced, most of them with the added benefit to be easy to execute, cost-effective, and environmental friendly. Cloud point extraction, single-drop microextraction, hollow fiber liquid phase microextraction, and dispersive liquid-liquid microextraction, are examples of liquid-liquid microextraction techniques that have emerged in recent years in the field of sample preparation and are being used increasingly. The major advantage of microextractive techniques is the use of only microliters of solvents instead of several hundred mililiters in the classical liquid-liquid extraction. In addition, due to the compatibility of the solvents used and the low volumes involved, samples are easily transferred to the next step of analysis, liquid or gas chromatography. The aim of this work is to review the application of liquid-liquid microextraction techniques in the analysis of pesticide residues in food and water and to compare its use with other well-established sample preparation techniques. Special emphasis will be given to articles published in the last four years. Principles, advantages and relative merits of each technique will be also summarized and discussed.

2. Analytical tools for determination of pesticide residues in food and water

Pesticide analysis is almost invariably accomplished by means of a chromatographic technique, either GC or LC coupled to universal (MS, MS/MS) or selective detectors (ECD, electron-capture detector; NPD, nitrogen phosphorus detector; FPD, flame photometric detector; UV, ultraviolet detector; and FLD, fluorimetric detector), following an adequate sample preparation step. Regardless the type of chromatographic technique employed, sample preparation remains as the limiting step to reach desired performance parameters, due to the low legally established levels and the complex nature of the matrices in which the target compounds are present typically in low amounts. As a rule, the physico-chemical methods used to obtain a pesticide extract able to be chromatographically analyzed consist in the extraction/isolation of the target analytes by an appropriate extraction technique followed by some purification and concentration steps. The classical procedures are often time consuming, laborious and environmental unfriendly, taking into account the large volume of organic solvents usually required. Recently, as referred in the Introduction section, new techniques have been introduced, offering consistently high enrichment factors and consequently higher sensitivity for the analytes of interest, together with a significant reduction of organic solvent consumption as well as extraction time. The most relevant techniques in this field are further detailed in the following sections.

2.1 Sample preparation

2.1.1 Cloud-point extraction (CPE)

Watanabe and collaborators, introduced in 1976 cloud-point extraction (CPE), a promising new separation and extraction technique, as an alternative to classical procedures with

organic solvents (Paleologos et al., 2005). CPE or micelle-mediated extraction, is based on the capacity exhibited by aqueous micellar solutions of some surfactants to form the cloud point, or turbidity, phenomenon that occur when the solution is heated or cooled above or below certain temperature. The temperature at which this phenomenon occurs is known as the cloud-point temperature or micelle-mediated extraction (Carabias-Martínez et al., 2000).
Surfactants are amphiphilic molecules, which have a polar moiety (the head), hydrophilic in nature, linked to a hydrophobic portion (the tail). In aqueous solution, and at low concentrations, surfactant molecules are found in monomer form, although dimers and trimers have also been detected (Paleologos et al., 2005).
When the surfactant concentration is increased above a certain threshold, called ''critical micellar concentration'' (CMC), the surfactant molecules become dynamically associated to form molecular aggregates of colloidal size. These aggregates, containing between 60 and - 100 monomers, are called micelles and are at equilibrium with a surfactant concentration in the solution close to the CMC. Depending on the nature and concentration of the surfactant, as well as on the solvent used, another series of structures may be formed, organized as inverse micelles, microemulsions, vesicles, monolayers, or bilayers (Carabias-Martínez et al., 2000).
To date, liquid–liquid phase separation based on non-ionic or zwitterionic surfactant micelles (i.e., CPE) are employed, while the use of charged surfactant species is still scarce (Paleologos et al., 2005). Sanz et al. (2004) used non-ionic surfactants such as polyoxyethylene 10 lauryl ether and oligoethylene glycolmonoalkyl ether (GenapolX-080) at 95ºC for 15 min to extract eight organophosphorus pesticide residues (chlorpyrifos, diazinon, dimethoate, ethoprophos, malathion, methidathion, parathion methyl and paration ethyl) from water, which were analyzed by HPLC-UV. The authors obtained a enrichment factor of 20, recoveries between 27 and 105%, and limits of detection (LOD) lower than 30 μg/L. In 2008, Santalad et al. presented a simple and rapid spectrophotometry method based on acid-induced anionic surfactant micelle-mediated extraction (acid-induced cloud-point extraction) coupled to derivatization with 2-naphthylamine-1-sulfonic acid to determine carbaryl residues in water and vegetables. In this work, sodium dodecyl sulphate (the extractant), was combined with 2-naphthylamine-1-sulfonic acid derivatization, allowing the extraction at low temperature (45ºC). The proposed method showed good analytical features with low LOD (50 μg/L), good precision with a relative standard deviation (RSD) of 2.3%, and high recoveries when applied in samples (85%).
Notwithstanding the capacity to concentrate the analytes and the good recoveries achieved with CPE, its application in the extraction of pesticide residues in food matrices is restricted, in part due to the physico-chemical properties of the surfactant. As it is viscous, it cannot be injected directly to conventional analytical instruments, so it has to be diluted with an aqueous or organic solvent to reduce its viscosity, thus impairing the anticipated theoretical preconcentration factors. Moreover, surfactant-bearing chromophores interfere with UV detection by overlapping with the analyte signal. This problem can be solved by diluting the surfactant-rich phase with an organic solvent prior to injection into the chromatographic column, increasing the portion of organic solvent in LC mobile phases or using fluorescence detection (Paleologos et al., 2005).

2.1.2 Single drop microextraction (SDME)

Drop-drop microextraction was first introduced, in 1996, by Liu & Dasgupta, (1996). They extracted sodium dodecyl sulphate ion pairs by a microdrop (1.3 μL) of a water-immiscible

organic solvent, suspended in a larger aqueous drop. At the same year, Jeannot and Cantwell introduced a technique that they termed as solvent microextraction in which the extraction medium was a droplet (8 μL) of 1-octanol held at the end of a Teflon rod and suspended in a stirred aqueous sample solution. After extraction for a prescribed time, the Teflon rod was withdrawn from the aqueous solution; the organic phase sampled with a microsyringe and injected into a GC system. In this work, the authors also proposed equilibrium and kinetic theories to explain this microextraction procedure. Subsequently, the technique was changed to allow simultaneous extraction and injection of analytes, by introducing as support a microsyringe, where the organic phase was suspended at the needle tip (Jeannot & Cantwell, 1997) (Figure 1).

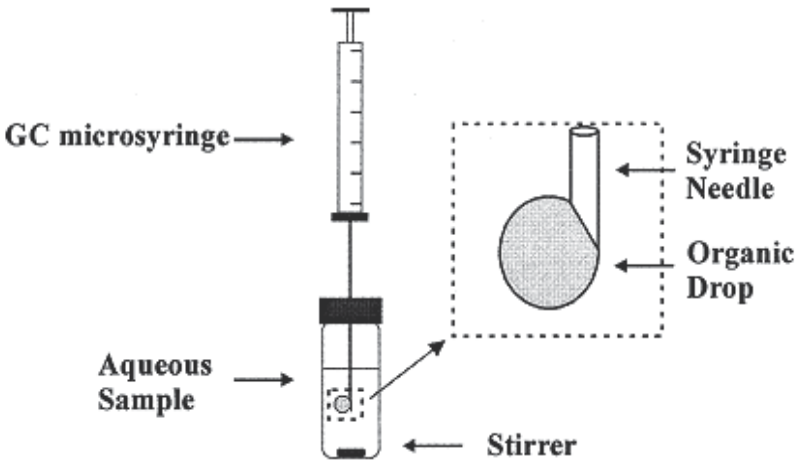

Fig. 1. Schematic illustration of direct immersion single-drop microextraction (from Xu et al., 2007).

One advantage of SDME over other liquid extraction techniques is the small volume of organic solvent required. Additionally, in this technique, analytes with high partition coefficient can reach high concentrations, since they are transferred by diffusion from a significant volume of sample (1-5 mL) to a small micro-extract (5-50 μL).

Since its introduction, different modes of SMDE have been developed, in order to improve extraction efficiency, such as direct SDME, headspace SDME (HS-SDME) and continuous-flow microextraction (CFME).

Direct SDME consists of suspending a microdrop of organic solvent at the tip of a syringe, which is immersed in the aqueous sample. An alternative approach was described as dynamic technique by He & Lee (1997), in which organic solvent repetitively forms a film inside the syringe barrel by continuously pulling and pushing of the syringe plunger. Extraction takes place between the sample solution and the organic film (He & Lee, 2006).

Direct SDME has extensively been used for the direct extraction of pesticide residues from aqueous samples (Table 1). Xiao et al. (2006) evaluated two types of SDME, static and dynamic, in extraction of six organophosphorus pesticides (OPPs) (dichlorvos, phorate, fenitrothion, malathion, parathion, quinalphos) from water and fruit juice. Significant parameters affecting SDME performance such as extractant solvent, solvent volume, stirring rate, sample pH and ionic strenght were evaluated. The authors verified that static SDME

procedure allowed an enrichment factor of the six OPPs nearly 100 fold, which were much better than the results obtained with the dynamic mode. The optimized static SDME procedure in conjugation with GC-FPD allowed good detection limits ranging from 0.21 to 0.56 μg/L. In the same year, Zhao et al. (2006) also optimized a SDME procedure for extraction of seven OPPs (ethoprophos, diazinon, parathion methyl, fenitrothion, malathion, isocarbophos and quinalphos) in orange juices with analysis under GC-FPD. An effective extraction was achieved by suspending during 15 min a 1.6 μL drop of toluene to the tip of a microsyringe immersed in a 5 mL donor aqueous solution with 5 % (w/v) NaCl and stirred at 400 rpm. The seven OPPs were extracted from orange juice samples with good limits of detection (below 5 μg/L). However, better detection limits for 13 OPPs pesticides (ranging from 0.001 to 0.005 μg/L) in water were obtained by Ahmadi et al. (2006) using SMDE with a modified 1.0 μL microsyringe and GC-FPD, compared to 10 μl microsyringe used in the works above referred. By using a 1.0 μL microsyringe the repeatability of the drop volume and the injection were improved, due to the maximum volume of microsyringe without dead volume. On the other hand, the modification of the needle tip caused increasing cross section of it and increasing adhesion force between needle tip and drop, thereby increasing drop stability and allowing a higher stirrer speed (up to 1700 rpm). The method used 0.9 μl of carbon tetrachloride as extractant solvent, 40 min of extraction time, stirring at 1300 rpm and no salt addition. The potential of SMDE was also investigated by Liu et al. (2006) in the extraction of four fungicides from water and wine samples. Additionally, SDME has been applied in the extraction of organochlorine pesticides (OCPs) in various matrices (Table 1). Qia & He (2006) introduced a funnel from SDME to extract 11 OCPs and 2 pyrethroid pesticides from tea samples and analyze by GC-ECD. More recently, Cortada et al. (2009a) proposed a SDME procedure comprising a 2 μL toluene microdrop exposed for 37 min to 10 mL aqueous sample without salt addition and stirred at 380 rpm to extract eight OCPs from wastewater followed by GC-MS analysis.

Contrary to the aqueous samples, vegetable and fruits, being mostly in solid or heterogeneous form do not allow direct extraction with SDME. However, it is possible to use SDME after a previous pretreatment. Nine OCPs (β-,λ-,α-, σ- BHC, dicofol, dieldrin, DDD, DDE, and DDT) were extracted with SDME from fresh vegetable (cabbage, cauliflower, Chinese cabbage) after an adequate mixture of sample aliquots with acetone using a ultra-sonic vibrator. An effective extraction was achieved by suspending a 1.0 μL mixed drop of *p*-xylene and acetone (8:2 w/v) to the tip of a microsyringe immersed in a 2 mL donor sample solution and stirred at 400 rpm (Zhang et al., 2008). SDME technique coupled with GC-NPD and GC-ECD has also been successfully applied for the determination of multiclass pesticides in vegetable samples (tomato and courgette) by Amvrazi & Tsiropoulos (2009). Donor sample solution preparation from solid vegetable tissues was achieved in one step with the minimum amount of organic solvent (10% acetone in water) and optimum SDME was accomplished using a toluene drop (1.6 μL) under mild stirring for 25 min.

HS-SDME is very similar to direct SDME except that a microdrop of a high boiling extracting solvent is exposed to the headspace of a sample. This technique allows rapid stirring of the sample solution with no adverse impact on the stability of the droplet. Additionally, as in headspace-solid phase microextraction (HS-SPME), non-volatile matrix interferences are strongly reduced, if not totally eliminated. In this mode, the analytes are distributed among three phases, the water sample, the headspace and the organic drop (Xu et al., 2007). Aqueous phase mass transfer is the rate determining step in the extraction

process as explained by Theis et al. (2001). Hence, a high stirring speed of the sample solution facilitates mass transfer among the three phases. A HS-SDME was optimized for the extraction of organochlorine and organophosphorous pesticide residues in food matrices (cucumbers and strawberries) (Kin & Huat, 2009). The extraction was achieved by exposing 1.5 μL toluene drop to the headspace of a 5 mL aqueous solution in a 15 mL vial and stirred at 800 rpm. The analytical parameters, such as linearity, precision, LOD, limits of quantification (LOQ), and recovery, were compared with those obtained by HS-SPME and solid-phase extraction. The mean recoveries for all three methods were all above 70% and below 104%. HS-SPME was the best method with the lowest LOD and LOQ values. Overall, the proposed HS-SDME- GC-ECD method was acceptable for the analysis of pesticide residues in food matrices.

CFME was introduced by Liu & Lee, 2000, in order to improve the mass transfer between aqueous and organic phases. The technique is based in the continually refreshing of the surface of the immobilized organic drop used as extractant solvent by a constant flow of sample solution delivered by an HPLC pumping system (Xu et al., 2007). Both diffusion and molecular momentum resulting from mechanical forces contribute to its effectiveness. With the use of an HPLC injection valve, precise control of the solvent drop size could be achieved, avoiding the introduction of undesirable air bubbles. Another advantage was the high enrichment factor that can be achieved, requiring smaller volumes of aqueous samples for extraction (Xu et al., 2007). He & Lee (2006) reported the combination of CFME with HPLC to extract and determine the widely-used organonitrogens and OPPs (simazine, fensulfothion, etridiazole, mepronil and bensulide) (Table 1). CFME employs a single organic solvent drop of carbon tetrachloride (3 μL) positioned at the tip of a polyether ether ketone (PEEK) tubing, which is immersed in a continuous flowing aqueous sample solution in a 0.5-mL glass chamber. The PEEK tubing acts as the organic drop holder and fluid delivery duct. Analytes are partitioned between the organic drop and the bulk sample solution. Important extraction parameters including type of solvent, volume, sample solution flow rate, extraction time, pH and the addition of salts were investigated. Detection limits lower than 4 μg/L were obtained for all analytes.

As mentioned above several parameters affect the rates and efficiencies of SDME techniques such as: i) analyte properties, ii) solvent acceptor, iii) drop volume, iv) agitation, v) ionic strength, vi) extraction time. A detailed discussion of these important parameters can be found in the literature (Jeannota et al., 2010). i) Analyte properties: low molecular weight, volatile and semi volatile analytes are extractable by headspace (HS-SDME). Direct immersion (DI-SDME) extraction is appropriate for non polar or moderately polar high molecular weight, semi volatile chemicals. Highly polar chemicals may need to be derivatized to ensure recovery, especially when the matrix is aqueous. ii) Extractant solvent: the extractant solvent in SDME is usually a pure or mixed hydrophobic solvent (*n*-hexane, benzene, toluene, dichloromethane, *n*-butanol, etc.), although some authors have reported the use of a hydrophilic solvent mixture as extractant solvent (*p*-xylene:acetone). iii) Drop volume: the use of a large drop results in an increase of analyte extracted. However, larger drops (>3 μL) are difficult to manipulate and less reliable. Difficulties with drop size variations are minimized if the drop size used is about 1 μL. iv) Ionic strength: addition of salts (such as NaCl or Na_2SO_4) to the sample may improve the extraction of analytes since high ionic strength reduces their water solubility. However, apart from the salting-out effect, the presence of salt can change the physical properties of the extraction film, thus reducing the diffusion rates of the analytes into the drop. v) Agitation of the sample: the

time required to thermodynamic equilibrium can be reduced by agitation. Three sample agitation methods are available: stirring, vibration and vortexing. Stirring, using a magnetic stir bar, is effective with stirring rates of 300–600 rpm for DI-SDME and 500–1000 rpm for HS-SDME. The limitations of higher stirring rates are the dislodgement of the drop by the sample solution or splashing when using headspace. Vibration and vortex stirring, used with some autosamplers, are also effective, with the limitation that the agitation cannot occur while the drop is exposed at the needle tip. vi) Extraction time: extraction efficiency increases with longer extraction times in most of SDME techniques. The extraction time should be enough to extract an adequate amount of analyte by the microdrops. Times between 5 and 45 min are commonly used, longer times may cause drop dissolution.
Despite its simplicity, easy implementation, and low cost, SDME techniques have some limitations, for example: i) direct immersion requires careful and intricate manual operation because of problems of drop dislodgment and instability; ii) complex matrices requires a pretreatment or extra filtration step; iii) sensitivity and precision of SDME methods even acceptable need further improvement. The main issue lies with the adverse consequences of prolonged extraction time and fast stirring rate, since they may result in drop dissolution and/or dislodgement; and iv) SDME is not yet suitable as routinely applicable online preconcentration procedure (Xu et al., 2007).

2.1.3 Hollow-fiber liquid-phase microextraction (HP-LPME)

Pedersen-Bjergaard & Rasmussen introduced hollow-fiber based liquid-phase microextraction (HP-LPME) in 1999, to improve the stability and reliability of SDME techniques (Pedersen-Bjergaard & Rasmussen, 1999). In HP-LPME the extracting phase was placed inside the lumen of a porous polypropylene hollow fiber. The fiber had a porosity of 70% with a pore size of 0.2 μm, a wall thickness of 200 μm and an internal diameter of 600 μm. A supported liquid membrane was formed by dipping the hollow fiber into a suitable organic solvent. The solvent penetrates the pores of the hollow fiber and bound by capillary forces to the polypropylene network comprising the fiber wall. The high porosity enabled immobilization of a considerable volume of solvent as a thin film, e.g. a 1 cm length of the fiber was able to immobilize ca. 8 μL of solvent as a 200 μm film within the polypropylene network. The extracting phase (acceptor solution) which was placed into the lumen of the fiber was mechanically protected inside the hollow fiber and it was separated from the sample by the supported liquid membrane (organic solvent), thus preventing its dissolution into the aqueous sample. In LPME (HP-LPME), analytes are extracted from an aqueous sample, through the organic solvent immobilized as supported liquid membrane (SLM), into the acceptor solution placed inside the lumen of the hollow fiber. Subsequently, the acceptor solution is removed by a micro-syringe and further analyzed (Pedersen-Bjergaard & Rasmussen, 2008). Chemical principles of HP-LPME are similar to those employed in supported liquid membrane (SLM), but the techniques differ in terms of instrumentation and operation.
According to the analyte to be extracted, HP-LPME can be performed either in two-phase or three-phase modes. In the two-phase LPME sampling mode, analyte is extracted from an aqueous sample (donor phase) through a water-immiscible solvent immobilized in the pores of the hollow fiber into the organic solvent (acceptor phase) present inside the hollow fiber (Figure 2). In the three-phase LPME sampling mode, analyte is extracted from an aqueous solution (donor phase) through the organic solvent immobilized in the pores of the hollow

Analytes	Sample	Extractant solvent	Volume of organic solvent (μL)	Stiring speed (rpm)	Extraction time (min)	Detector	LOD/LOQ	Recovery %	EF	References
Organochlorine (1) Triazole (1) Azole (2)	Water (river) and wine (red wine)	Toluene	1.6	800	30	GC-ECD	LOD: 0.0009-0.0960 μg/L LOQ: n.r.	80-102	57-105	Liu et al., 2006
Organophosphosphate (7)	Juice (orange)	Toluene	1.6	400	15	GC-FPD	LOD: 0.98-2.20 μg/L LOQ: n.r.	76-108	n.r.	Zhao et al., 2006
Organophosphosphate (6)	Water (lake) and juices (apple, orange and pear)	Toluene	1.5	600	20	GC-FPD	LOD: 0.21-0.56 μg/L LOQ: n.r.	78-114	n.r.	Xiao et al., 2006
Organophosphate (13)	Water (farm, river and well)	Carbon tetrachloride	0.9	1300	40	GC-FPD	LOD: 0.002-0.020 μg/L LOQ: n.r.	91-104	552-831	Ahmadi et al., 2006
Triazine (1) Organophosphate (2) Thiadiazole (1) Benzaniliide (1)	Water	Carbon tetrachloride	3.0	n.a.	10	HPLC-UV	LOD: 0.6-4.0 μg/L LOQ: n.r.	77-106	n.r.	He & Lee, 2006
Organochlorine (9)	Vegetable (cabbage, cauliflower, Chinese cabbage)	Acetone:p xylene (2:8v/v)	1.0	400	30	GC-MS	LOD: 0.05-0.20 μg/L LOQ: n.r.	63-100	3-232	Zhang et al., 2008
Organophosphate (9) Anilinopyrimidine (1) Dicarboximide (1) Triadiazine (1) Strobilurin (1) Juvenile hormone mimic (1)	Vegetables (courgette, tomato)	Toluene	1.6	350	25	GC-NPD/ECD	LOD: 0.03-30 μg/kg LOQ: n.r.	28-109	100-400	Amvrazi & Tsiropoulos 2009
Organochlorine (18)	Water and wastewater	Toluene	2.0	380	37	GC-MS	LOD: 0.022-0.101 μg/L LOQ: 0.074-0.337 μg/L	41-107	3-232	Cortada et al., 2009a

EF, enrichment factor; n.r., not reported; n.a., no adjustment

Table 1. Applications of SDME in the extraction of pesticide residues.

fiber (organic phase) into another aqueous phase (acceptor phase) present inside the lumen of the hollow fiber (Figure 2). The organic phase serves in this case as a barrier between the acceptor and the donor aqueous solutions, preventing mixing of these two phases. Whereas two-phase mode has been mainly used for hydrophobic compounds, further analyzed by GC, three-phase mode has been preferably used for ionisable compounds, using LC or capillary electrophoresis (CE) as analytical techniques (Psillakis & Kalogerakis, 2003).

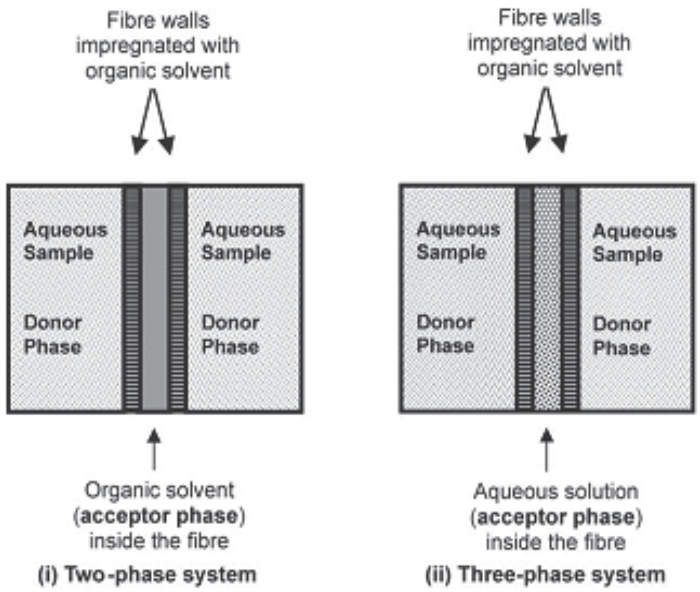

Fig. 2. Schematic illustration of 2- and 3-phase LPME (from Pedersen-Bjergaard & Rasmussen, 2008).

HP-LPME even providing high enrichment, an easy cleanup, low solvent consumption and making possible the direct analysis by chromatography of the acceptor phase requires long extraction times, which is perhaps the major disadvantage of the technique. Normally, extraction time range between 15 and 45 min for sample volumes below 2 mL, whereas for 1 L samples even 2 h may be required to reach equilibrium (Pedersen-Bjergaard & Rasmussen, 2008).

Recently, some proposals have been made in order to speed up the throughput of the procedure, either by treating many samples in parallel, carrying out the extraction under non-equilibrium condition (Ho et al. 2002), or using the so called dynamic hollow fiber protected liquid phase microextraction (DHFP-LPME). The latter technique was successful applied by Huang & Huang (2006) in the extraction of OCPs from green tea leaves and ready-to-drink tea prior to GC–ECD analysis. In this work, six OCPs (heptachlor, aldrin, endosulfan, *p,p'*-DDE, dieldrin and *o,p'*-DDT) were extracted and concentrated to a volume of 3 μL of organic extracting solvent (1-octanol) confined within a 1.5 cm length of hollow fiber. The effects of extractant solvent, extraction time and temperature, sample agitation, plunger speed, and salt concentration on the extraction performance were investigated. Good enrichments were achieved (34–297 fold) with this method, and good repeatabilities of extraction were obtained, with RSDs below 12.57%. Detection limits were below 1 μg/L for ready-to-drink tea and below 1 μg/g for green tea leaves. The application of HP-LPME to a

large number of pesticides representatives of several chemical classes was reported by Bolaños et al. 2008. In this study 50 pesticides were extracted from alcoholic beverages (wine and beer) to a volume of 5 µl of organic extracting solvent (1-octanol) confined within a 2 cm length of hollow fiber followed by ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS), without any further clean-up step. Using optimized conditions, low detection limits (0.01–5.6 µg/L) and acceptable linearity (R^2 > 0.95) were obtained. Recently, a liquid-phase microextraction (LPME) based on polypropylene hollow fiber was evaluated for the extraction of the fungicides (thiabendazole, carbendazim and imazalil) from orange juices (Barahona et al., 2010). Each sample aliquot (3 mL) was previously alkalinized with NaOH until reach a pH of 10-11, and the analytes were further extracted through a supported liquid membrane (SLM) of 2-octanone into 20 µL of a stagnant aqueous solution of 10 mM HCl inside the lumen of the hollow fibre. Subsequently, the acceptor solution was directly subjected to analysis by LC-MS and capillary electrophoresis (CE). The LC-MS provided better sensitivity than CE allowing a LODs below 0.1 µg/L.

As described in the works above mentioned several parameters should be optimized in order to obtain the maximum efficiency such as i) fiber, ii) organic solvent, iii) extraction time, iv) temperature, v) agitation, vi) ionic strength and vii) pH (Psillakis & Kalogerakis, 2003). i) Fiber: the fiber should be hydrophobic and compatible with the organic solvents used. Such requirements are met by fibers based on polypropylene; most of them have 600 mm of inner diameter, compatible with the volumes (µL) of the acceptor solution required for microextraction. ii) Organic solvent: a fundamental step in the optimization of the LPME methods is the selection of the organic solvent. Some properties need to be considered in their choice including: water-immiscibility, to prevent the organic phase dissolution in the aqueous (donor) phase; low volatility, to avoid organic phase loss during extraction; compatibility with the fiber used; easy immobilization within the pores of the hollow fiber; and high solubility for target analytes. iii) Extraction time: mass-transfer is a time-dependent process, increasing with the time of extraction. In practice to ensure high sample throughputs sampling times are shorter than the total chromatographic run time. iv) Agitation: agitation of the sample is routinely applied to accelerate the extraction kinetics. Increasing the agitation rate of the donor solution enhances extraction, the diffusion of analytes through the interfacial layer of the hollow fiber is facilitated, and the repeatability of the extraction method is improved. v) Temperature: with increasing temperature, the diffusion coefficients also increase in response to decreased viscosity. Thus the time required to reach equilibrium decrease. On the other hand, partition coefficients for the acceptor phase decrease, reducing the amount of analyte extracted. Therefore, the speed of extraction could be improved at costs of a loss of sensitivity. Typically, LPME is performed at room temperature in order to avoid possible bubbles problems and evaporation of the solvent during extraction, since the amount of solvent used is very small (20 µL). vi) Ionic strength: depending on the nature of the target analytes, addition of salt to the sample solution can decrease their solubility and therefore enhance extraction because of the salting-out effect, in particular for polar analytes. Among the salts mainly used sodium chloride is the most common. vii) pH: sample pH is crucial for efficient extraction of acidic and basic analytes. pH adjusting results in a greater ratio of distribution, ensures high enrichment factors and high recovery of the analytes of interest. Adjustments in pH can increase the extraction efficiency, since both the balance dissociation and the solubility of acids and bases are directly affected by sample pH.

HP-LPME provides in general an acceptable sensitivity in the analysis of pesticide residues. However, extraction procedure requires the presence of the analytes in liquid solutions, being its application usually restricted to liquid samples. Moreover the technique is difficult or even impossible to automate, the time of extraction could be considered too long and the operator skills should be high in order to get reproducible results.

2.1.4 Dispersive liquid-liquid microextraction (DLLME)

Dispersive liquid-liquid microextraction (DLLME) was developed by Assai and co-workers in 2006 (Rezaee et al., 2006). Consists in the rapid addition to an aqueous sample (in a conical test tube) of a mixture of two selected solvents (few microliters of a water-immiscible high density extractant solvent jointly with a dispersive solvent with high miscibility in both extractant and water phases). The aim is to form a cloudy solution of small droplets of extractant solvent which are dispersed throughout the aqueous phase. In consequence of the very large surface area formed between the two phases, hydrophobic solutes are rapidly and efficiently enriched in the extractant solvent and, after centrifugation, they can be determined in the phase settled at the bottom of the tube. The resultant sedimented phase is read for direct analysis by GC or LC.

Since its introduction, DLLME has gained popularity as a simple, fast and reliable tool for sampling preparation of a variety of analytes, as can be seen in recent reviews (Xiao-Huan et al., 2009; Ojeda & Rojas, 2009; Rezaee et al. 2010; Herrera-Herrera et al., 2010). DLLME has extensively been used for direct extraction of pesticides from aqueous samples such water, fruit juice and wine (Table 2). The first study using DLLME in pesticide residues was applied in the extraction of 13 OPPs (phorate, diazinon, disolfotane, methyl parathion, sumithion, chloropyrifos, malathion, fenthion, profenphose, ethion, phosalone, azinphose-methyl, co-ral) from river water (Berijani et al., 2006). In this study a mixture of 12.0 μL of chlorobenzene (extractant solvent) and 1.00 mL of acetone (dispersive solvent) was rapidly injected in 5 mL of aqueous sample. The sedimented phase (about 5 μL) collected after centrifugation (2 min at 5000 rpm) was analyzed by GC-FPD. Some important parameters, such as kind of extractant and dispersive solvents and their volumes, extraction time, temperature and salt effect were investigated. Under the optimized conditions, enrichment factors and extraction recoveries were high, ranging between 789–1070 and 78.9–107%, respectively. LODs ranged between 3 and 20 pg/mL for most of the analytes. Other classes of pesticides were extracted by DLLME from water such as triazine herbicides, amide herbicides, phenylurea herbicides, organochlorines, pyretroids and carbamates (Table 2). In most of the reported studies only one chemical class of pesticides was evaluated, being the number of pesticide residues scarce (less than eighteen analytes). However, in a recent publication different classes of pesticides namely triazole fungicides, isoxazolidinone herbicides and carbamates were simultaneously evaluated, although the number of analytes pertaining at each class has been reduced (three) (Caldas et al., 2010). After optimization of the parameters that influence the extraction efficiency, such as the type and volume of the dispersive and extractant solvents, extraction time, speed of centrifugation, pH and addition of salt, the extraction of pesticide residues from 5 mL of water was achieved with a mixture of 2.0 mL acetonitrile (dispersive solvent) containing 60 μL of carbon tetrachloride (extractant solvent), followed by centrifugation at 2000 rpm for 5 min; the analysis was performed by LC-MS/MS. The recoveries of pesticides in water at spiking levels between 0.02 and 2.0 μg/L ranged from 62.7% to 120.0%. RSDs varied between 1.9% and 9.1%. LOQs of the method considering a 50-fold preconcentration step were 0.02 μg/L. The LODs of the method were not reported in this study.

The application of the DLLME procedure in the extraction of pesticide residues in food samples is reported in only few papers, probably due to the complexity of food matrices (Table 2). Montes et al. (2009) used DLLME for preconcentration of seven fungicides (metalaxyl-M, penconazole, folpet, diniconazole, propiconazole, difenoconazole and azoxystrobin) in wine samples after extraction with SPE. A direct use of DLLME as extraction procedure followed by GC-MS analysis was performed by Cunha et al. (2009) to determine 24 pesticide residues, belonging at eight different chemical classes, in juice fruits. In order to avoid the precipitation of some components of the matrix, which make unsuitable the application of DLLME as referred by Montes et al (2009), samples were centrifuged prior extraction. As can be seen in Figure 3, the optimized DLLME procedure

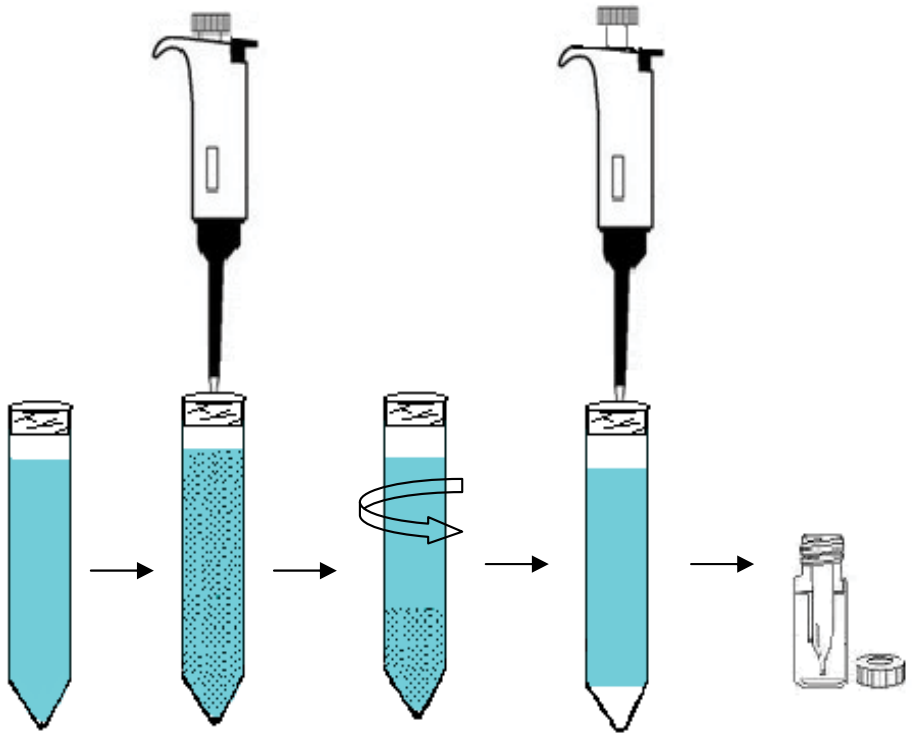

Fig. 3. Diagram of the dispersive liquid–liquid microextraction procedure used by Cunha et al. (2009).

consisted in the formation of a cloudy solution promoted by the fast addition to the sample (5 mL) of a mixture of carbon tetrachloride (extractant solvent, 100 µL) and acetone (dispersive solvent, 400 µL). The tiny droplets formed and dispersed were sedimented (85 µL) in the bottom of the conical test tube after centrifugation at 2000 rpm for 2 min. More than the parameters that influence the extraction efficiency of DLLME such as type and

volume of extractant solvent, type and volume of dispersive solvent and salt addition, other factors that could restrict the analytical performance, such as matrix effects or robustness of the method were evaluated according the Sanco guidelines (2007). Under the optimized conditions mean recoveries for apple juice spiked at three concentration levels ranged from 60% to 105% and the intra-repeatability ranged from 1% to 21%. The LODs of the 24 pesticides ranged from 0.06 to 2.20 μg/L. In 2 of a total of 28 analysed fruit juice samples residues of captan were found, although at levels below the maximum legal limit established by European Union (Figure 4).

DLLME is more suitable for the extraction of analytes from aqueous samples; nonetheless, some authors have applied this process in solid samples after an adequate pretreatment. Zhao et al. (2007) applied DLLME as a concentration procedure after a previous extraction with QuEChERS of OPPs (ethoprophos, parathion methyl, fenitrothion, malathion, chlorpyrifos and profenofos) from watermelon and cucumber. Hence, 1 mL of the extract obtained after homogenization of 10 g of sample with 10 mL of acetonitrile, 4 g $MgSO_4$, and 1 g NaCl, was added with 27 μL of chlorobenzene and rapidly injected in 5 mL of water. Then 1 μL of 18 μL of sedimented phase obtained by centrifugation of the mixture at 4000 rpm for 3 min was analyzed by GC-FPD. The optimized method allowed recoveries between 67 and 111%, repeatability between 2 and 9% and LODs ranging from 0.010 to 0.190 μg/kg, for all the target pesticides. In other study, Zang et al. (2008) applied the DLLME procedure directly in the extraction of captan, folpet and captafol from apples. The developed procedure consisted in the injection of a mixture containing chlorobenzene (extractive), and acetone (dispersive) directly into an aqueous extract of apple samples, obtained after homogenization with a solution of zinc acetate dehydrate and dilution with water. Under the optimum conditions, high enrichment factors for the targets were achieved ranging from 824 to 912. The recoveries of fungicides in apples ranged from 93.0 to 109.5% and the RSD ranged from 3.8 to 4.9%. The LODs were between 3.0 and 8.0 μg/kg.

To date, the majority of the applications related to DLLME involve the use of solvents of high, density commonly chlorinated solvents (e.g. chlorobenzene, carbon tetrachloride and tetrachloroethylene) as extractant solvents. However, the use of ionic liquids (IL) as extractants has been found to be especially important in DLLME as well as in other microextraction procedures (in order to replace the volatile ones used during sample preparation procedures) because of their negligible vapor pressure, good solubility for organic and inorganic compounds, no flammability, high thermal stability, wide temperature range as a liquid phase, etc. (Han & Armstrong, 2007; Ravelo-Pérez et al., 2009). One of the main drawback of the use of IL in DLLME is the impossibility to make use of GC in the analysis, due to the adverse effects of these solvents in the chromatographic system. IL-DLLME has been applied in the extraction of a high variety of pesticides in water and food matrices such as fruits and honey, as can be seen in Table 2. DLLME based on IL was initially applied by Zhou et al. (2008a), to extract five pyrethroid pesticides (cyhalothrin, deltamethrin, fenvalerate, taufluvalinate and biphenthrin) in different types of water samples (tap, river and reservoir water, and groundwater). In this study, the sample (10 mL) was heated at 80 ºC after addition of 45 μL of 1-hexyl-3 methylimidazolium hexafluorophosphate [C6MIM][PF6]. The IL mixed with the solution entirely at this temperature and thereafter the solution was cooled with ice-water for a certain time. The IL and the aqueous phase were separated after centrifugation and the IL phase injected into the

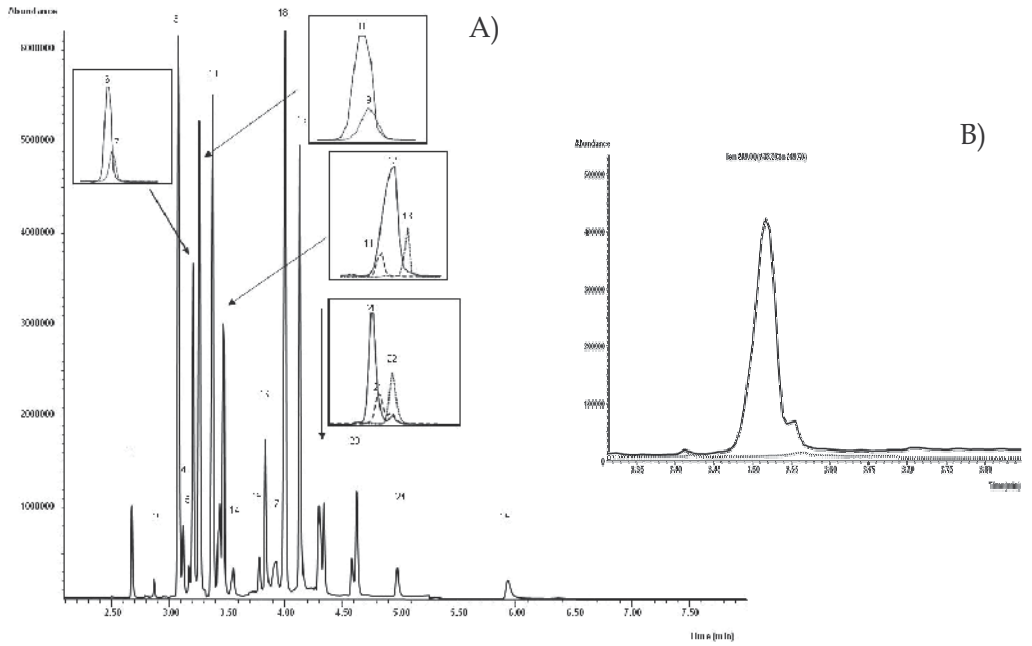

Fig. 4.A) Chromatogram of spiked blank apple juice with 24 OPPs B) Overlay of extracted ion chromatograms in SIM mode for captan (ion 149) obtained in not contaminated (- - -) and contaminated (—) (0.541 µg/L) apple juice samples using DLLEM extraction and MDGC-MS analysis (from Cunha et al., 2009).

HPLC-UV. In this study good recoveries were obtained (76.7– 135.6%) and LODs were in the range 0.28–0.6 µg/L. In a further work, the same group used a similar procedure, using [C6MIM][PF6] as extractant solvent in DLLME at 80ºC for determine traces of methylparathion and phoxim in water (Zhou et al., 2008b). A new IL-DLLME procedure was introduced by Liu et al. (2009) for the extraction of four insecticides (fipronil, chlorfenapyr, buprofezin, and hexythiazox) from water. The proposed procedure combined extraction and concentration of the analytes into one step, avoiding heating and cooling steps, so reducing extraction time. Thus, a mixture of 0.052 g [C6MIM][PF6] and 0.50 mL methanol (dispersive solvent) was quickly injected into the sample (5.0 mL). Then, the mixture was centrifugated at 4000 rpm for 10.0 min, and 19 µL of sedimented phase were diluted with 50 µL methanol and 10 µL of the misture analysed by HPLC-UV. Under the optimized conditions, good enrichment factors (209–276) and accepted recoveries (79–110%) were obtained for the extraction of the target analytes in water samples. The LODs for the four insecticides ranged from 0.53 to 1.28 µg/L.

The application of IL-DLLME to solid samples is scarce as referred above for the classical DLLME. Usually, it is necessary a previous pretreatment of the sample in order to obtain an aqueous extract before extraction. In a recent work Wang et al. (2010) developed an IL-DLLME/HPLC-UV method for the extraction and determination of triazines in honey. A mixture of 175 µL of [C6MIM][PF6] (extractant solvent) and 50 µL of 10% Triton X 114 (dispersive solvent) was rapidly injected into 20 mL aqueous honey sample, obtained by dissolution of 2 g of honey with 20 mL of water. The detection limits for chlortoluron,

prometon, propazine, linuron and prebane were 6.92, 5.84, 8.55, 8.59 and 5.31 μg/kg, respectively.

Another type of extractant solvents used in DLLME are low density solvents such as undecanol, 1-dodecanol, 2-dodecanol and n-hexadecane, which are usually less toxic than the chlorinated solvents. An interesting work was developed by Leong & Huang (2009) for the determination of OCPs in water samples. The method is based on the solidification of a floating organic drop (DLLME-SFO) and it is combined with GC-ECD. The dispersive solvent (200 μL of acetonitrile) containing 10 μL of hexadecane (HEX) was rapidly injected into 5.0 mL water sample. After centrifugation, the fine HEX droplets (6±0.5 μL) floating at the top of the screw-capped tube were solidified through ice and then transferred into a vial to be injected into GC. Under optimum conditions, enrichment factors and extraction recoveries are high ranging between 37–872 and 82.9–102.5%, respectively. LODs ranged between 0.011 and 0.110 μg/L for most of the analytes. Recently Chen et al. (2010) reported a low-density extractant solvent-based, termed solvent terminated (ST) DLLME to determine carbamate pesticides (carbofuran, tsumacide, isoprocarb, and pirimicarb) in water by GC-MS/MS. Hence, 0.50 mL of acetonitrile containing 15 μL of toluene were rapidly injected in 5 mL of water. After dispersing, the obtained emulsion was quickly cleared into two phases when an aliquot of acetonitrile (0.5 mL) was introduced as a chemical demulsifier into the aqueous bulk. Therefore, the developed procedure does not need centrifugation to achieve phase separation. Under the optimized conditions, the LODs for all the target carbamate pesticides were in the range of 0.001–0.50 μg/L and the precisions were in the range of 2.3–6.8%.

In order to achieve such a wide range of applications, several parameters have to be taken into account to optimize DLLME to extract pesticide residues, such as i) type and volume of extractant solvent, ii) type and volume of dispersive solvent, iii) extraction time, and iv) effect of salt addition. i) Extractant solvent: the extractant solvents should be immiscible with water, and they must possess both good solubility for analytes and good chromatographic behavior. They can either have higher or lower density than water and the volume used ranged between 10 to 100 μL. Lower volumes of extractant solvent enhance enrichment factor, although reducing the volume of sedimented phase, could give problems of reproducibility. ii) Dispersive solvent: the dispersive solvent should be miscible with both aqueous sample and extractant solvent and possess the capacity to decrease the interfacial tension of extractant solvent in order to make the droplet size smaller, increasing the extraction efficiency. Acetone, methanol and acetonitrile can be used as dispersive solvents at volumes ranging from 0.5 mL to 2 mL. iii) Extraction time: in DLLME after mixture of the three components (sample, extractant and dispersive solvent) the equilibrium is achieved in few seconds due to the large contact surface between tiny drops of extractant solvent and the sample. Nevertheless, in most of the studies the extraction time ranged from 1 to 5 min. iv) Salt addition: salt addition can improve extraction yield in DLLME, particularly for those analytes with lower solubility, as a result of a "salting out" effect. This effect is prevailing in DLLME when NaCl is employed.

DLLME has generally showed a very good performance to extract pesticide residues from water and aqueous extracts of food samples, but it is desirable to extend this application to more complex matrices and to a large number of pesticide residues using standard guidelines for the validation of the methods.

Analytes	Sample	Extractant solvent	Dispersive solvent	Detector	LOD/LOQ	Recovery %	EF	References
Organophosphate (13)	Water (river and farm)	Chlorobenzene (12 μL)	Acetone (1.0 mL)	GC-FPD	LOD: 0.003-0.020 μg/L LOQ: n.r.	93-118	789-1070	Berijani et al., 2006
Organophosphate (6)	Watermelon and cucumber	Chlorobenzene (27 μL)	Acetonitrile (1.0 mL)	GC-FPD	LOD: 0.5-20 μg/kg LOQ: n.r.	67-111	41-50	Zhao et al., 2007
N-methylcarbamate (1)	Water (river and lake)	Tetrachloroethane (20 μL)	Methanol (0.5 mL)	HPLC-UV	LOD: 1.0 μg/L LOQ: n.r.	93-97	70.7	Wei et al., 2007
Organophosphate (1) Phathalimide (1) Carboximide (1)	Apples	Chlorobenzene (9 μL)	Acetone (1.0 mL)	GC-ECD	LOD: 3.0-8.0 μg/kg LOQ: n.r.	93-110	824-912	Zhang et al., 2008
Pyrethroid (3)	Water (river, well and tap)	Chlorobenzene (15 μL)	Acetone (1 mL)	GC-ECD	LOD: 0.04-0.10 μg/L LOQ: n.r.	76-116	708-1087	Xiao-Huan et al., 2008
Pyrethroid (5)	Water (tap, river, reservoir and ground)	$[C_6MIM][PF_6]$ (45 μL)	n.a.	HLPC-UV	LOD: 0.28-0.6 μg/L LOQ: n.r.	77-136	n.r.	Zhou et al., 2008a
Organophosphate (2)	Water (rain, river, reservoir and ground)	$[C_6MIM][PF_6]$ (50 μL)	n.a.	HLPC-UV	LOD: 0.17-0.29 μg/L LOQ: n.r.	88-104	50	Zhou et al., 2008b
Organophosphate (5) Carbamate (1)	Water (tap, lake, river, well and farm) green tea and tea leaves	Carbon tetrachloride (10 μL)	Methanol (0.8 mL)	GC-FPD	LOD: 0.21-3.05 μg/L LOQ: n.r.	79-117	176-946	Xiong & Hu, 2008

Table 2. Applications of DLLME in the extraction of pesticide residues

Analytes	Sample	Extractant solvent	Dispersive solvent	Detector	LOD/LOQ	Recovery %	EF	References
Organophosphate (13) Pyrethroid (3) Phathalimide (1) Dicarboximide (1) Phenylamide (1) Cyclodiene (1) Anilino-pyrimidine (1) Strobilurin (1)	Juice (apple)	Carbon tetrachloride (100 µL)	Acetone (0.4 mL)	GC-MS	LOD: 0.06-2.20 µg/L LOQ: 0.2-7.3 µg/L	60-105	35-62	Cunha et al., 2009
Carbamate (5)	Water (surface)	Trichloromethane (40 µL)	Acetonitrile (1 mL)	HPLC-DAD	LOD: 0.1-0.4 µg/L LOQ: n.r.	86-97	80-177	Liu et al., 2009b
Phenyurea (8)	Water (river, tap and well)	Carbon disul de (103 µL) and toluene (45 µL)	Acetone (2 mL)	HPLC-UV/DAD	LOD: 0.01-0.5 µg/L LOQ: n.r.	86-109	11-118	Saraji & Tansazan, 2009
Carbamate (1) Organophosphate (1)	Water (river and tap) and juice (apple, peach and grape)	Tetrachloroethane (15 µL)	Acetonitrile (1.0 mL)	HPLC-FLD	LOD: 0.0123-0.016 µg/L LOQ: n.r.	80-118	n.r.	Fu et al., 2009
Organochlorine (5)	Water (river, sea and reservoir)	Tetrachloroethylene (5.2 µL)	tert-butyl methyl ether (7.8 µL)	GS-MS	LOD: 0.0004-0.0025 µg/L LOQ: n.r.	54-119	1885-2648	Tsai & Huang, 2009
Pyrazole (1) Thiazolidine (2) Pyrrole (1)	Water (tap, lake and fountain)	$[C_6MIM][PF_6]$ (0.052 g)	Methanol (0.5 mL)	HPLC-DAD	LOD: 0.53-1.28 µg/L LOQ: n.r.	79-106	209-276	Liu et al., 2009a
Organophosphate (4)	Water (river, tap, rain and well)	$[C_8MIM][PF_6]$ (35 µL)	Methanol (1.0 mL)	HPLC-UV	LOD: 0.1- 5.0 µg/L LOQ: n.r.	87-118	200	He et al., 2009

Table 2. Applications of DLLME in extraction of pesticide residue (cont.)

Analytes	Sample	Extractant solvent	Dispersive solvent	Detector	LOD/LOQ	Recovery %	EF	References
Benzimidazole (1) Carbamate (2) Dicarboximide (1) Quinazoline (1) Triazole (1) Diphenyl ether (1) Thiazolidine (1)	Bananas	[C_6MIM][PF_6] (88 mg)	Methanol (0.714 mL)	HPLC-DAD	LOD: 0.320-4.66 μg/kg LOQ: n.r.	53-97%	n.r.	Ravelo-Pérez et al., 2009
Organochlorine (18)	Water (river, surface and tap) and wastewater	Tetrachloroethylene (10 μL)	Acetone (1.0 mL)	GC–MS	LOD: 0.001-0.025 μg/L LOQ: n.r.	56-120	46-316	Cortada et al., 2009b
Organophosphate (1) Strobilurin (1) Phenylamide (1) Triazole (1) Conazole (1) Azole (1)	Wine (red and white)	1,1,1-trichloethane (100 μL)	Acetone (1 mL)	GS-MS	LOD: n.r. LOQ: 0.020-.020 μg/L	78-107	156-254	Montes et al., 2009
Organochlorine (6)	Water (lake and tap)	Hexadecane (10 μL)	Acetonitrile (0.2 mL)	GC-ECD	LOD: 0.011–0.109 μg/L LOQ: n.r.	83-103	37-872	Leong & Huang, 2009
Organophosphate (10)	Tea	N-hexane (24 μL)	Acetonitrile (0.5 mL)	GC-FPD	LODs: 0.030-1.00 μg/kg LOQ: n.r.	83-117	n.r.	Moinfar & Hosseini, 2009
Triazole (2) Carbamate (1)	Water (tap)	Carbon tetrachloride (60 μL)	Acetonitrile (2.0 mL)	LC-MS/MS	LOD: n.r. LOQ: 0.02 μg/L	63-120	50	Caldas et al., 2010
Carbamate (4)	Water (lake)	Toluene (50 μL)	Acetonitrile (1.0 mL)	GS-MS	LOD: 0.001-0.050 μg/L LOQ: n.r.	95-104	n.r.	Chen et al., 2010
Triazine (5)	Honey	[C_6MIM][PF_6] (175 μL) and 10% Triton X 114 (50 μL)	n.a	HPLC-DAD	LOD: 5.31–8.59 μg/kg LOQ: n.r.	60-133	n.r.	Wang et al., 2010

EF, enrichment factor; n.r., not reported; n.a. no adjustment

Table 2. Applications of DLLME in extraction of pesticide residue (cont.)

2.2. Analysis

The determination of pesticide residues in water and food matrices has traditionally been performed by GC, due the high number of theoretical plates of the columns employed and the variety and selectivity capabilities of the detectors than can be coupled such as ECD, NPD, and FPD. Among the detectors used, MS is the preferred tool for determination of multi class pesticide residues because it permits: i) the simultaneous quantification and identification of detected analytes; ii) the detection of a wide range of analytes independently of its elemental composition; iii) mass-spectrometric resolution of co-eluting peaks; and iv) potentially faster analysis time (Cunha et al., 2010).

To increase sample throughput during GC analysis, which would consequently reduce the laboratory operating costs, several approaches were evaluated such as the reduction of: column length, column inner diameter or column stationary film thickness; and the utilization of fast temperature programming, low-pressure and multicapillary columns (Maštovská & Lehotay 2003). In practice a combination of two or more approaches is very often applied to enhance the speeding-up effect with the less sacrifice in sample capacity and/or separation efficiency. Sample capacity influences the limit of detection and the sensitivity, for example. Separation efficiency influences performance characteristics such as selectivity, detection limit (through the level of chemical noise) and, of course, accuracy of the analytical results. Multidimensional GC system with Deans switch heart-cutting represents a very interesting technical solution, which not only responds adequately to the demand of increased speed of analysis, capacity and separation efficiency, but also provided an enhancement in robustness. This technique is based essentially on the transfer of selected effluent fractions from a first to a second column for MS analysis and transfer of fractions without analytical interest to a restrictor column for waste (see Figure 5) (Cunha et al., 2009; Cunha & Fernandes, 2010). A devoted transfer device (Deans switch), situated between the two columns, enables the entire procedure.

Recently a dual GC column system involving a short wide-bore capillary column connected by a Deans switch device to a narrower and longer second chromatographic column was successful applied in determination of 24 pesticide residues in fruit juice (Cunha et al., 2009). This system allowed a gain in the speed of chromatographic analysis, providing an efficient sample injection and column introduction of the analytes with limited interferences, high sample capacity, and sharp and symmetric peak shapes without loss of resolution.

Notwithstanding the recent advances in GC-MS systems, the analysis of polar, non-volatile or/and thermally labile pesticides by this technique is limited, usually requiring chemical derivatization. LC-MS/MS has become a standard approach in developed countries to expand the range of pesticides quantified and identified in complex matrices.

3. Conclusions

Microextraction methods usually require both smaller sample size and organic solvent volumes when compared with the conventional methods. The main advantages of these procedures are the high degree of enrichment for the analytes in complex matrices, which enable detection limits down to the levels required by the regulatory bodies to the analysis of pesticide residues in water and food. Additionally, given the compatibility of the solvents used, and the low volumes involved, the procedures are easily associated with gas or liquid chromatography. Most of microextraction applications are employed in aqueous samples for the extraction of nonpolar or moderately polar high molecular weight analytes. Although

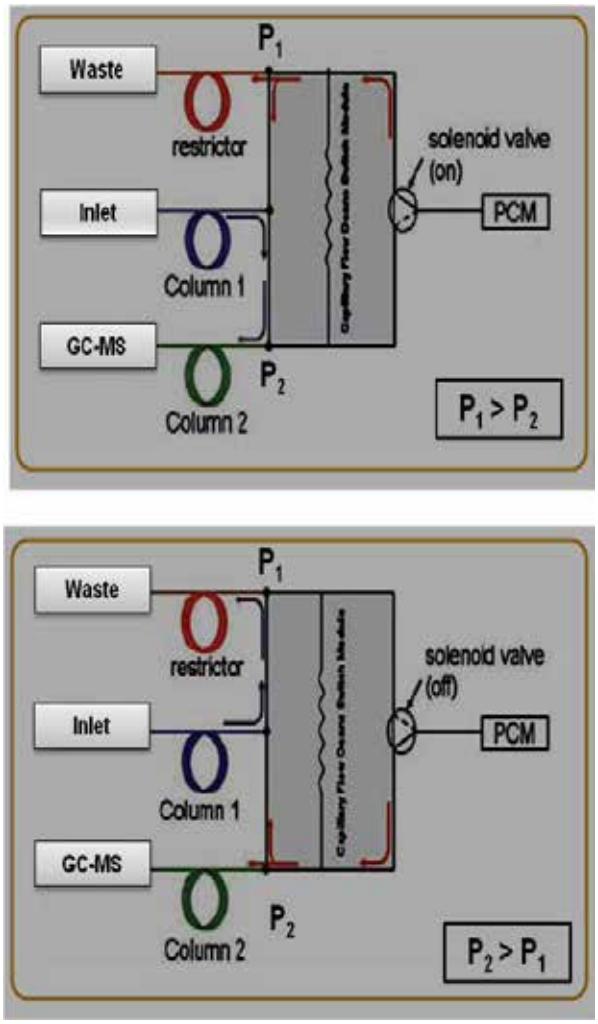

Fig. 5. Deans switch GC–MS system. (A) The solenoid valve is in the on position, allowing effluent to flow to the 2D GC separation column prior to MS detection. (B) The solenoid valve is in the off position and effluent from the primary column is flowing to the exit gas line. (Adapted from Agilent).

some attempts were made for the extraction of analytes in solid matrices and also for the extraction of polar analytes, is still expected an increment along this line in the future. On other hand, despite their high-throughput, the automation of most of microextraction procedures presented seems to be very difficult and has not yet been achieved, thus new developments in this area are required.

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2

Sample Preparation in the Analysis of Pesticides Residue in Food by Chromatographic Techniques

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1. Introduction

Food samples present an enormous challenge to analytical chemists in their efforts to determine residues of pesticides at trace levels to satisfy food safety regulations in EU, USA and Japan. The wide array of food matrices from liquids to solids require different sample preparation techniques for accurate and reproducible results with chromatographic techniques such as Gas chromatography(GC) and High Performance Liquid Chromatography (HPLC). In addition, there exists a wide range of pesticides which are used legally for crop protection and their residue content in food must be accurately monitored for safe consumption. The GC and HPLC techniques with different types of detector systems can provide such analysis at trace levels to fulfill the maximum residue levels(MRL) as per the food safety regulations in these countries. However, the accurate and reproducible results often depend upon the sample preparation techniques associated with the different food matrices.

Sample preparation has always been regarded as the bottleneck in the analytical laboratory performing numerous analyses, but it is the key to accurate analysis. In this regard, as per pesticide residue analysis, where not only the physical volume of the analyses can be enormous but also the number of pesticides involved can range from a selected few to a broad spectrum depending on the food source. This will usually necessitate the employment of different sample preparation methods for different targeted pesticides as well as the multitude of food matrices.

It has been estimated that the sample preparation step in most determinations consume approximately 60 – 70 % of the total time required for the analysis. It must be able to produce analytically accurate results and be economically efficient for routine analysis. In addition, it must be safe and easy to perform.

Most sample preparation procedures for GC and HPLC determination follow the basic steps as outlined below:

1. The food sample is homogenized or blended to obtain a uniform matrix.
2. This will be followed by extraction of the pesticide residue with solvents.
3. A cleanup step is employed to remove interfering matrix components from the GC or HPLC chromatograms.

4. The elution and/or fractionation of the extracted analytes.
5. Concentrate the eluent and re-constitute in a solvent which is compatible with the GC or HPLC conditions.
6. Finally, the solution containing the pesticide can be introduced into the GC or HPLC.

The different types of sample preparation for such analyses will be presented . These are liquid-liquid extraction, solid phase extraction, solid phase microextraction, single drop microextraction, liquid-solid extraction, microwave assisted solvent extraction, supercritical fluid extraction, dispersive liquid-liquid microextraction and accelerated solvent extraction.

2. Liquid-liquid Extraction (LLE)

Analytes in solutions or liquid samples can be extracted by direct partitioning with an immiscible solvent. Liquid-liquid extraction (LLE) is based on the relative solubility of an analyte in two immiscible phases and is governed by the equilibrium distribution/partition coefficient. Extraction of an analyte is achieved by the differences in the solubilising power (polarity) of the two immiscible liquid phases.

LLE is traditionally one of the most common methods of extraction, particularly for organic compounds from aqueous matrices. Typically a separating funnel is used and the two immiscible phases are mixed by shaking and then allowed to separate. To avoid emulsions, in some cases, a salt may be added and centrifugation can be used if necessary. Alternatively a matrix solid-phase dispersion(MSPD) approach can be used to avoid emulsions. Both layers can be collected for further analysis. To ensure the complete extraction of an analyte into the required phase, multiple extractions may be necessary. Due to the limited selectivity, particularly for trace level analysis, there is a need for cleanup or analyte enrichment and concentration steps prior to instrumental analysis.

In the case of multiresidue methods, the extracting solvent has to be suitable for the extraction of compounds within a wide polarity range from a variety of matrices containing different amounts of water, fats, sugars and other substances. The usual way for extracting pesticide residues from the sample is by thorough disintegration of the matrix in a high speed homogenizer in the presence of the solvent or solvent mixture. In this way, even the AOAC method, which is one of the most commonly instituted methods, has been modified. The original methods which were extraction with acetonitrile, followed by liquid-liquid partitioning with petroleum ether/dichloromethane and a laborious florisil column cleanup, was modified in 1985 to include acetone instead of acetonitrile (Torres *et al*., 1996).

Acetone extraction is usually preferred since it is suitable for both non-polar and polar pesticides, as has been demonstrated in many comparative studies performed by GC and HPLC. In addition, acetone has low toxicity, is easy to purify, evaporate and filter and is inexpensive. Fruit and vegetable extracts in acetone are usually cleaner than those obtained with other solvents of similar polarity (Torres *et al*., 1996).

A rapid and efficient multiresidue extraction procedure using ethyl acetate and sodium sulphate, followed by GPC on an SX-3 column, was first reported by Roos *et al*. (1987). Recoveries better than 90% were obtained for organochlorine(OC) and organophophorous (OP) pesticides, fungicides and chlorobiphenyls. The ethyl acetate and sodium sulphate extraction without further cleanup was applied as a screening method for the analysis of eight OP pesticides with varying polarities in different types of vegetables using gas chromatography coupled to the flame photometric and nitrogen-phosphorous(GC-FPD, GC-NPD) detectors. With the use of specific detectors, interfering chromatographic peaks were

reduced and the analysis time and solvent usage were also minimized, resulting in lower-cost analyses (Cai *et al*., 1995).

In another study, Castro *et al*. (2002) developed a rapid LLE method for the determination of endosulfan isomers and endosulfan-sulfate in plant samples. Tomato leaf samples were homogenized with ethyl acetate and extracts cleaned-up on an aluminium oxide column. The pesticides were eluted with a hexane-ethyl acetate (80:20, v/v) mixture. Recoveries obtained from plant samples were higher than 78% with an RSD lower than 14% and detection limits were 0.02 μg/g for each pesticide. Barriada-Pereira *et al*. (2004) compared the use of cartridges filled with four different sorbents: florisil, a tandem of florisil and alumina, silica, and carbon black to clean up plant leaf extracts prior to OC pesticides determination. Carbon black was shown to be the preferred sorbent, providing colorless eluates, cleaner chromatograms and fewer interferences. Jansson *et al*. (2004) used the National Food Administration (NFA) ethyl acetate extraction to determine 57 different pesticides and metabolites in a wide variety of fruits and vegetables by LC-MS/MS. The recoveries obtained were in the range 70 – 100%. The proposed method is quick and straightforward and no additional clean-up steps are needed.

3. Solid-phase Extraction (SPE)

Solid phase extraction (SPE) was developed in the mid-1970 as an alternative approach to LLE for separation, purification, pre-concentration and solvent exchange of solutes for solution (Thurman and Mills, 1998). SPE can be used directly as an extraction technique for liquid matrices, or as a cleanup method for solvent extracts.

An SPE method always consists of three to four successive steps, as illustrated in Figure 1. First, the solid sorbent should be conditioned using an appropriate solvent. This step is crucial, as it enables the wetting of the packing material and the solvation of the functional groups. In addition, it removes possible impurities initially contained in the sorbent or the packaging. Also, this step removes the air present in the column and fills the void volume with solvent. The nature of the conditioning solvent depends on the type of the solid sorbent. Typically, for reversed phase sorbent, methanol is frequently used, followed by water or an aqueous buffer whose pH and ionic strength are similar to that of the sample. Precautionary steps are taken to prevent the solid sorbent from drying in between the conditioning and the sample treatment steps, otherwise the analytes will not be efficiently retained giving rise to poor recoveries. If the sorbent is dry for more than several minutes, it must be reconditioned.

The second step is the percolation of the sample through the solid sorbent. Depending on the system used, the volumes used can range from 1 mL to 1 L. The sample may be applied to the column by gravity, pumping, aspirated by vacuum or by an automated system. The sample flow rate through the sorbent should be low enough to enable efficient retention of the analytes, and high enough to avoid excessive retention. During this step, the analytes are concentrated on the sorbent. Even though the matrix components may also be retained by the solid sorbent, some of them could pass through, thus enabling some purification (matrix separation) of the sample.

The third step (which is optional) may be the washing of the solid sorbent with an appropriate solvent, having low elution strength, to eliminate matrix components which have been retained by the solid sorbent, without displacing the analytes. A drying step may also be advisable, especially for aqueous matrices, to remove traces of water from the solid

sorbent. This will eliminate the presence of water in the final extract, which, in some cases, may hinder the subsequent concentration of the extract and the analysis.

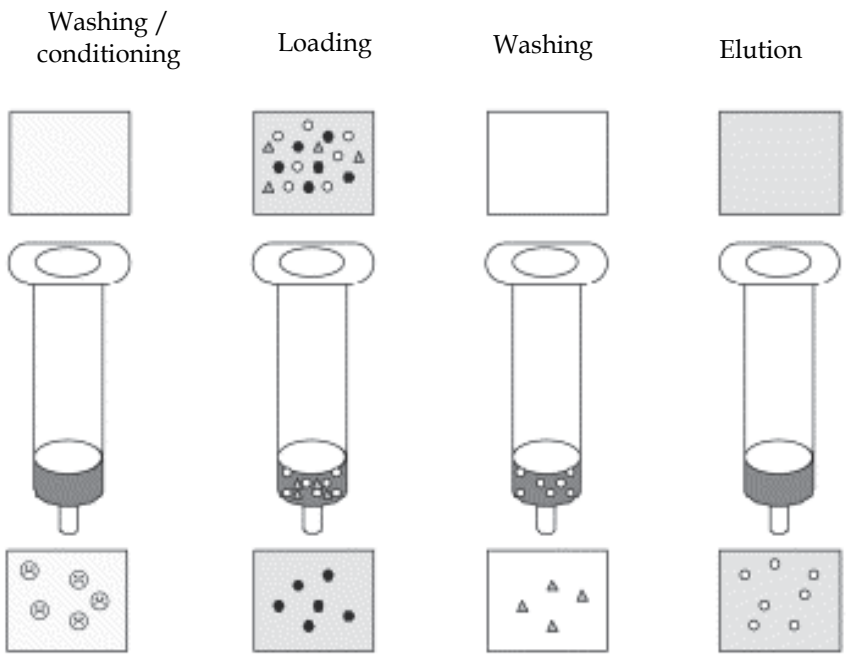

Fig. 1. SPE Operation Procedures

The final step is the elution of the analytes of interest by an appropriate solvent, without removing the retained matrix components. The solvent volume should be adjusted so that quantitative recovery of the analytes is achieved with a subsequent low dilution. In addition, the flow rate should be correctly adjusted to ensure efficient elution. It is often recommended that the solvent volume be fractionated into two aliquots, and to allow the solvent to soak into the solid sorbent before the elution.

The SPE cartridge possesses two important features, standardization and hence greater reproducibility, which includes a wide range of phases, from normal phase, reversed phase to ion-exchange materials thus enabling aqueous solutions to be treated and employing additional trapping mechanisms.

The sorbents come in different packaging: filled micro-columns, cartridge, syringe barrels and discs. The disposable sorbent containers are illustrated in Figure 2. Although the cartridges are for single use only and disposable, thus representing a significant consumable cost, this has been shown to be much lower than the cost of chemicals and the manpower needed for the corresponding traditional solvent extraction methods. Other types of SPE have also been developed, including flat disks with the stationary phase particles supported on a mesh, enabling very large volumes to be rapidly extracted. Recent use of high flow rates through extraction cartridges has been shown to give improved extraction but such "turbulent flow extractions" were very similar to conventional extractions.

Many of the published methods for pesticide determination in fresh fruits and vegetables use a combination of two or more commercially available SPE columns for cleanup in the

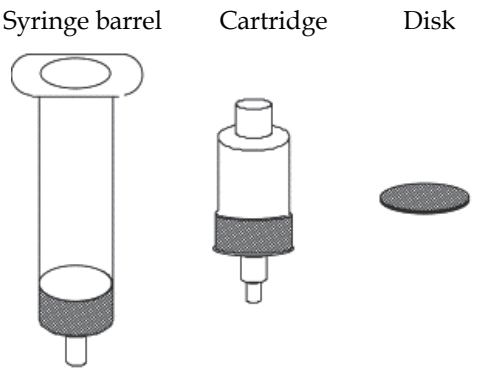

Fig. 2. Disposable SPE Sorbent Containers

normal-phase (NP) mode. Weak anion-exchange sorbents such as primary secondary amine (PSA), aminopropyl (NH_2), or diethylaminopropyl (DEA) modified silica are often used for cleanup of food samples together with strong anion-exchange sorbents (SAX) (Sharif *et al*., 2006). Other SPE cleanup approaches include the combination of GCB (graphitized carbon black) and PSA columns (Abhilash *et al*., 2007). In addition, there are other applications using reversed-phase (RP) SPE for pre-concentration / cleanup of pesticide residues from fruit and vegetable samples.

Before the SPE technique can be applied to a solid matrix such as fruits and vegetables, a separate homogenization step and often filtration, sonication, centrifugation and liquid-liquid cleanup are required. Stajnbaher and Zupancic-Kralj (2003) used solid-phase extraction on a highly cross-linked polystryrene divinylbenzene column (LiChrolut EN) for the simultaneous isolation of 90 pesticides of different physico-chemical properties from fruits and vegetables and pre-concentration of the pesticides from the water-diluted acetone extract. It only used small volumes of solvent per sample (30 ml acetone and 14 ml ethyl acetate, 6 ml methanol). The majority of pesticide recoveries for various fruits and vegetables exceeded 80% in the concentration range from 0.01 to 0.50 mg/kg.

Hernandez *et al*. (2006) determined pesticide residues in fruits and vegetables by SPE. 20 g of homogenized sample was mixed in 60 mL of methanol:water (80:20, v/v) 0.1% HCOOH. The mixture was extracted for 2 min at 8000 rpm and filtered. The filtrate was diluted with MeOH:H2O (80:20, v/v) 0.1% HCOOH to 100 mL. Then, the extract was diluted eight times with LC-grade water. 2.5 mL aliquot was taken and diluted to 20 mL. 5 mL of diluted extract was passed through a SPE cartridge, preconditioned with 5 mL of MeOH, 5 mL of methanol:methyl tert buthyl ether (10:90, v/v) 0.1% HCOOH, 5 mL of MeOH 0.1% HCOOH and finally with 5 mL of acidified water. The cartridge was dried for 1 hour before the sample was loaded and eluted with 5 mL MeOH:MTBE (10:90, v/v) 0.1% HCOOH. The recoveries ranged from 70% to 110% with satisfactory precision (< 15%) that could be used for the accurate determination of 52 pesticides and metabolites in one single determination step at the 0.01 mg/kg level.

Shimelis *et al*. (2007) evaluated the performance of different sorbents for cleanup of oily vegetable matrices. The authors highlighted the use of a dual-layer SPE, using a combination of PSA with GCB for sample cleanup during multi-residue pesticide screening of agricultural and food products. The retention of fatty acids by the PSA sorbent was quantified and the effect of the elution solvent on the retention of fatty acid on the SPE

cartridge was evaluated. The use of stronger elution solvents to elute certain pesticides from graphitized carbon was shown to interfere with the capacity of PSA to bind fatty acids. The ability of the PSA to retain fatty acids was found to be highly dependent on the conditioning and elution protocols. A necessity to use a stronger solvent in order to elute polar pesticides from dual-layer GCB/PSA SPE weaken the capacity of the PSA for removal of fatty acids from the samples. Therefore, practical applications of dual-layer GCB/PSA cartridges should be limited to food samples with lower levels of fatty acids.

Stajnbaher and Zupancic-Kralji (2008) determined 24 pesticides representing different chemical classess (OPPs, OCPs, carbamates and pyrethroids) in fruits and vegetables using GC-MS. The samples were homogenized and extracted by adding 18mL acetone. The sample was thoroughly mixed in a vortex for 2 min and the extract was centrifuged for 10 min at 4000 rpm. The supernatant was transferred to a volumetric tube and acetone:water (2:1, v/v) mixture was added. After shaking, a 2.5 mL aliquot corresponding to 1g of sample was transferred to a 15 mL reservoir of a funnel shaped SPE column filled with 150 mg of LiChrolut EN sorbent which was washed with 4 mL of ethyl acetate and preconditioned with 4 mL of methanol followed by 5 mL of deionized water. The pesticides retained were then eluted with 4 mL of ethyl acetate. Recoveries were found to be between 70% and 110% for most of the pesticides. It was reported that the miniaturized SPE method in connection with programmed temperature vaporizing (PTV)-based large volume injection was faster in sample preparation and economically beneficial as it reduced the costs of the SPE material and use of solvents.

In comparison with traditional liquid extraction techniques, SPE is simpler, more convenient, and easier to automate. In addition, SPE possesses other distinct advantages including: (1) requires a lower volume of solvent than traditional liquid-liquid extractions; (2) involves simple manipulations which are not time consuming and makes it possible for field treatment of samples; (3) the SPE cartridges can be used for short-term storage of the compounds; (4) provides high enhancement factors proportional to the volume of water passed through the SPE cartridges. One of the drawbacks of the SPE method is that the packing must be uniform to avoid poor efficiency and although the pre-packed commercial cartridges are now considered reliable, solid and oily components in a sample matrix may plug the SPE cartridge or block pores in the sorbent causing it to become overloaded and also automated systems can have difficulties with reproducibility for some sample types. The sample matrix can also affect the ability of the sorbent to extract the analyte due to competition for retention. Many traditional sorbents are limited in terms of selectivity and insufficient retention of very polar compounds can pose a problem. The use of hydrophilic materials for the improved extraction of the more polar compounds by SPE was detailed by Fontanals *et al*. (2005). A comprehensive review, covering trends, method development, coupled with liquid chromatography and different types of SPE sorbent materials was published by Hennion (1999) and some examples of the use of SPE in food analysis were given in a review by Buldini *et al*. (2002).

4. Solid-phase Microextraction (SPME)

Solid-phase microextraction (SPME), was developed by Pawliszyn and co-workers in 1990 in an attempt to redress the limitations inherent in SPE and LLE (Kataoka et al., 2000). It is a new sample preparation technique using a fused-silica fiber that is coated on the outside with an appropriate stationary phase. The analyte in the sample is directly extracted and

concentrated onto the fiber coating. The method saves preparation time, solvent usage and disposal costs, and can improve the detection limits (Pawliszyn, 1997). It has been used routinely in combination with GC and HPLC, and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semivolatile organic compounds from environmental, biological and food samples (Eisert and Levsen, 1996; Pawliszyn, 1997; Prosen and Zupancic-Kralj, 1999). The main advantages of SPME extraction compared to solvent extraction are the reduction in solvent use, the combination of sampling and extraction into one step and the ability to examine smaller sample sizes. It can also have high sensitivity and can be used for polar and non-polar analytes in a wide range of matrices by linking to both GC and LC.

There are currently three SPME modes that require either fused-silica fibers or GC capillary columns. Headspace (HS) and direct immersion (DI) SPME are the two fiber extraction modes, while the in-tube SPME mode is applied in the LC or HPLC instrument.

In the DI-SPME mode, the fiber is inserted into the sample medium and the analytes are transported directly to the extraction phase. For aqueous matrices, more efficient agitation techniques, such as fast sample flow, rapid fiber or vial movement, stirring or sonication are required. These actions are undertaken to reduce the effect caused by the "depletion zone" which occurs close to the fiber as a result of fluid shielding and slow diffusion of analytes in the liquid media. DI-SPME is the most common mode for pesticide analysis, and is conducted by directly inserting the fiber into the sample matrix. A method for the determination of seven OP pesticides in fruits and fruit juice samples was developed and validated by Simplicio and Boas (1999). Mean recoveries were all above 75.9% and below 102.6% for juice and between 70% and 99% for the fruit samples. Limits of detection of the method for fruits and fruit juice matrices were below 2 μg/kg for all pesticides. Beltran *et al*. (2003) has developed a DI-SPME method for the determination of seven pyrethroid pesticides in tomatoes and strawberries. Detection limits for tomato and strawberry samples were between 0.003 and 0.025 mg/kg with RSD values of less than 25%. Residues of metobromuron, monolinuron and linuron herbicides and their aniline homologs in carrots, onions and potatoes have been quantified with DI-SPME with the polyacrylate (PA) fiber. A juice was obtained from samples, then diluted, added with sodium chloride and buffered. Recoveries obtained were between 76 – 95% with RSD values of less than 10% (Berrada *et al*., 2004). Sagratini *et al*. (2007) developed a new analysis method to detect carbamates and phenylurea pesticide residues in fruit juices using DI-SPME coupled with LC/MS and LC/QIT-MS. The pesticide residues present in watery matrices such as fruit juices were extracted using three types of fibers: 50-μm Carbowax/templated resin (CW/TPR), 60-μm poly(dimethylsiloxane) /divinylbenzene (PDMS/DVB) and 85-μm polyacrylate. After extraction, the desorption (in a static mode) was performed in the specific interface chamber SPME/HPLC, previously filled with 70% methanol and 30% water. The best recoveries, evaluated at two fortification levels (0.2 and 0.5 mg kg^{-1}) in fruit juices, were obtained using PDMS/DVB and CW/TPR fibers, and ranged from 25 to 82% (monolinuron, diuron and diethofencarb), with relative standard deviations (RSDs) from 1 to 17%. All the limits of quantification (LOQs) were in the range of 0.005–0.05 μg ml^{-1} which are equal to, or lower than the maximum residue limits (MRLs) established by the Italian and Spanish legislations.

A new vanguard–rearguard analytical method for determining 54 pesticide residues in different fruit juices (natural and commercial orange, peach and pineapple juices were tested) is proposed by Cortés-Aguado *et al*. (2008). First, pesticides are quickly extracted with ethyl acetate in a test tube, transferred to a mixture of water:acetone 9:1 (v/v), and

isolated by solid-phase microextraction (SPME). Only 1 mL of juice sample is required for the analysis. The combination of a solvent and SPME extractions offer a significant selectivity and sensitivity with a proven reduction of false-positive and false-negative cases. The use of a vanguard–rearguard strategy can reduce by 50%, the total time required for routine determination of juice samples in a laboratory following the accepted strategy of identification, confirmation and quantification of the pesticides in the samples by a conventional analytical method.

In the headspace sampling mode, the analyte is transported through a layer of gas before reaching the coating. This protects the fiber coating from damage by high molecular weight substances and other non-volatile concomitants present in the liquid sample matrix, such as humic materials or proteins. The amount of analyte extracted at equilibrium using DI or HS sampling are identical as long as the sample and gaseous headspace volumes are the same. This is a result of the equilibrium concentration being independent of the fiber location in the sample/headspace system. If the above condition is not satisfied, a significant sensitivity difference between the direct and headspace technique exists only for very volatile analytes. The choice of sampling mode has a significant impact on the extraction kinetics. When the fiber coating is in the headspace, the analytes are removed from the headspace first, followed by indirect extraction from the matrix. Therefore, volatile analytes are extracted faster than semivolatile components since they are at a higher concentration in the headspace, which contributes to faster mass transport rates through the headspace. The temperature has a significant effect on the kinetics of the process by determining the vapor pressure of the analytes. The equilibrium times for volatile components are shorter in the headspace SPME mode than for direct extraction under similar agitation conditions. This outcome occurs as a result of two factors: a substantial portion of the analyte is in the headspace prior to extraction, and the diffusion coefficients in the gas phase are about four orders of magnitude greater than in the liquid media. Navalon *et al*. (2002) determined the fungicides, pyrimethanil and kresoxim-methyl in green groceries by HS-SPME. The analysis yielded good reproducibility with the RSD values between 7.4% and 15%. Lambropoulou and Albanis (2003) extracted and quantified seven OP pesticide residues in strawberries and cherries in the HS-SPME at an LOD < 13 μg/kg. HS-SPME has been used to quantify eight pesticides in wine and fruit juices (Zambonin *et al*., 2004).

Chai M.K., Tan, G.H., & Asha, L. (2008) and Chai, M.K. and Tan, G.H. (2009) optimized and evaluated the headspace solid-phase microextraction (HS-SPME) for the simultaneous determination of multiclass pesticide residues in fruits and vegetables by GC-ECD. The average recoveries obtained for each pesticide ranged between 71% and 98% at three fortification levels with the relative standard deviation of less than 5%. Repeatability (0.3–3.7%) and intermediate precision (0.8–2.5%) were shown to be satisfactory. The limits of detection (0.01–1 μg L−1) and the limits of quantification (0.05–5 μg L−1) of these pesticides were much lower than the maximum residue levels (MRL), allowed for fruits and vegetables in Malaysia.

In-tube SPME using an open tubular capillary column as the SPME device was developed to couple directly with an HPLC or LC-MS. It is suitable for automation, and can continuously perform extraction, desorption and injection using a standard autosampler. With the in-tube SPME technique, organic compounds in aqueous samples are directly extracted from the sample into the internally coated stationary phase of a capillary column, and then desorbed by introducing a moving stream of mobile phase or static desorption solvent when the analytes are more strongly absorbed onto the capillary coating. The capillaries selected have

coatings similar to those of commercially available SPME fibers. The capillary column is placed between the injector loop and the injection needle of the HPLC autosampler. While the injection syringe repeatedly draws and ejects samples from the vial under computer control, the analytes partition from the sample matrix into the stationary phase until equilibrium is reached.

Subsequently, the extracted analytes are directly desorbed from the capillary coating by mobile phase flow or by aspirating a desorption solvent. The desorbed analytes are transported to the HPLC column for separation, and then detected with the UV or mass selective detection. Mitani *et al*. (2003) applied an automated on-line method for the determination of the isoflavones, daidzein and genistein in soybean foods by using in-tube SPME coupled to HPLC. The detection limits obtained were 0.4 – 0.5 ng/mL and the recoveries were above 97%. Another potential advantage of in-tube SPME is that it can be easily coupled to miniaturized chromatographic systems thus enhancing the sensitivity. This has been illustrated for triazines by Chafer-Pericas *et al*. (2006). The limits of detections obtained for such pesticides were about 250 – 500 times lower than those achieved by using on-fibre SPME combined with conventional LC.

The fiber used in SPME is coated with a thin polymeric film, which concentrates the organic analytes during absorption or adsorption from the sample matrix. There are two mechanisms, absorption or adsorption according to the nature of the fiber. If the fiber is a liquid phase, the analyte are extracted by absorption; if the fiber is a porous particle blend, the analytes are extracted by adsorption. Absorption is a non-competitive process where analyte dissolve into the bulk of the liquid, whereas adsorption is a competitive process where analytes bind to the surface of the solid (Pawliszyn, 1999). In the adsorption case, there are a limited number of sites where analytes can bind to. When all the sites are occupied, the fiber is saturated. Therefore the linear range of the adsorption-type fibers is smaller than the one for absorption-type fibers. In a competitive process, analytes of higher affinity for the coating can displace analytes of lower affinity for the fiber. A large number of fiber coatings based on solid sorbents are now available, in addition to the original general-purpose poly(dimethylsiloxane) (PDMS) and poly(acrilate) (PA) coated fibers, namely: PDMS/divinylbenzene (DVB), Carbowax/DVB, Carbowax/template resin (TR), Carbowax/PDMS, and DVB/Carboxen/PDMS-coated fibers. Extraction of analytes by the new porous polymer SPME fibers with mixed coating is primarily based on adsorption rather than absorption. Some of these porous polymer SPME fibers with bipolar characteristics can be very useful for the simultaneous analysis of pesticides, enlarging the spectrum of SPME applications (Cai *et al*., 2006).

Menezes-Filhoa *et al*. (2010) developed a method for the simultaneous analysis of 14 pesticide residues (clofentezine, carbofuran, diazinon, methyl parathion, malathion, fenthion, thiabendazole, imazalil, bifenthrin, permethrin, prochloraz, pyraclostrobin, difenoconazole and azoxystrobin) in mango fruit, based on SPME coupled to GC–MS. Different parameters of the method were evaluated. The best results were obtained using polyacrylate fiber and direct immersion mode at 50 ^{o}C for 30 min, along with stirring at 250rpm and desorption for 5 min at 280 ^{o}C. The method was validated using mango samples spiked with pesticides at concentration levels ranging from 33.3 to 333.3 μgkg^{-1}. The average recoveries (n = 3) for the lowest concentration level ranged from 71.6 to 117.5%, with RSD between 3.1and 12.3%, respectively. Detection and quantification limits ranged from 1.0 to 3.3 μgkg^{-1} and from 3.33 to 33.33 μgkg^{-1}, respectively.

Amongst the advantages it should be stated that the SPME method has a higher degree of automation when compared to other more laborious protocols. The procedure can be completely automated using an autosampler with SPME equipment. Other advantages of SPME are the inherent high sensitivity and the absence of solvents and sample pre-treatment required, thus minimizing the sample manipulation and contamination. The main disadvantages include poor fiber-to-fiber reproducibility, and poor precision and ruggedness on the determination. The technique is limited to relatively semi-volatile or volatile compounds, and matrix-effects showed up in complex matrices. Finally, relatively expensive consumables and a dedicated and skilled optimization of different experimental conditions and parameters are required, making the SPME method not as straightforward for multi-residue method development.

5. Matrix Solid-phase Dispersion (MSPD)

Since its introduction in 1989, matrix solid phase dispersion (MSPD) has been cited as the extraction method employed in over 250 studies (Barker, 2007). It has proven to be an efficient and somewhat generic technique for the isolation of a wide range of drugs, pesticides, naturally occurring constituents and other compounds for a wide variety of complex plant and animal samples. MSPD combines aspects of several analytical techniques, performing sample disruption while dispersing the components of the sample on and into a solid support, thereby generating a chromatographic material that possesses a particular character for the extraction of compounds form the dispersed sample.

In the MSPD process, a sample (liquid, semi-solid or solid) is placed in a glass or agate mortar containing an appropriate bonded-phase or other solid support material such as octadecylsiloxane (ODS) and derivatized silica (C_{18}) or other suitable support materials (Figure 3).

The solid support and sample are manually blended together using a glass or agate pestle, a step that takes about 30 seconds. When blending is complete, the sample is then packed into an empty column or on top of a solid-phase extraction (SPE) sorbent without any further drying or cleanup prior to elution. The column is often an empty syringe barrel or a cartridge with a stainless-steel or polypropylene frit, cellulose filter or a plug of silanized glass wool at the bottom. A second frit or plug is often placed on top of the sample before compression with a syringe plunger. The main difference between MSPD and SPE is that the sample is dispersed throughout the column and not retained in only the first few millimeters. As regards elution, there are two possibilities: (a) the target analytes are retained on the column and interfering compounds are eluted in a washing step, followed by the target analytes being eluted by a different solvent; or (b) the interfering matrix components are selectively retained on the column and the target analytes directly eluted. Finally, additional cleanup is performed or the sample is directly analyzed. Sometimes, the MSPD column is coupled on line with an SPE column or, as in several recent applications; the SPE sorbent is packed in the bottom part of the MSPD column to remove interfering matrix components (Kristenson *et al*., 2006).

Several factors have been examined for their effects in the MSPD extraction. These include:

a. the effects of average particle size diameter, where as expected, very small particle sizes (3 - 10 μm) would lead to extended solvent elution times and the need for excessive pressures or vacuum to obtain an adequate flow. A blend of silicas possessing a range

of particle sizes (40 - 100 μm) works quite well and such materials also tend to be less expensive.

b. the character of the bonded-phase. Depending on the polarity of the phase chosen, various effects on the results may be observed. Applications requiring a lipophilic bonded-phase employ C_{18} and C_8 materials interchangeably.

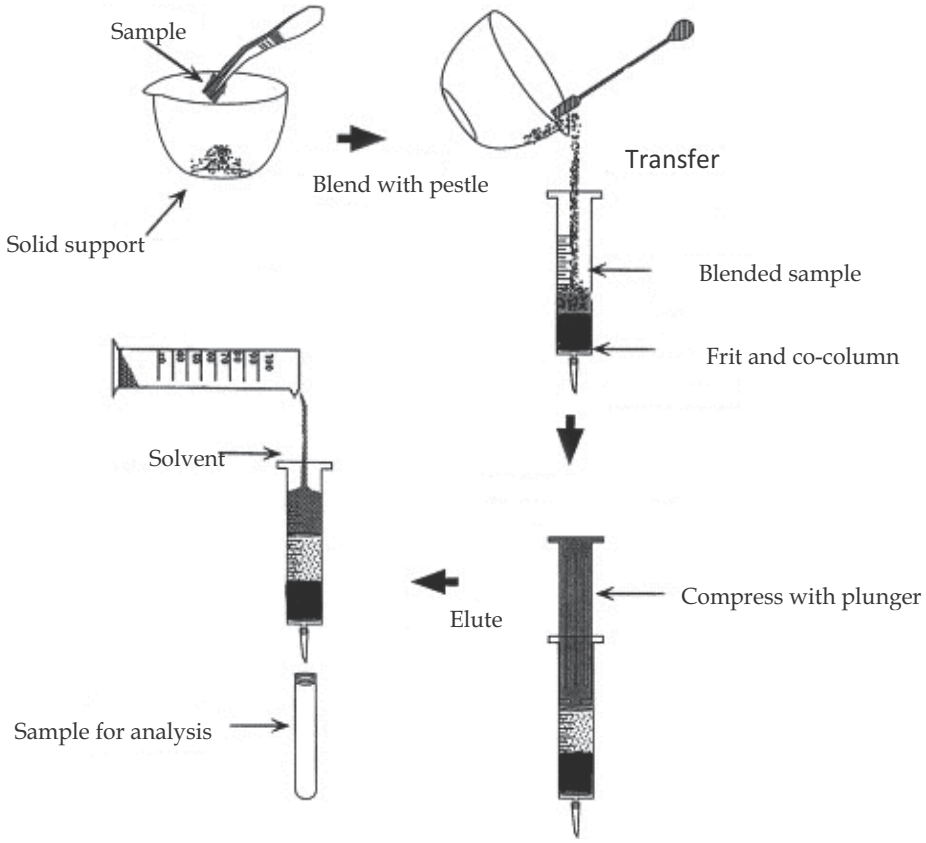

Fig. 3. MSPD Extraction Procedures (Barker, 2007)

c. the use of underivatized silica or other solid support materials. Use of unmodified or underivatized solids, such as sand to blend samples do not work in exactly the same manner as originally described for the bonded-phase solid support, such as ODS. Silica-based support materials (derivatized silica, silica gel, sand, florisil) are still being used almost exclusively in MSPD. Blasco *et al*. (2004) have demonstrated the use of an activated carbon fiber for the isolation of dithiocarbamates from fruits, vegetables and cereals.

d. the best proportion ratio of sample to solid support material. The most often applied is 1 to 4, respectively, but it can vary from application to application. This ratio is dependent on the method employed. Both smaller and greater ratios have been used successfully.

e. Chemical modification of the matrix or matrix solid support blend. Addition of chelating agents such as acids and bases at the time of blending would affect the

distribution and elution of target analytes from the sample. The solution profile of matrix components is likewise affected.

f. The optimum choice of eluent and the sequence of their application to a column. The elution solvent sequence is to isolate the analyte or further clean the column of interfering substances with each solvent step. MSPD columns permit isolation of analytes with different polarities or the entire chemical classes of compounds in a single solvent, making MSPD amenable to multiresidue analysis on a single sample. Several recent studies have reported the use of hot water as an eluting solvent as well as the addition of pressure, which is known as pressurized-liquid extraction (PLE) or accelerated solvent extraction (ASE) (Bogialli *et al*., 2004). Such applications demonstrate the potential to make extraction methods based on MSPD free of hazardous solvents and even less expensive to perform.

g. The elution volume. It has been observed that for an 8 ml elution of a 2 g MSPD column blended with 0.5 g sample, the target analytes usually elute in the first 4 ml, which is approximately one column volume. This will vary for each application and should be examined to reduce the use of solvent and the unintended co-elution of potential interferences.

h. The effect of the sample matrix itself. All the components of the sample are dispersed throughout the column, covering much of the bonded-phase solid support surface, creating a new phase that can have dramatic effects on isolation in going from one matrix to another (Barker, 2000a; Barker, 2000b).

Kristenson *et al*. (2001) developed a miniaturized automated MSPD method for extracting pesticides from apples, pears and grapes. Only 25 mg of sample and 0.1 ml ethyl acetate were used and the extracts were analyzed by GC-MS without any further purification. In terms of recovery, C_{18}, C_8 and silica were compared for use as dispersants. The best results were obtained by using C_{18}. The LODs were 4 - 90 μg/kg. Bogialli *et al*. (2004) developed a simple, rapid and specific method for analyzing seven widely used carbamate insecticides in fruits and vegetables. After matrix deposition on crystobalite (sand), the analytes were extracted with water, heated to 50 - 100 °C. At 50 °C, recoveries were between 76 to 99 %.

A method based on MSPD and GC was proposed for the determination of OC and pyrethroid insecticides in tea leaves (Hu *et al*., 2005). After evaluating various extraction conditions, Hu *et al*. (2005) found that the best compromise in terms of recovery and cleanup was the use of Florisil as the dispersant and hexane-dichloromethane (DCM) as the extractant. LODs of the method ranged between 2 and 60 ng/g, which are lower than the MRLs set by the EU. Barker *et al*. (2000b), Bogialli and Corcia (2007) detailed a number of applications of MSPD for the analysis of residues and Kristenson *et al*. (2006) detailed advances in the technique.

Recently, Cunha *et al*. (2007) proposed a similar approach for the determination of phosmet and its metabolites in olives using C_{18} and $MgSO_4$ as sorbent and acetonitrile as eluting solvent. They found that C_{18} and $MgSO_4$ as matrix sorbents have advantageous effects on extraction yields compared with polar sorbents such as silica, alumina, Florisil or aminopropyl. No additional cleanup step was proposed. A single-step extraction and purification method was developed for the separation of 26 OCPs, 3 pyrethroid pesticides and 6 PCBs from fatty foods of either animal or vegetable origin (portions of meat adipose tissues, meat products, milk and milk products, cheese, eggs, etc.) (Kodba *et al*., 2007). The method included homogenization of the isolated fat and DE (celite). Separation was achieved using a mini Pasteur pipette where a MSPD was carried out with only 5 mL of

dimethyl sulphoxide (DMSO) as the eluting solvent. A Pasteur pipette was joined to a pre-packed slurry filled Florisil column, where the LLE and adsorption chromatography successively took place. Recoveries for PCBs were from 81% to 86% and for OCPs 68–94% but one, which gave lower, and more variable recoveries. Excellent recoveries were obtained for pyrethroid pesticides, mostly more than 80%. The method was applied to 509 fatty samples for monitoring these compounds.

An ultrasonic-assisted MSPD method employing C_8 as sorbent was developed for extracting and cleaning-up 15 OPs and 9 triazines in fruits (Ramos *et al*., 2008), in order to increase process efficiency. The method performances were compared between those of a conventional MSPD with those of a heat-assisted MSPD. The employment of a sonoreactor allowed the reduction of the sonication time to 1–3 min, and consequently overcomes the possible analyte degradation associated with increased temperatures occurring in longer sonication times. The low method detection limits(MDLs) of the ultrasound-assisted MSPD method ensured proper determination of maximum allowed residue levels for all, except for dimethoate and disulfuton. All the evaluated samples, such as apples, pears and apricots, showed a low or no matrix effect with this method.

Silve *et al*. (2008) proposed a simple and effective extraction method based on MSPD to determine dimethoate, malathion, lufenuron, carbofuran, 3-hydroxycarbofuran, thiabendazole, difenoconazole and trichlorfon in coconut pulp using gas-chromatography-mass spectrometry. Different parameters of the method were evaluated, such as type of sorbent (C_{18}, alumina, silica gel and Florisil), the amount of sorbent and eluent (dichloromethane, acetone ethyl acetate, acetonitrile, n-hexane and n-hexane:ethyl acetate (1:1, v/v). The best results were obtained using 0.5 g of coconut pulp, 1.0g of C_{18} as dispersant sorbent, 1.0 g of Florisil as cleanup sorbent and acetonitrile saturated with n-hexane as the eluting solvent.

Analysis of pesticides belonging to different classes was performed by LC–MS/MS after MSPD using diatomaceous earth as dispersant and dichloromethane as eluent (Radišić *et al*. 2009). Significant matrix effects observed for most of the pesticides tested were eliminated using matrix-matched calibration plots. Recoveries were in the range 71–118%, with RSDs between 5–15%. It was determined that the pH had a decisive influence on the carbendazim recovery, while its influence was not so prominent for other tested pesticides. The highest recoveries for carbendazim were obtained with the pH-value adjusted to 6, and a slight increase in recoveries of other pesticides was also observed.

The main advantages of MSPD extraction are that besides requiring only small amounts of sample and solvents, it is rapid, inexpensive and can be carried out under mild extraction conditions (room temperature and atmospheric pressure) and provides acceptable yield and selectivity, and thus, in turn, decreases environmental contamination and improves worker safety. Moreover, the flexibility and versatility of MSPD allows the application of the process to a wide variety of analytes and biological and environmental matrices. In fact, MSPD has shown its feasibility not only for solid or semi-solid samples, but also for the viscous samples (milk, blood, etc.). For these reasons, the employment of MSPD which was first introduced in 1989, has still grown in recent years. Although useful for the analysis of trace contaminants in food, particularly as an aid or an alternative to LLE or solid phase extraction, the MSPD technique is not easily automated and could be time-consuming for a large number sample size. Although the MSPD extracts are clean enough for direct instrumental analysis, a further cleanup step is often required, particularly with fatty matrices.

6. Dispersive Liquid-liquid Microextraction (DLLME)

Dispersive liquid-liquid microextraction (DLLME) is developed by Razaee *et al*. (2006). The method is based on the ternary component solvent system. A mixture of a water-immiscible extraction solvent dissolved in a water-miscible disperser solvent is injected rapidly into an aqueous sample. A cloudy solution consisting of fine droplets of the extraction solvent dispersed into an aqueous phase is formed. Due to the considerably large surface area between the extraction solvent and the aqueous sample, the extraction of the analytes is achieved quickly. Then centrifugation takes place, and the extraction solvent with the analytes is sedimented and analysed by an appropriate method (Berijani *et al*., 2006).
DLLME is a miniaturized liquid-liquid extraction (LLE) using microliter volumes of extraction solvent, which is based on the equilibrium distribution process of the target analytes between sample solution and extraction solvent. The enrichment factor and extraction recovery are calculated as follows (Berijani *et al*., 2006; Rezaee *et al*., 2006):

$$F = C_{sed}/C_o$$

$$R=(C_{sed}\ V_{sed})/(C_o\ V_{aq})$$

Where F, C_{sed} and C_o are the enrichment factor, the analyte concentration in the sediment, and the initial concentration of analyte in the aqueous sample, respectively; R, V_{sed} and V_{aq} are the extraction recovery, the volume of the sediment phase, and the volume of the aqueous sample, respectively.
The extraction efficiency for the target analyte by DLLME is influenced by many factors, such as the type of extraction and dispersive solvent, and their volume, the extraction time and salt addition (Kozani *et al*., 2007).
The selection of an appropriate extraction solvent is a major parameter for DLLME process. The extraction solvent should satisfy two conditions: one is the higher density of the extraction solvent than that of water, which makes it possible to separate extraction solvent from aqueous phase by centrifugation; the other is the extraction capability of extraction solvent for the compounds of interest, good chromatographic behavior, and low solubility of extraction solvent in water (Kozani *et al*., 2007; Farahani *et al*., 2007). The extraction solvent volume has a large effect on the enrichment factor. With the increase of the extraction solvent volume, the final organic phase obtained by centrifugation is increased, resulting in a decrease of the concentration of the target analyte in the organic phase. Although the extraction recovery remains constant, the enrichment factor will be decreased, leading to a decrease in the sensitivity of the determination for the target compounds. Therefore, the selection of the optimal extraction solvent volume should result in both the high enrichment factor and adequate volume for the subsequent determination after centrifugation (Kozani *et al*., 2007; Farahani *et al*., 2007).
The disperser solvent is soluble in the extraction solvent and should be miscible in water, thus enabling the extraction solvent to be dispersed as fine particles in the aqueous phase to form a cloudy solution (water/disperser solvent/extraction solvent). In such a case, the surface area between extraction solvent and aqueous phase (sample) can be infinitely large, thus increasing the extraction efficiency. The disperser solvent volume directly affects the formation of the cloudy solution, the degree of the dispersion of the extraction solvent in the aqueous phase, and subsequently, the extraction efficiency (Fattahi *et al*., 2007).

In DLLME, the extraction time is defined as the interval between injecting the mixture of disperser solvent and extraction solvent and centrifugation. It is was found that the extraction time has little effect on the extraction efficiency of DLLME (Farahani *et al*., 2007; Fattahi *et al*., 2007). This is because the extraction solvent can be evenly dispersed after the formation of the cloudy solution, the transition of the analyte from aqueous phase (sample) to extraction phase can be very fast, and the equilibrium state can be subsequently achieved very quickly, resulting in a very short extraction time needed for equilibrium. A short extraction time is an advantage associated with the DLLME technique (Farahani *et al*., 2007; Fattahi *et al*., 2007).

Pesticide analysis is probably the field in which DLLME has found its major applications. Different sources of water (mainly tap, river, well and lake waters) were selected as the matrix for the DLLME. In few cases, food matrices (Zhao et al., 2007; Cunha et al., 2009; Fu et al., 2009; Moinfar et al., 2009; Ravelo-Pe´rez et al., 2009a; Ravelo-Pe´rez et al., 2009b) have also been analyzed, and to a lesser extent, probably because of the complexity of the samples and also because of the need to develop a previous pretreatment procedure based mainly on solvent or water extraction.

Zhao *et al*. (2007) demonstrated that there were no significant differences when acetonitrile and chlorobenzene were employed as the disperser and extraction solvents, respectively, to extract six OPPs (ethoprophos, parathion methyl, fenitrothion, malathion, chlorpyrifos and profenofos) from watermelon and cucumber. In this study, sample pretreatment consisted of adding 10 mL of acetonitrile to 10 g of sample together with 4 g of anhydrous $MgSO_4$ and 1 g of NaCl. The mixture was then shaken in a vortex mixer and centrifuged. In the DLLME procedure, 27 mL of chlorobenzene (extraction solvent) were added to 1 mL of acetonitrile taken from the previous extraction step and the mixture was introduced into 5 mL of purified water. After shaking and centrifuging, the sedimented chlorobenzene phase was collected and injected into the GC-FPD system. Recoveries for each target analyte were in the range between 67 and 111%. The RSD varied between 2 and 9% (n = 3). LODs were found ranging from 0.010 to 0.190 μg/kg for all the target pesticides. Compared with the conventional sample preparation method, the proposed method has the advantage of being quick and easy to operate, and having high-enrichment factors and low consumption of organic solvents.

Fu *et al*. (2009) developed a DLLME method to determine carbamate (carbaryl) and organophosphorus (triazophos) pesticide residues in water and fruit juices. Using the optimum extraction conditions -extraction solvent: tetrachloroethane, 15.0 μL; dispersive solvent: acetonitrile, 1.0 mL; no addition of salt and extraction time below 5 s, the enrichment factors for the carbaryl and triazophos were 87.3 and 275.6, respectively. The linearity was obtained in the concentration range of 0.1–1000 $ngmL^{-1}$ with correlation coefficients from 0.9991 to 0.9999. The LODs, ranged from 12.3 to 16.0 pg mL^{-1}. The RSDs, for 10 ng mL^{-1} of carbaryl and 20 ng mL^{-1} of triazophos varied from 1.38% to 2.74% (n = 6). The relative recoveries of fruit juice samples were in the range of 86.3–105.3%.

In the past decade, a new type of solvent, ionic liquids (IL), has been introduced into analytical chemistry as an extractant (Poole and Poole, 2010). ILs are low-melting salts that form liquids composed entirely of ions, which have generally been found to be less toxic, less volatile and less contaminating than conventional solvents. These salts have also been used as extraction solvents in DLLME for extracting pesticides in fruit samples (Ravelo-Pe´rez et al. 2009a; Ravelo-Pe´rez et al. 2009b)

The first application of IL-DLLME for the extraction of pesticides from matrices other than water was recently developed by Ravelo-Pe´rez et al. (2009a, 2009b) for the extraction of fruit extracts. In these cases, disperser solvents were used together with the IL. In the first of these studies (Ravelo-Pe´rez et al. 2009a), parameters affecting the IL-DLLME of eight pesticides (i.e. thiophanate-methyl, carbofuran, carbaryl, tebuconazole, iprodione, oxyfluorfen, hexythiazox and fenazaquin) were optimized by means of an experimental design (central composite design). The selected parameters were sample pH, NaCl percentage, IL amount (1-hexyl-3-methylimidazolium hexafluorophosphate)- [HMIm][PF_6]) and methanol volume (disperser solvent). The final procedure in this study consisted of the ultrasonic- assisted extraction of 1 g of homogenized bananas with acetonitrile using different salts ($MgSO_4$, NaCl, sodium hydrogen citrate sesquihydrate and sodium citrate tribasic dihydrate) to improve the recoveries. After centrifugation and evaporation of the supernatant, reconstitution of the extract in water at pH 2.7 provided the best media to develop the IL-DLLME procedure, which used 88 mg of [HMIm][PF_6], 714 L of methanol and 28.9% (w/v) of NaCl. The combination of these two procedures (acetonitrile extraction of the fruit and reconstitution of the evaporated extract in water) provided a suitable arrangement that allowed application of DLLME as part of the sample-pretreatment procedure for extraction of pesticides from complex samples. Mean recovery percentages were 53–97% with RSD values below 8.7%, which represent LODs of 0.320–4.66 μg/kg, well below maximum residue limits of the European Union (EU MRLs). In the later study Ravelo-Pe´rez et al. (2009b) extended the application of the method for the extraction of the same group of pesticides from grapes and plums. Mean recovery values were 72–100% for table grapes and 66–105% for plums, with LODs very similar to those reported in the earlier study, which are aslo below the EU MRLs. In both these studies, the method was also applied to the analysis of commercial fruit samples, in which residues of pesticides could be found in some samples, with some exceeding the MRLs.

Compared to other techniques, DLLME is characterized by very short extraction times, mainly because of the large surface area between the solvent and the aqueous phase. Other advantages are simplicity of operation, low cost, and high recovery and enrichment factors, offering potential for ultra-trace analysis. However, the main drawback of DLLME is that its efficiency is restricted by solvent selection to systems capable of forming a dispersive phase, somewhat limiting its range of application by sample. However, the introduction of new solvents (e.g., ILs, which are numerous) may provide a new alternative. Automation of DLLME seems to be very difficult and has not yet been achieved, although an attempt was made for the analysis of inorganic species (Anthemidis and Ioannou, 2009).

7. Stir-bar Sorptive Extraction (SBSE)

Stir bar sorptive extraction (SBSE) was developed by Baltussen *et al*. (1999) to overcome the limited extraction capacity of SPME fibers. A glass stirrer bar is coated with a potentially thick bonded absorbent layer (polydimethylsiloxane – PDMS) to give a large surface area of stationary phase, leading to a higher phase ratio and hence a better recovery and sample capacity (Figure 4). The advantages of sorptive extraction using PDMS include predictable enrichment, the absence of displacement effects, inertness, and rapid thermal desorption at mild temperature. Stir bar sorptive extraction of a liquid sample is performed by placing a suitable amount of sample in a headspace vial. The stir bar is added and the sample is stirred, typically for 30 - 240 min. the extraction time is controlled kinetically, determined by

sample volume, stirring speed, and stir bar dimensions and must be optimized for a given application.

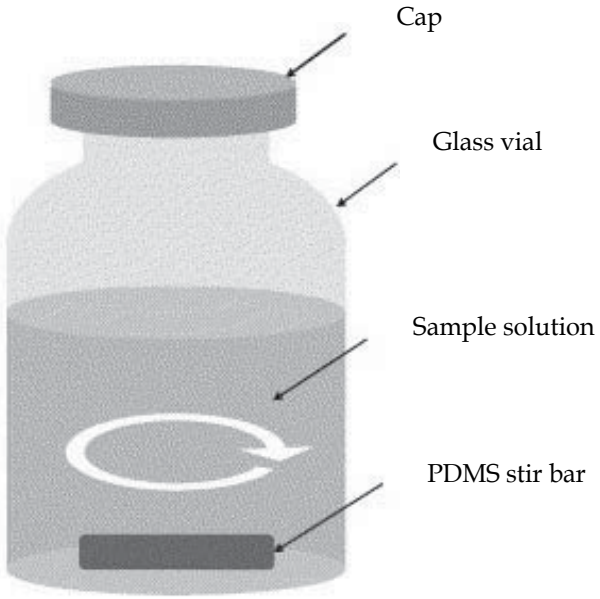

Fig. 4. Schematic Diagram of a SBSE Setup

Normally, SBSE is applied to the extraction of aqueous samples containing low concentrations of organic compounds. For samples containing high concentrations of solvents, the solutions should be diluted before extraction. For the extraction of highly non-polar solutes, an organic modifier is added to minimize wall adsorption. Thus, the optimization of the organic modifier concentration is necessary.

After extraction, the stir bar is removed, then placed on a clean tissue paper, rinsed with distilled water to remove water droplets, and introduced in a thermal desorption unit. This step will avoid the formation of non-volatile material during the thermal desorption step. Rinsing would not cause any solute loss, because the adsorbed solutes are present inside the PDMS phase. After thermal desorption, the stir bars can be reused. Typically, the lifetime of a single stir bar is approximately 20 to 50 extractions, depending on the matrix (David and Sandra, 2007).

Since SBSE using PDMS coating is similar to liquid-liquid extraction using a non-polar solvent, the technique is mainly used for non-fatty matrices (< 3% fat). The analysis of pesticides in fruits and vegetables (Sandra *et al*., 2003; Juan-Garcia *et al*., 2004; Juan-Garcia *et al*., 2005; Zuin *et al*., 2006) has been described. After homogenization, the fruit and vegetable samples are extracted using a water miscible solvent. An aliquot of the extract is diluted with water and followed by SBSE. Both LC-MS desorption and thermal desorption GC-MS have been used.

Sandra et al. (2003) used SBSE with thermal desorption capillary GC-MS for the screening of pesticides (OPPs and OCPs) in fruits, vegetables and baby food. A 10 mm stir bar coated with 0.5 mm PDMS was used. The recoveries for spiked samples ranged between 43-75%. The coupling of SBSE with RTL-GC-MS operated in the scan mode could monitor simultaneously about 300 pesticides present in fruits, vegetables and baby food. The detection limits from mg/kg to the sub µg/kg level were obtained.

Juan-Garcia *et al*. (2004) studied the detection of fungicide residues in grapes by LC-MS. Two procedures based on SPE and SBSE have been assessed for extracting these compounds in grapes. The recoveries obtained by SPE in samples spiked at the LOQ level ranged from 60 to 100% with RSDs from 7 to 17%. With the SBSE the recoveries obtained from samples spiked at the LOQ level were between 15 and 100% and the RSDs between 10 and 19%. The LOQs of most compounds are better via the SPE (0.003–0.01 mg kg^{-1}) than by SBSE (0.01 mg kg^{-1} for all fungicides).

Guan et al. (2008) prepared a novel poly(phthalazine ether sulfone ketone) (PPESK) film and coated this on stir bars with a thickness of 250 μm for sorptive extraction. The PPESK coated stir bar has high thermostability (290 °C) and a long lifetime (50 repeated use). The extraction properties of this stir bar were evaluated for the extraction of both polar and semi-polar analytes, including organochlorine compounds and organophosphorus pesticides. The PPESK stir bar showed higher affinity towards polar compounds than that of a PDMS coated stir bar and a higher sample load compared with the corresponding PPESK fiber. It was applied to the determination of organophosphorus pesticides in juices by gas chromatographic analysis. Limits of detection for organophosphorus pesticides were in the range of 0.17–2.25 ng L^{-1} and 2.47–10.3 ng L^{-1} in grape and peach juice, respectively, using the flame thermionic detector (FTD), with precisions of less than 20% RSD.

Although SBSE is widely applied in environmental and food analysis, it has also some limitations or drawbacks. One of the drawbacks is related to the fact that the coated stir-bar cannot be directly desorbed in a simple split/splitless injection port of a gas chromatograph. Hence the analyte has to be back extracted into a suitable solvent, which adds an additional step to the overall analytical method, or a specially designed Thermal Desorption Unit (TDU) has to be used. Moreover, operations like removing the stir-bar from the sample, rinsing and drying are usually performed manually, which is laborious and can introduce errors. Automation of these steps is possible but this increases the cost and complexity of the hardware involved. However, the most important limitations of SBSE are related to the coating of stir-bars. The non polar PDMS is at present the only polymer commercially available as a coating for stir-bars. Recovery of polar analytes is poor and often in situ derivatisation is applied to increase extraction yields. Stir-bars coated with materials with better affinity to polar compounds would improve SBSE flexibility and selectivity while maintaining its concentration capability. New approaches or concentrating materials are therefore required to overcome the above-mentioned limitation and to extend the range of applications. Up to now, developments of novel stir-bars have been reported with limited references. One of the methods developed was to use dual-phase-coated stir-bars, which combine two or more sampling materials with different concentration capabilities (Bicchi *et al*. 2005). These new stir-bars consist of a short PDMS tube at both ends with two magnetic stoppers, whose inner cavity is packed with different types of adsorbents such as activated carbon. Dual-phase stir-bars with carbon have been shown to improve the recovery of volatile and polar compounds compared to the conventional PDMS stir-bar.

8. Single-drop Microextraction (SDME)

Recently, alternative but SPME related concepts have been introduced for sample extraction. The use of a single droplet for extraction purposes was first recommended in the mid-1990s (Mester and Sturgeon, 2005). Figure 5 shows one possible embodiment of the SDME technique employing a microsyringe. The syringe needle is used to pierce the septum of a

closed container. When the tip of the needle is in the desired position (in the aqueous phase or in the headspace) a hanging droplet of solvent is exposed to the matrix by depressing the plunger of the syringe. After extraction is completed, the droplet is withdrawn into the syringe barrel by lifting the plunger. The extracted samples can then be submitted directly to GC analysis. Thus the system requires two discrete parts: the first for extraction and the second for injection.

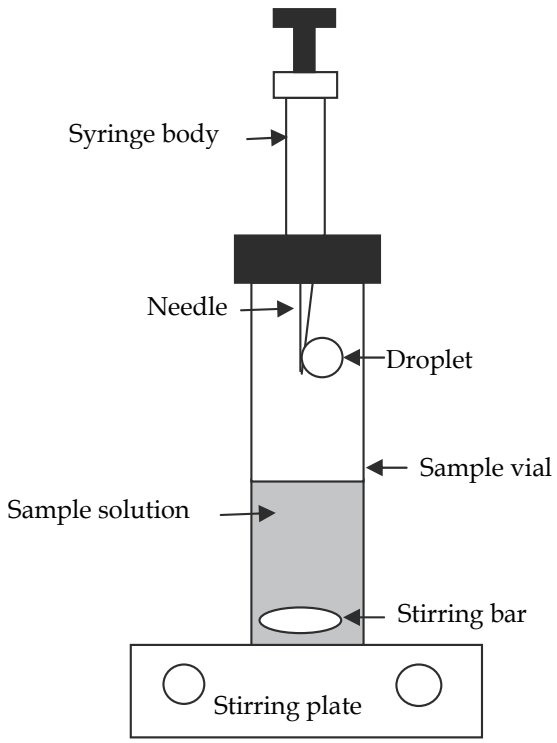

Fig. 5. Schematic Diagram of a SDME Setup (Mester and Sturgeon, 2005)

The properties of the analyte and its matrix will determine whether direct immersion (DI-SDME) or headspace (HS-SDME) extraction is appropriate. Thus, one must consider the volatility (boiling point), ionization (for acids and bases) and polarity of the analyte and matrix. HS-SDME is appropriate for most polar and non-polar, lower molecular weight, volatile and semivolatile compounds. DI-SDME extraction is appropriate for non-polar or moderately polar higher molecular weight, semivolatile chemicals. There are some important restrictions on the selection of a particular extracting solvent. When extracting from an aqueous solution, the solvent needs to be water immiscible. The solvent needs to have a boiling point high enough that it will not evaporate, but also appropriate for the chromatographic system. It needs to have a high enough viscosity to cling onto the tip of a syringe needle, but not so viscous that the diffusion rate of the analyte into the drop affects extraction time significantly. The intermolecular attraction characteristics of the solvent must also be compatible with the analyte being extracted. Toluene appears to be the most commonly used acceptor phase, because it has high solubility for the target analytes, is immiscible in water and stable enough over the extraction time. Based on this solvent as the

acceptor phase, several methods were validated and applied to the determination of OP and OC pesticides in solid samples (Xiao *et al*., 2006; Zhao, E. *et al*., 2006b). Carbon tetrachloride has also been successfully applied to the extraction of OP pesticides (Ahmadi *et al*., 2006); this solvent is, however, more prone to dissolve or become dislodged when long extraction periods are used. Isooctane and n-hexane have been also used for the determination of OP and OC pesticides (Zhao, L. and Lee, 2001; Lopez-Blanco *et al*., 2003). In the SDME procedure, solvent volumes lower than 3 μL are commonly used, due to the instability of the microdrop at higher values as well as to the good compatibility with the GC instruments.

SDME involves dynamic partitioning of the target compounds between the acceptor phase and the sample solution, and the extraction efficiency depends on the mass transfer of analyte from the aqueous phase to the organic solvent phase. Since the mass transfer is a time-dependent process, a graph representing the relationship between peak area and extraction time is typically reported. Generally, extraction yield increases over relatively long exposure times. Since SDME is not an exhaustive extraction technique, it is not always practical to match extraction time with extraction equilibrium, because the potential for solvent loss due to dissolution increases with time. Therefore, extraction times are rarely set at equilibrium but rather at a point where sensitivity and precision are maximized over an acceptable experimental time. For pesticide analysis, extraction times of 15-30 min are usually selected.

Agitation is a critical parameter in SDME procedures. The mass transfer of the target compounds to the organic solvent can be enhanced by agitation of the sample solution, thereby reducing the time required to attain thermodynamic equilibrium. However, excessive agitation could result in a dislodgement of the acceptor phase and difficulties in analyte quantification, especially with prolonged exposure times.

The "salting out" effect was studied, and the results showed that high salt concentrations in the aqueous samples usually decrease the diffusion of analytes toward the organic phase thus impairing the extraction. This effect is more pronounced in the case of SDME and thus most of the studies have been performed without or with a small amount of salt addition (Zhao, L. and Lee, 2001; Xiao *et al*., 2006; Ahmadi *et al*., 2006). Caution should be taken when high salt concentrations are used in the sample matrix, since under these conditions, in combination with the agitation of the sample, the formation of air bubbles was promoted, increasing the incidence of drop loss or dislodgement of organic solvent.

Generally, the studies of SDME in the determination of pesticides in food samples are very limited because of their complex matrices (Xiao *et al*., 2006; Zhao, E. *et al*., 2006b; Zhang *et al*., 2008; Amvrazia & Tsiropoulos, 2009a).

Xiao *et al*., 2006 developed a single-drop microextraction (SDME) procedure for the analysis of organophosphorus pesticides (OPPs) in fruit juice by gas chromatography (GC) with flame photometric detection (GC-FPD). Two types of SDME mode, static and cycle-flow SDME, were evaluated. The enrichment factors for six OPPs in static SDME were nearly 100-fold (except for dichlorvos 23-fold), which were much better than that in cycle-flow SDME. Therefore, static SDME with tributyl phosphate (TBP) as the internal standard was selected for the real sample analysis. A 100-fold dilution of fruit juice samples is adequate to determine levels of most pesticides below the MRLs because of the low limits of detection of the method. The recoveries for the spiked juice samples were from 77.7 to 113.6%.

An approach for the extraction of 9 kinds of organochlorine pesticides (OCPs) from vegetable samples (cabbage, cauliflower, Chinese cabbage) coupling single-drop microextraction with gas chromatography–mass spectrometry was presented by Zhang *et al.*

(2008). An effective extraction was achieved by suspending a 1.00 μL mixed drop of p-xylene and acetone (8:2, v/v) to the tip of a microsyringe immersed in a 2 mL donor aqueous solution and stirred at 400 rpm. The relative recoveries were from 63.3 to 100%, with repeatability ranging from 8.74 to 18.9% (R.S.D.). In contrast to some common acceptor solvents, a novel combination of liquids comprising p-xylene and acetone showed better extractions and lower detection limits (0.05 ng $mL^{-1)}$ for organochlorine pesticides.

Amvrazia & Tsiropoulos (2009a) evaluated the single-drop microextraction (SDME) technique coupled with GC-NPD and GC-ECD for the determination of14 types of multi-class pesticides in vegetables (tomato and courgette). The optimum sample preparation was achieved with the use of a mixture of acetone/H_2O (10/90, v/v) in a donor sample solution and subsequent SDME using a toluene drop (1.6 μL) under mild stirring for 25 min. The efficiency of the extraction process was studied in fortified tomato and courgette samples and the matrix effect assessment performed showed that quantification should be performed using a standard curve of spiked vegetable samples since certain matrix components as observed in the tomato analysis, may enhance pesticide recoveries via SDME. The proposed method showed good linearity, limits of detection at the sub-μg kg^{-1} level and high precision (RSD <15%) and was applied successfuly in real vegetable samples showing that SDME can be a promising way for sample preparation in pesticide residue analysis.

Due to its simplicity, ease of implementation, and insignificant startup cost, SDME is accessible to virtually all laboratories. However, it has some limitations, for example: (a) in its most basic form, direct immersion mode it requires careful and elaborate manual operation because of the problem of drop dislodgment and instability; (b) the SDME is affected by the presence of humic acids or suspended solids indicating that it has a limited advantage in complex matrices, in which extra filtration of the sample is necessary; (c) notwithstanding the acceptable analytical performance mentioned above, the sensitivity and the precision of SDME methods can be further improved. The main issue lies with the adverse consequences of prolonged extraction time and fast stirring rates, since they may result in drop dissolution and dislodgement; (d) SDME is not yet suitable as a routine online pre-concentration procedure. Although some progress has been made to automate SDME, cost considerations will mean that the approach will not be widely accessible (Xu *et al*., 2007).

9. Pressurized Fluid Extraction (PFE)

This technique, also named accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE), is a solid-liquid extraction process performed in closed vessels at relatively elevated temperature, usually 80 to 200 ^{o}C, and elevated pressures, between 10 and 20 MPa conditions for short time periods (5-10min). Therefore, PFE is quite similar to supercritical fluid extraction(SFE) but CO_2 is replaced by organic solvents to mitigate potential polarity problems. Extraction is carried out under pressure to maintain the conventional organic solvents in its liquid state, but extracting at temperatures well above their atmospheric boiling points. Therefore, the solvent is still below its critical condition during PFE but has enhanced solvation power and low viscosities and hence allows higher diffusion rates for the analytes. In this way the extraction efficiency increases, minimizing the amount of solvent needed and expediting the extraction process. The time required for extraction is independent of the sample mass and the efficiency of extraction is mainly dependent on the temperature. Figure 6 shows a schematic diagram of a PFE system.

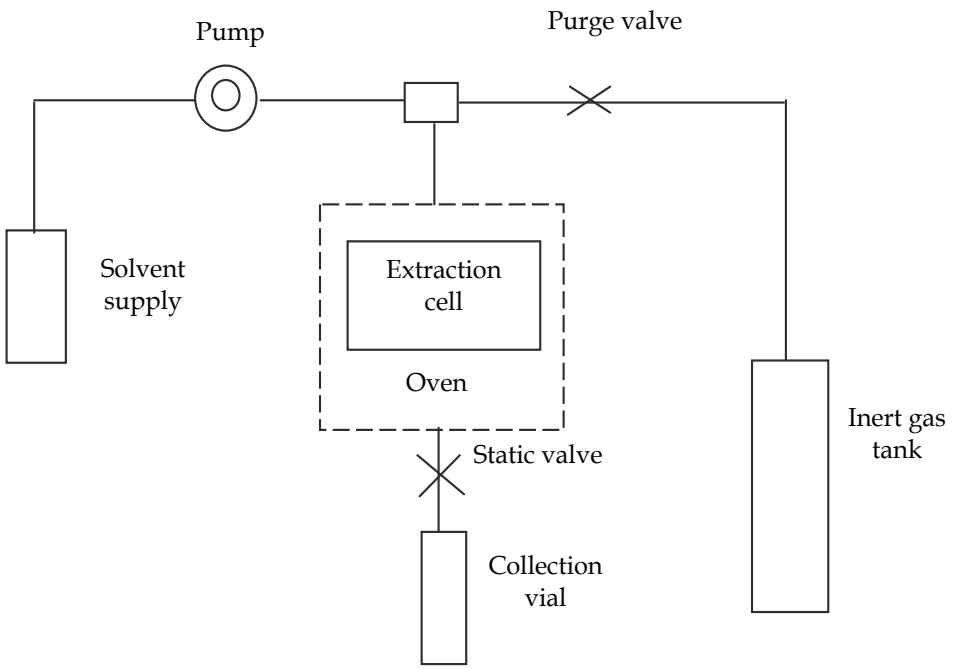

Fig. 6. Schematic Diagram of a PFE System (Buldini *et al*., 2002)

Both static and flow through extraction systems can be used. In the static extraction mode, the sample is loaded in an inert cell and pressurized with a solvent heated above its boiling point for some time. The extract is then automatically removed and transferred to a vial. In the flow through extraction mode, a fresh solvent is continuously introduced to the sample. This improves the extraction efficiency but, the extract is subsequently diluted. The extract is pushed into the collection vial by a second aliquot of solvent inserted into the extraction cell and this second aliquot is then collected into the same vial by pushing it with an inert gas flow. The whole process takes approximately 15-20 min.

In PFE, the pressure is applied to maintain the solvent in its liquid state. This reduces the number of parameters that need to be optimized to achieve efficient extractions compared with SFE. The main parameters to consider now are temperature and time and this reduces the time devoted to method development and optimization of the extraction procedure. The method set up is generally straightforward because the same solvent recommended in the official and routine Soxhlet methods can be used. Therefore, PFE is an attractive technique because it is fast (e.g. extraction time approximately 15 min per sample), uses less solvent volume (15-40 ml), no filtration is required after extraction, the instrumentation allows extraction in unattended operation and different sample sizes can be accommodated. The two main disadvantages of PFE include limited selectivity because it usually requires further cleanup of the extract obtained and higher initial cost than SFE or microwave-assisted extraction (MAE) systems.

Tao *et al*. (2004) applied PFE for extracting DDT and its metabolites from wheat with hexane/acetone (1:1, v/v) at 120 ^{o}C and a pressure of 101 MPa. Moreno *et al*. (2006) investigated the extraction of 65 pesticides including OC pesticides from greasy vegetable

matrices such as avocado using PFE with ethyl acetate/cyclohexane (1:1, v/v) at 120 °C and a pressure of 12 MPa.

Chuang *et al*. (2001) investigated the use of PFE for the analysis of pesticides in baby food but observed matrix interferences due to the high level of fat present in the samples. Although widely used as an initial extraction for solid samples, for trace analysis, post-extraction procedures for analyte enrichment/concentration are often required. SPE can be coupled to the extractor outlet to enable cleanup and concentration. Coupling of PFE to other cleanup steps was used for the determination of pesticides in foods (Herrera *et al*. 2002).

When water is employed as the extraction solvent in PFE, a different terminology is used to highlight the fact that water is an environmental-friendly solvent. Thus, terms such as pressurized hot water extraction, subcritical water extraction (SWE), superheated water extraction and high temperature water extraction can be found in the literature (Ramos *et al*., 2002; Smith, 2003; Carabias-Martinez *et al*., 2005). Because the polarity of water decreases markedly as the temperature is increased, superheated water at 100 – 200 °C, under a relatively low pressure can act as a medium to non-polar solvent (ethanol or acetone) and is an efficient extraction solvent for many analytes (Ramos *et al*., 2002; Smith, 2003; Carabias-Martinez *et al*., 2005). A review of the technique, including several applications was given by Smith in 2002.

A limitation in extracting with hot water is the inability to recover compounds that are hydrophobic, thermally labile, or easily hydrolyzed. Besides, one of the disadvantages of SWE, particularly for trace analysis is that the extract obtained is a dilute aqueous solution. This means that a further concentration / extraction step is often required prior to analysis (such as liquid-liquid extraction or solid-phase extraction). To avoid the additional cleanup, a trapping agent can be added to the extraction vessel, such as an SPE disc, which is then subsequently extracted. Commercial PLE systems can be used and it is possible to link SWE to LC, by trapping analytes onto a cartridge, prior to elution with the mobile phase. Phase transfer catalysis can be used to enable in situ derivatisation and concentration of the product into an organic solvent (Chienthavorn *et al*. 2006)

10. Microwave-assisted Extraction (MAE)

MAE uses microwave radiation (0.3 – 300 GHz) as the source of heating a solid-solvent sample mixture. Due to the particular effects of microwaves on the matter namely, dipole rotation and ionic conductance, heating with microwaves is instantaneous and occurs in the bulk of the sample, leading to very fast extraction. The heat generated in the sample by the microwave field requires the presence of a dielectric compound. The greater the dielectric constant, the more thermal energy is released and the more rapid would be the heating for a given frequency. Consequently, the effect of microwave energy is strongly dependent on the nature of both the solvent and the solid matrix. Usually, the extraction solvent has a high dielectric constant, so that it strongly absorbs the microwave energy. However, in some cases especially for thermo labile compounds, the microwave may be absorbed only by the matrix, resulting in heating of the sample and the release of the solutes into the cold solvent. Therefore, the nature of the solvent is of great importance in MAE: it should selectively and efficiently solubilize the analytes in the sample but, at the same time, it should absorb the microwave without leading to a strong heating to avoid eventual degradation of the analyte compounds. Thus, it is common practice to use a binary solvent mixture (e.g. hexane-

acetone, 1:1) where only one of the solvents is absorbing the microwave energy. Other important parameters affecting the extraction process are the applied power, the temperature and the extraction time. Moreover, the water content of the sample needs to be carefully controlled to avoid excessive heating, thus allowing reproducible results.
The application of microwave energy to the samples may be performed either in closed vessels with pressure and temperature control (pressurized MAE) or in open vessels at atmospheric pressure (focused MAE). In focused MAE method, the temperature is limited by the boiling point of the solvent at atmospheric pressure, but in pressurized MAE the temperature may be elevated by applying an adequate pressure (Dean. 2000).
The technique has proven to be better than soxhlet extraction by reducing the solvent consumption and extraction time (Diagne *et al*., 2002; Barriada-Pereira *et al*., 2003). Usually sample sizes range from 0.5 to 10 g and 10 ml of solvent is sufficient for the extraction time from less than 1 to 10 min. The same laboratory microwave unit previously described for sample digestion is used, so reducing costs; the simultaneous extraction of many different samples is also possible without any mutual interference.
Cai *et al*. (2003) used MAE to extract OC pesticides from Chinese teas before solid-phase microextraction followed by GC-ECD analysis. The recoveries of MAE were compared with those of ultrasonic extraction and the results showed that MAE provided better recoveries (efficiencies) and shorter extraction times than ultrasonic extraction.
Recently, MAE has been proposed for the extraction of pesticide residues in avocado, avocado oil and olive oil (Fuentes *et al*. 2008, Hernandez-Borges *et al*. 2008). Fuentes *et al*. (2008) proposed a method using MAE to assist the liquid-liquid extraction of pesticide in avocado oil and olive oil. An additional cleanup step using an Envi-carb SPE cartridge was proposed. The method is relatively simple, low solvent consuming and has a good throughput of samples (10 samples can be analyzed in 4 hrs). Hernandez-Borges *et al.* (2008) proposed a MAE of abamectin residues in avocado. Homogenized avocado samples were extracted once with 20 mL acetonitrile:water 4:1 (v/v) in a microwave oven for 26 min at 700 W with a maximum temperature of 80 ºC. An additional cleanup step was performed using a C_{18} SPE cartridge.
MAE also limits contamination or absorption from the vessel, due to direct heating of the sample. The main advantages of microwave pre-treatment are the low temperature requirement, high extraction rate, complete automation and the possibility of simultaneously extracting many different samples at the same time with little interference. However, MAE has also several drawbacks such as the extract must be filtered after extraction, polar solvents are needed, cleanup of extracts may be necessary and the equipment is moderately expensive.

11. Supercritical Fluid Extraction (SFE)

A general trend in the isolation of pesticide residues is to decrease the consumption of expensive and toxic organic solvents and to increase the availability of a broad range of analytes and matrices. A possible solution is to use supercritical fluid extraction (SFE). SFE uses liquids such as compressed carbon dioxide (CO_2) as an extracting phase that is capable of removing less volatile compounds at ambient temperature. Supercritical fluids possess both gas-like mass transfer and liquid-like solvating characteristics.
SFE utilizes commercially available equipment where the fluid is pumped, at a pressure above its critical point (7.38 mPa & 31.1 ºC), with the sample placed in an inert extraction cell. The temperature of the cell is increased to overcome the critical point of the fluid. After

depressurization, the analytes are collected in a small volume of organic solvent or on a solid-phase filled cartridge (solid adsorbent trap). Extraction can be performed in the static, dynamic or recirculating mode: in the static extraction mode, the cell containing the sample is filled with the supercritical fluid, pressurized and allowed to equilibrate; using the dynamic mode, the supercritical fluid is passed through the extraction cell continuously; finally in the recirculating mode the same fluid is repeatedly pumped through the sample and, after the required number of cycles, it is pumped out to the collection system (Figure 7). One of the most interesting properties of these fluids is the direct relationship of solvent strength to density. Since the density of the fluid is a function of its temperature and pressure, precise control of these parameters allows a solvent with a narrow window of solvating strength to be obtained. It is possible, therefore, to substitute a variety of conventional solvents with a single supercritical fluid. For instance, supercritical carbon dioxide at 7.515 MPa and 80 ^{o}C (d 0.15 g/ml) is characterized by a solvating strength similar to gases, such as pentane, while at 38.265 MPa and 40 ^{o}C (d 0.95 g/ml) its solvating strength resembles liquids, such as dichloromethane, carbon tetrachloride, toluene or benzene. When selecting the extraction pressure, it should be kept in mind that as the pressure increases, higher molecular weight compounds become soluble, while as the pressure decreases, the supercritical fluid loses some of its solvent strength. If the pressure is reduced to atmospheric values, the fluid loses practically all of its solvating ability and the extracted compounds fall out of the solution (Buldine *et al*., 2002).

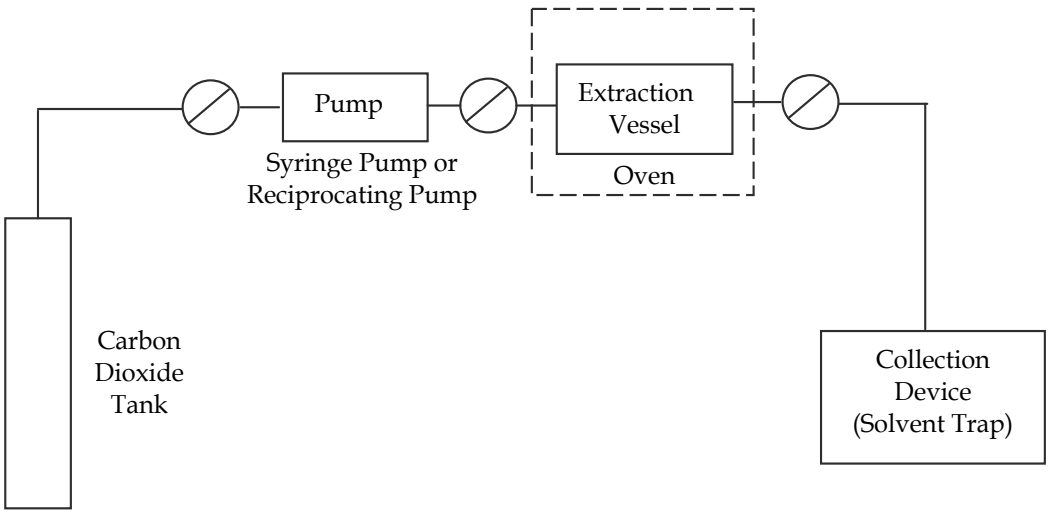

Fig. 7. Schematic Diagram of a SFE System (Fidalgo-Used *et al*., 2007)

Water is a poor choice for this technique because of its high critical temperature and pressure. The most widely used supercritical fluid is carbon dioxide which is characterized by low critical values and low chemical reactivity. Carbon dioxide is easily obtained in extremely pure form at a reasonable cost, and it has low viscosity, non-inflammable, environment-friendly, high diffusion rate with a high volatility and it can be separated from the collected analytes without incurring disposal problems. Nitrous oxide would be a good supercritical fluid, but it is highly inflammable. Ammonia is a polar substance with good solvent strength, but it is chemically reactive and corrosive. The hydrocarbons are usually inflammable and are not viable for analytical SFE. A common practice in SFE, which has to

be taken into consideration in connection with the physicochemical properties of supercritical fluids, is the use of modifiers (co-solvents). There are compounds that are added to the primary fluid to enhance extraction efficiency. For example, the addition of a small percentage (1 – 10%) of methanol to carbon dioxide expands its extraction range to include more polar analytes (Buldine *et al*., 2002).

King et al. (1993) applied SFE with carbon dioxide for the selective isolation of organochlorine, organophosphorus and organonitrogen pesticides from contaminated cereals. The resulting extracts were cleaned-up by GPC and GC-FPD and used for quantification. A determination method for 56 different pesticides was reported by Lehotay and Garcia (1997). The sample was frozen and a drying agent consisting of magnesium sulfate was mixed and homogenized with a small amount of dry ice. The sample was extracted with supercritical CO_2, trapped with C_{18} bonded silica, eluted with acetone, and subsequently analyzed by GC ion-trap mass spectrometry. Magnesium sulfate as a drying agent was mixed with the sample to get rid of water, and gave a high recovery for methamidophos as well as for other pesticides.

Highly polar pesticides such as the phosphorothioates and phosphoramidothioates showed very low recoveries by the supercritical CO_2 extraction method (e.g., acephate, omethoate and vamidothion). Generally, a modifier is added to the supercritical CO_2 to improve the extraction yield. Stefani *et al*. (1997) worked on many extraction methods using two steps, such as two subsequent extractions of the same sample without the addition of a polar solvent to supercritical CO_2. The two steps were similar except for the volume of the trap solvent. Celite and anhydrous calcined sodium sulfate were added as drying agents to the samples. The optimization of SFE on several organochlorine and organophosphorus pesticides in samples with high water content such as strawberry was performed. Lyophilization and addition of anhydrous sodium sulfate were examined to solve the problem caused by the water content of vegetable samples (Nerin *et al*., 1998). SFE efficiency is affected by a wide range of parameters such as the nature of the supercritical fluid, temperature and pressure, extraction time, the shape of the extraction cell, the sample particle size, the matrix type, the moisture content of the matrix and the analyte collection system. Due to these numerous parameters affecting the extraction efficiencies, SFE affords a high degree of selectivity and the extracts are relatively clean. However, the presence of water and fat in food samples can require extensive sample preparation and the development of more on-line cleanup procedures for SFE should enable further applications for food analysis to be developed. For example, sorbents, such as alumina, florisil and silica, can be placed in the extraction cell, or used as a cleanup following extraction to increase selectivity. Sorbents in the extraction cell can also be used for 'inverse' SFE extraction, in which interfering compounds are removed by a weak supercritical extraction fluid, leaving the analyte trapped on the sorbent for subsequent extraction under stronger conditions (King, 1998). Besides, the need to control so many operating parameters makes SFE optimization tedious and difficult in practice. Other disadvantages of the SFE technique include: limited sample size and high cost of the equipment.

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3

Analytical Methods for Viable and Rapid Determination of Organochlorine Pesticides in Water and Soil Samples

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1. Introduction

Organochlorine pesticides (OCPs) are the potential group of chemicals used to improve agricultural productivity. The extensive use of pesticides to improve agricultural productivity played an important role in the last century. These compounds have been applied for decades in preventing, repelling or mitigating the effects of pests. OCPs are one of the most persistent organic pollutants present in the environment. Although most of OCPs have been banned in many countries because of mutagenic and carcinogenic effects, they and their metabolites are still present in the environment owing to their persistence and lipophilic properties. The toxicity, potential bioaccumulation and non-biodegradability of these compounds represent risks to the environment (FAO/WHO, 1989).

Maximum admissible concentration (MAC) of pesticides and related products for drinking water is 0.1 μg L^{-1} for individual pesticides and 0.5 μg L^{-1} for total concentrations given by the European Union (EU) Drinking Water Directives (EEC, 1980). Additionally, pesticides residue in surface water must be less than 1–3 μg L^{-1}. Moreover, because of their hydrophobicity and persistence, OCPs accumulate in soils where they are likely to be retained for many years (FAO/WHO, 1989). Therefore, determination and monitoring of OCPs in different environmental matrices are important for environment, especially for human health. Consequently, residue analyses of OCPs in waters and soils by developing analytical procedure continue to be an active area of research in recent years (Santos & Galceran, 2004).

Trace analysis of OCPs in water is usually performed by gas chromatography (GC) combined with a previous an extraction or a pre-concentration step including traditional liquid–liquid extraction (LLE) (Barcelo´, 1993, Fatoki & Awofolu, 2003; Tahboub et al., 2005), solid phase extraction (SPE) (Aguilar et al., 1996; 1997), solid phase microextraction (SPME) (Page & Lacroix, 1997; Aguilar et al., 1999; Tomkins & Barnard, 2002; Li et al., 2003; Dong et al., 2005) and the more recently developed liquid phase microextraction under different names, i.e., dispersive liquid–liquid microextraction (DLLME) (Cortada et al., 2009a; Leong & Huang, 2009; Tsai & Huang, 2009), liquid-phase microextraction (LPME) (Huang & Huang, 2007; Farahani et al., 2008), single-drop microextraction (SDME) (Cortada et al., 2009b), polymer-coated hollow fiber microextraction (PC-HFME) (Basheer et al., 2004), stir bar sorptive extraction (SBSE) (Leo´n et al., 2003; Pe´rez-Carrera et al., 2007), ultrasound

assisted emulsification-microextraction (USAEME) (Ozcan et al., 2009a), vortex assisted liquid-liquid microextraction (VALLME) (Ozcan, 2010). Among these methods, LLE and SPE are the oldest procedures for the extraction of OCPs from aqueous matrices. LLE is probably the most widely used method for the extraction of OCPs from aqueous samples (Barcelo´, 1993, Fatoki & Awofolu, 2003; Tahboub et al., 2005). However, LLE needs relatively large volumes of organic solvents and samples and it is time-consuming as well as a labor-intensive method, and hazardous to health and environment. The LLE method has some complications such as the formation of stable emulsions. SPE has been used as an alternative method to LLE for the extraction of OCPs from water samples because it uses less solvent and is less time-consuming than LLE. Nevertheless SPE demands a large volume of organic solvents and samples. However, SPE is a relatively expensive method. In this method, analytes may be adsorbed, and complex matrices can cause settling in cartridges (Leong & Huang, 2009; Ozcan et al., 2009a; Quayle et al., 1997). LLE and SPE methods complicate and difficultly in automation. Using large amounts of organic solvents can cause environmental pollution and health hazards for laboratory personel and extra operational costs for waste treatment (Sarafraz-Yazdi & Amiri 2010).

Therefore, in order to overcome disadvantages of these methods, an efficient, fast, easy, economical and comparable sample preparation method such as solid-phase microextraction (SPME) and different modes of liquid–liquid microextraction (LLME), termed as liquid-phase microextraction (LPME) or solvent microextraction (SME) for example single drop microextraction (SDME), dispersive liquid–liquid microextraction (DLLME), ultrasound assisted emulsification-microextraction (USAEME) and vortex assisted liquid-liquid microextraction (VALLME) have been developed in recent years.

Among these methods, SPME is based on the partitioning of analytes between sample matrixes and the polymer-coated fibre. While SPME has some important advantages such as rapid, simple, solvent free, the main disadvantages of SPME method are relatively high price and fragile coating layer of fiber. Fiber also can degrade with time and the partial loss of stationary phase can cause co-elution with the analytes. In addition, sample carry-over has been frequently reported for SPME method (Psillakis et al., 2003). Liquid–liquid micro-extraction is based on the distribution of the analytes between a microvolume of organic solvent and the aqueous solution (He & Lee, 1997; Jeannot & Cantwell, 1996; 1997). These alternative techniques such as SDME, LPME, DLLME have advantages, such as short extraction time, small volumes of solvent and water requirement, rapid, easy, and low cost. Compared to the SPME, SDME has many advantages including no sample carry-over, wide selection of available solvents, simplicity and ease of use, short pre-concentration time, requiring no conditioning (as is the case with the fibre in the SPME), no need for instrument modification, etc. Nevertheless, these techniques also have some drawbacks. For example, SDME method has difficulty to automate, instability of droplet, and relative low precisions (Xu et al., 2007). In comparison to the traditional LLE and SPE, LPME procedure has many advantages including wide selection of available solvents, low cost, simplicity and ease of use, minimal solvent use, short pre-concentration time and possibility of automation. Furthermore compared to the SPME, LPME has also advantages, such as no sample carry-over, requiring no conditioning, no need for instrument modification, etc. (Khajeh et al., 2006). Nevertheless, some drawbacks, such as instability of droplet and relative low precision were reported for LPME procedure (Xu et al., 2007). DLLME is based on the formation of tiny droplets of the extractant in the sample solution using water-immiscible organic solvent (extractant) dissolved in a water-miscible organic dispersive solvent. The advantages of DLLME could be given as rapid, simple, short extraction time, low cost, high

recovery of analytes. However, general drawbacks of this method are difficult to automate and it requires using a dispersive solvent which usually decreases the partition coefficient of analytes into the extraction solvent (Rezaee et al., 2006; Pena-Pereira et al., 2009).

USAEME procedure combines micro-extraction system and ultrasonic radiation in one step. Ultrasonic radiation is a powerful means for acceleration of various steps in analytical procedure for both solid and liquid samples (Priego-Lo´pez & Luque de Castro, 2003; Aydin et al., 2006; Tor et al., 2006a; 2006b; Ozcan et al., 2009a; 2009b; 2009c; 2010). USAEME technique leads to an increment in the extraction efficiency in a minimum amount of time (Luque de Castro & Priego-Capote, 2006; 2007). Some other advantages of USAEME are viable, simple, rapid, low cost, and it needs less amount of sample and extraction solvent (Ozcan et al., 2009a; Saleh et al., 2009; Luque de Castro & Priego-Capote, 2007). However, the most important disadvantage of this method is that excessive ultrasound energy may degrade the analytes in water and may cause irreversible damages to the properties of analytes (Luque de Castro & Priego-Capote, 2007; Sanchez-Prado et al., 2008). A novel extraction technique, which is called as vortex-assisted liquid–liquid microextraction (VALLME) has recently been developed. In this method, dispersion of the solvent phase into the aqueous solution has been provided by using vortex mixing and miniaturization approach has been achieved using a micro volume of extraction solvent. VALLME is a fast, repeatable and efficient method and it requires quite small volume of extraction solvent.

The analysis of trace levels of organic pollutants in complex matrices such as soil, sediment usually requires several steps. An extraction step is followed by a clean-up of the extract prior to the chromatographic analysis. Extraction is a critical sample preparation step for the analysis of OCPs in soil samples because these hydrophobic compounds are strongly sorbed to the soil material. Various extraction procedures including soxhlet (Wobst et al., 1999; Fatoki & Awofolu, 2003; Bakan & Ariman, 2004), shaking flask (Kolb et al., 1995; Pozo et al., 2001; Nawab et al., 2003), sonication (Babic et al., 1998; Gonçalves & Alpendurada, 2005; Banjoo & Nelson, 2005; Castro et al., 2001), microwave assisted extraction (MAE) (Camel, 2000; Ericsson & Colmsjo, 2000; Pino et al., 2000; Jayaraman et al., 2001), super critical fluid extraction (SFE) (Reindl & Hofler, 1994; Barnabas et al., 1995; Koinnecke et al., 1997; Benner, 1998; Morselli et al., 1999) and pressured liquid extraction (Lundstedt et al., 2000; Ramos et al., 2000; Richter, 2000) can be used for the extraction of target compounds from soil. Moreover, determination of OCPs in soil can be carried out by using German standard method (DFG S-19 multimethod) (DFG, 1987) and ISO 10382 (ISO, 2002). The preference of each technique mainly depends on the efficiency, recovery, reproducibility, minimal solvent use, simplicity and ease of use. Soxhlet extraction is considered to be the standard method used for the extraction of OCPs from soils. The soxhlet and shaking flask extractions are time consuming and require large volume of organic solvents (Bøwadt et al., 1995; Hartonen et al., 1997; Schantz et al., 1998). Therefore, in order to reduce the extraction time, amount of solvent required, as well as sample amount, new extraction procedures, i.e., supercritical fluid extraction (SFE) (Bøwadt & Hawthorne, 1995), microwave assisted extraction (MAE) (Eskilsson & Björklund, 2000) and accelerated solvent extraction (ASE) (Björklund et al., 2000) etc., have been developed as alternative techniques. More recent procedures, i.e., SFE, MAE and ASE, gave shorter extraction time and reduced solvent consumption because these extraction procedures are working at high temperatures above the boiling point of the solvent. Except for SFE, reconcentration and clean-up steps have to be performed for MAE and ASE procedures. On the other hand, time and cost needed for SFE are quite high as well as for ASE (Berset et al., 1999).

Therefore, nowadays, modern methods have been proposed to solve time and solvent consuming problems as an alternative to traditional methods (Berset et al., 1999). Ultrasonic extraction procedure has lower equipment cost, ease of operation, little or no sample preparation (e.g. wet sediments) and lower extraction temperature, etc. Therefore, it can be also used to extract the target compounds from soil as an alternative to common Soxhlet and shaking flask extraction (Sporring et al., 2005; Aydin et al., 2006; Tor et al., 2006).
Ultrasonication is being used more and more in analytical chemistry, enabling different steps in the analytical process, particularly in sample preparation, such as the extraction of organic and inorganic compounds from different matrices (Ashler et al., 2001; Aydin et al., 2006; Ozcan et al., 2008; Mierzwa et al., 1997). This type of energy is of great help in the pre-treatment of samples as it facilitates and accelerates operations such as the extraction of organic and inorganic compounds. In ultrasound-assisted LLE, it facilitates the emulsification phenomenon and accelerates the mass-transfer process between two immiscible phases. The most widely accepted mechanism for ultrasound-assisted emulsification is based on the cavitation effect. The implosion bubbles generated by the cavitation phenomenon produces intensive shockwaves in the surrounding liquid and high velocity liquid jets. Such microjets can cause droplet disruption in the vicinity of collapsing bubbles and thus, improve emulsification by generating smaller droplet size of the dispersed phase right after disruption (Luque de Castro & Priego-Capote 2006). Submicron droplet-size results in significant enlargement of the contact surface between both immiscible liquids improving the mass-transfer between the phases. Additionally, ultrasonication offers several advantages that make it an ideal method for pre-treating a large number of samples. These advantages include high extraction efficiency, lower equipment costs, ease of operation and lower extraction temperatures, etc. Therefore, in this chapter, the application of ultrasonic extraction procedures for residue analysis of OCPs in water and soil samples was described. The applicability of the ultrasonic extraction was evaluated by comparison with traditional extraction methods (LLE and SPE for water samples, shaking flask, soxhlet extraction and large-scale ultrasonic extraction for soil).

2. Aim of the study

Because of the toxicity, potential bioaccumulation and non-biodegradability of OCPs represent risks to the environment. Thus, analysis of different environmental samples for OCPs is of importance for environment health.
At present, more than 60% of registered pesticides and/or their metabolites can be analyzed by using gas chromatography (GC). GC equipped with electron capture detector (ECD) is the most widely used technique especially for the determination of OCPs in different matrices (Santos & Galceran, 2004; Bruner, 1993). Chromatographic analysis usually follows the tedious sample preparation to extract the pollutants from environmental matrices (i.e. soil, sediment, air, water). For the isolation of target compounds from matrices various, extraction and clean-up procedures have been employed. The isolation of OCPs from environmental samples is often difficult and time consuming. The preference of each technique used for the extraction of OCPs from both water and soil samples mainly depends on solvent and time consumption, ease of operation, etc. Therefore, nowadays, modern methods have been proposed to solve time and solvent consuming problems as an alternative to traditional methods. A miniaturisation strategy has been successfully applied on the various extraction procedures, to establish a viable, rapid and easy procedure for

residue analyses of some organic pollutants in soil and sediment samples as well as reducing sample and solvent requirements.

Ultrasound assisted extraction offers several advantages that make it an ideal method for pre-treating a large number of samples. These advantages include high extraction efficiency, lower equipment costs, ease of operation and lower extraction temperatures, etc. Therefore, in this chapter the application of miniaturised ultrasonic extraction procedures for residue analysis of OCPs in water and soil samples was described. The miniaturised ultrasonic extraction applied to the water samples is also called as ultrasound asssisted emulsification microextraction (USAEME). The applicability of the miniaturised ultrasonic extraction was evaluated by comparison with traditional extraction methods (shaking flask, soxhlet extraction and large-scale ultrasonic extraction of soil samples, while LLE and SPE of water samples).

3. Experimental

3.1 Reagents and solvents

All chemicals used were of analytical grade. OCPs mixed standard including α, β, γ and δ-hexachlorocyclohexane (HCH), heptachlor, heptachlor epoxide, dieldrin, aldrin, endrin, endrin aldehyde, endrin ketone, endosulfan I, endosulfan II, endosulfan sulfate, p,p'-DDE, p,p'-DDD, p,p'-DDT, methoxychlor were from Accustandard Co. (New Haven, CT, USA). Solvents of residue grade purity including acetone, dichloromethane, chloroform, 1,2-dichlorobenzene, 1,2,4-trichlorobenzene, *n*-hexane, methanol, ethylacetate were obtained from Merck Co. (Darmstadt, Germany). Sodium chloride and sodium sulfate were also from Merck Co. Octadecyl (C_{18}) SPE cartridges were obtained from J&T Baker (Deventer, Holland). Alumina 90 active, neutral, [(0.063–0.200 mm), (70–230 mesh ASTM)] was also from Merck Co. Standard stock solution 10 mg L^{-1} of mixed OCPs was prepared in methanol. All solutions were stored in the dark at 4 ^{o}C. Working solutions were prepared by dilution of standard stock solution with distilled water.

3.2 Chromatographic analysis

The determination of the OCPs was performed by GC/μ-ECD (Agilent Technologies, CA, USA). The features and operating conditions of GC/μ-ECD system were as follows: GC Agilent 6890 N installed with HP-5 5% phenylmethyl siloxane fused silica capillary column (30 m length, 0.32 mm i.d. and 0.25 μm film thickness). The split/splitless injector was set at 280 °C and operated in the splitless mode (purge delay 1 min, purge flow 30.1 mL min^{-1}). Detector temperature was set at 320 °C. The injection was performed by an Agilent 7683 B series automatic injector. The temperature program was as follow: initial column temperature 60 °C, 40 °C min^{-1} to 160 °C, 5 °C min^{-1} to 300 °C, hold at 300 °C for 5 min (run time 35.5 min). Helium (purity 99.999%) was used as carrier gas at flow rate of 2.5 mL min^{-1}.

3.3 Clean-up procedure

The activation and deactivation of the column sorbent material, aluminum oxide, was performed as follows. The aluminum oxide was activated at 210 ^{o}C for 4 h. It was allowed to cool down in a desiccator and then deactivation and homogenization were carried out by adding certain amounts of deionized water (5%) and shaking the sorbents in a horizontal shaker at 210 rpm for 2 h. The preparation of the traditional clean-up column filled with 10 g of deactivated column sorbent material was described in a previous paper (Tor et al., 2006).

The clean-up column, length of 30 cm and 1 cm of internal diameter, was prepared according to slurry packing technique (Jaouen-Madoulet et al., 2000). The extract, reduced in volume to 1 mL, was transferred quantitatively onto the top of the column. The elution of OCPs was carried out with 100 mL of *n*-hexane/ethylacetate (1/1, v/v), then the extract was concentrated to exactly 1 mL using a rotary evaporator (Buchi B-160 Vocabox, Switzerland) and nitrogen stream prior to GC/μ-ECD analysis. The micro-scale clean-up column consisted of a pasteur pipette, length of 10 and 0.5 cm internal diameter, fitted at its base with a plug of glass wool. 0.5 g aliquot of 5% deactivated aluminium oxide was filled into the pasteur pipette and conditioned with 5 mL of *n*-hexane. After miniaturized ultrasonic extraction, the extract was reduced in volume to 300 μL and it was transferred top of the column. The target compounds were eluted from the pipette under gravity (flow-rate of approximately 2 drop s^{-1}) with 5 mL of *n*-hexane/ethylacetate (7/3, v/v). The eluate was concentrated to 300 μL prior to GC/μ-ECD analysis.

3.4 Liquid-liquid extraction

Liquid-liquid extraction (LLE) procedure was adopted from US EPA Method 3510C (US EPA, 1996). 200 mL of water sample was placed in a 250 mL capacity of separatory funnel. The extraction was carried out three times with 20 mL of dichloromethane. The extracts were combined and dried with anhydrous sodium sulfate. The resulting extract was concentrated to exactly 1 mL using a rotary evaporator (Buchi B-160 Vocabox, Flawil 1, Switzerland) and gentle nitrogen stream. Then, GC/μ-ECD analysis was performed as described in Section 3.2.

3.5 Solid-phase extraction

SPE procedure was carried out as described by Aydin et al. (2004). Octadecyl (C_{18}) SPE cartridge was used for the extraction of OCPs from water sample. The cartridge was consecutively washed with 10 mL of methanol and 8 mL of *n*-hexane/ethyl acetate (5/3, v/v). Then, it was conditioned with 10 mL of methanol and 2×5 mL of distilled water. 200 mL of water sample was passed through the cartridge under vacuum. After the cartridge was dried for 10 min by maintaining vacuum, elution of OCPs from the cartridge was carried out with 10 mL of *n*-hexane/ethyl acetate (7/3, v/v). The extract was dried with sodium sulfate and concentrated to exactly 1 mL by a rotary evaporator and under gentle nitrogen stream. Then, GC/μ-ECD analysis was performed as described in Section 3.2.

3.6 Shake flask extraction

For shake flask extraction, a 10 g of soil sample was suspended in 50 mL of petroleum ether/acetone mixture (1/1, v/v) and shaken on a horizontal shaker for 12 h. Then, the extract was filtered and concentrated to exactly 1 mL by using the rotary evaporator and nitrogen stream, respectively (Tor et al., 2006; Aydin et al., 2006). The concentrated extract was transferred onto the traditional clean-up column and elution was performed as described in Section 3.3. GC/μ-ECD analysis was performed as described in Section 3.2.

3.7 Soxhlet extraction

For soxhlet extraction, a 10 g of soil sample was put into the extraction thimble and extracted with 150 mL of petroleum ether/acetone mixture (1/1, v/v) for 18 h (Tor et al., 2006; Aydin et al., 2006). The extract was reduced to exactly 1 mL using the rotary

evaporator and under a gentle stream of nitrogen. The concentrated extract was transferred onto the traditional clean-up column and elution was performed as described in Section 3.3. GC/μ-ECD analysis was performed as described in Section 3.2.

3.8 Large-scale ultrasonic extraction

Ultrasonic extraction of 10 g of soil sample was performed twice by using 25 mL of petroleum ether and acetone mixture (1/1, v/v) for 20 min (Tor et al., 2006a). After extraction steps, extracts were filtered and combined in a round bottom flask. The extract was reduced to exactly 1 mL using the rotary evaporator and under a gentle stream of nitrogen. The concentrated extract was transferred onto the traditional clean-up column and elution was performed as described in Section 3.3. GC/μ-ECD analysis was performed as described in Section 3.2.

3.9 Miniaturised ultrasonic extraction of OCPs from water samples

A 10 mL water sample was placed in a 10 mL glass-centrifuge tube. As an extraction solvent, 200 μL chloroform was added into the water sample and mixed. The resulting mixture was immersed into an ultrasonic bath (frequency 35 kHz, 320W, Super RK 510, Sonorex, Bandelin, Germany) for 15 min at 25 ◦C. During the sonication, the solution became turbid due to the dispersion of fine chloroform droplets into the aqueous bulk. The emulsification phenomenon favoured the mass-transfer process of OCPs from the aqueous bulk to the organic phase. The emulsion was centrifuged at 4000 rpm for 5 minutes to disrupt the emulsions and separate the solvent from the aqueous phase. After centrifugation, extraction solvent was removed from the bottom of the tube by using a 250 μL Hamilton syringe (Hamilton Bonaduz AG, Switzerland) and transferred into the microvial. Then, GC/μ-ECD analysis was performed as described in Section 3.2.

3.10 Miniaturised ultrasonic extraction of OCPs from soil samples

A 0.5 g soil sample was sonicated three times for 5 minutes with 5 mL of mixture of acetone-petroleum ether (1/1, v/v) in the ultrasonic bath. The extracts were combined and were filtered by using Whatman filter paper. The filtrates were reduced to 1 mL with the rotary evaporator (Buchi B-160 Vacobox, Switzerland) and adjusted to exactly 300 μL by using a gentle nitrogen stream. The concentrated extract was transferred onto the micro-scale clean-up column and elution was performed as described in Section 3.3. Then, GC/μ-ECD analysis was performed as described in Section 3.2.

3.11 Real water and soil samples

The efficiency of the ultrasonic extraction of OCPs from different water samples (i.e., tap water, well water, lake water, domestic and industrial wastewater samples) was compared with traditional LLE and SPE procedures. Tap water was obtained from the laboratory and well water came from deep-ground water in Konya (Turkey). Lake water was taken from Cavuscugol in Ilgın (Turkey). The domestic and industrial wastewater samples were taken from the sewage system in residential area and industrial zone in Konya (Turkey), respectively. All samples were collected free of air bubbles in glass containers and they were stored in the dark at 4 ◦C. Tap and well water samples were analysed without previous treatment or filtration. The lake water, domestic and industrial wastewater samples were filtered through a membrane filter with 0.45 μm pore size before the extraction procedures.

The efficiency of the ultrasonic extraction of OCPs from the real soil samples was also compared with traditional soxhlet, shake-flask and large-scale ultrasonic extraction procedures on the real soil samples. Real soil samples were also obtained from the Department of Soil, Agricultural Faculty of Selcuk University (Konya, Turkey). The textures of the soil samples were as follows. Sample A, sand: 42.2%, silt: 31.5%, clay: 24.5, organic matter: 1.80%, pH (0.01 M $CaCl_2$): 7.2 and maximum water capacity: 20.4%. Sample B, sand: 49.2%, silt: 32.3%, clay: 18.5%, organic matter: 1.90%, pH (0.01 M $CaCl_2$): 6.5, and maximum water capacity: 19.6%. Sample C, sand: 39.9%, silt: 35.0%, clay: 23.8%, organic matter: 1.36%, pH (0.01 M $CaCl_2$): 7.3, and maximum water capacity: 24.1%.

4. Results and discussions

4.1 Water analysis

The recovery experiments were carried out for the evaluation of the miniaturised ultrasonic extraction efficiency of selected OCPs in water samples. After choice of the most suitable solvent and solvent volume, several other parameters including extraction time, centrifugation time and ionic strength of the water sample were optimized. The applicability of ultrasonic solvent extraction procedure was also compared with LLE and SPE methods on the real water samples.

At the beginning of the experiments, the extraction efficiency of dichloromethane, 1,2-dichlorobenzene, 1,2,4-tichlorobenzene, chloroform and bromoform was determined. The choice of extraction solvent is critical for developing an efficient ultrasonic solvent extraction procedure since physico-chemical properties of the solvent govern the emulsification phenomenon, and consequently, the extraction efficiency. Moreover, the extraction solvent should have good affinity for target compounds and it should have excellent gas chromatographic behavior. 10 mL aliquots of distilled water including 2 μg L^{-1} of each OCP were extracted by using 100 μL of each solvent in ultrasonic bath for 5 min at 25 °C. During the sonication, the solution became turbid due to the dispersion of fine solvent droplets into the aqueous bulk. The emulsification phenomenon favored the mass-transfer process of OCPs from the aqueous bulk to the organic phase. Emulsification was observed in all cases with the exception of dichloromethane. Dichloromethane was completely dissolved in the aqueous solution. The results revealed that chloroform was of the highest extraction efficiency among the examined solvents.

In the second set of experiments, the optimum volume of solvent was determined. The main effect of the ultrasound in LLE is that the fragmentation of one of the phases to form emulsions with submicron droplet size that enormously extend the contact surface between both liquids (Abismail et al., 1999). Therefore, it is expected that increasing the volume of chloroform from 50 to 300 μL increases the number of submicron droplet. Hence, the higher mass-transfer or extraction efficiency is obtained. This optimization experiment was carried out using chloroform, which gave the highest recovery for the pesticides studied. In order to determine the optimum volume of chloroform, 10 mL fortified distilled water was extracted by means of ultrasound for 5 min with 50, 100, 200 and 300 μL of chloroform. 50 μL chloroform were completely dissolved in the aqueous solution. The results showed that the recoveries increased with chloroform volume from 100 to 200 μL. Then, a decrease in the recoveries was generally observed when the solvent volume was increased to 300 μL. Increasing the extraction solvent (chloroform) volume from 100 to 200 μL resulted in higher

extraction efficiency. However, increasing the solvent volume 300 μL caused a decrease in the response of the detector, and the unfavorable effect of larger solvent volume was because of a dilution effect of the analytes in the resulting organic phase. Therefore, in the present study, 200 μL of chloroform was selected for further optimization experiments.

After choice of chloroform and 200 μL as the optimum extraction solvent and extraction solvent volume, respectively, several other factors affecting the ultrasonic extraction procedure, such as extraction time (denoted as factor 1), centrifugation time (denoted as factor 2) and ionic strength of the sample (denoted as factor 3) were optimized by using a 2^3 factorial experimental design. The corresponding levels (low and high level) for factors 1–3 were 5 and 15 min, 5 and 10 min, 0 and 10%, respectively. All the experiments were performed in duplicate and randomized. After each extraction, the emulsion was centrifuged for 5 min at 4000 rpm. Then, extraction solvent was removed from the bottom of the tube by using a 250 μL syringe and transferred into the micro vial. Then, GC/μ-ECD analysis was performed as described in Section 3.2.

After processing the data by analysis of variance (ANOVA) using Tool Pak in Microsoft Excel, the ANOVA tables were constructed to test the significance of the effect of each factor on the extraction efficiency. At significance level of 5%, the factor with *F*- value over critical *F*-value (5.318) has a significant effect on the extraction efficiency.

As it is seen in Table 1, for all compounds, the significant parameters were extraction time (factor 1) and ionic strength of the sample (factor 3). However, centrifugation time (factor 2) was not significant. Additionally, interactions between the extraction time and centrifugation time (factor 1 and 2), between the extraction time and ionic strength (factor 1 and 3) were found to be significant. Lastly, interaction between the centrifugation time and ionic strength (factor 2 and 3) was also significant.

Time plays an important role in the emulsification and mass-transfer phenomena. Extraction time has a positive sign, so 15 min is better than 5 min for the extraction. Like SPME, liquid–liquid micro-extraction procedures are processes dependent on equilibrium rather than exhaustive extraction (Zhao & Lee, 2001; Tor & Aydin, 2006). The amount of analyte extracted at a given time depends upon the mass transfer of analyte from the aqueous phase to the organic solvent phase. This procedure requires a period of time for equilibrium to be established. For present study, it was observed that the recoveries increased with increasing extraction time from 5 to 15 min. Therefore, 15 min was chosen as the extraction time for further studies.

Centrifugation was required to break down the emulsion and accelerate the phase-separation process. As a result, increasing centrifugation time does not influence the extraction efficiency. Thus, 5 min was selected as the centrifugation time to get a satisfactory biphasic system.

Ionic strength of the sample had negative sign for the studied OCPs. As is well known, ionic strength affects the partitioning coefficients of analytes between an aqueous and organic phase. On the other hand, as the ionic strength of the medium increases, the viscosity and density of the solution increase. This causes a diminishing in the efficiency of the mass-transfer process and, consequently, the extraction efficiency of the procedure (Regueiro et al., 2008). Additionally, the ultrasound waves can be absorbed and dispersed in a viscous medium as calorific energy; thus, the cavitation process could be withdrawn reducing the emulsification phenomenon (Mason & Lorimer, 2002). In this study, an increase in the ionic strength of the sample from 0 to 10% decreased the extraction efficiency. Therefore, no sodium chloride was added to the samples for further studies.

Experiment no	Codified parameters			No codified parameters			Average recovery (%)
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3	
1-9	-	-	-	5	5	0	93
2-10	+	-	-	15	5	0	98
3-11	-	+	-	5	10	0	90
4-12	+	+	-	15	10	0	98
5-13	-	-	+	5	5	10	64
6-14	+	-	+	15	5	10	65
7-15	-	+	+	5	10	10	70
8-16	+	+	+	15	10	10	70
Factor 1, extraction time, Factor 2, centrifugation time, Factor 3, ionic strength of the sample							

Table 1. Design matrix for factorial design and average recoveries of OCPs for the effect of parameters on the miniaturised ultrasonic extraction procedure (Ozcan et al., 2009a).

In addition, interaction between factor 1 and 2 was positive and interactions between factor 1 and 3 and factor 2 and 3 were negative. According to the results, the optimum conditions for miniaturised ultrasonic extraction of OCPs from water were chosen as follows: for chloroform as extraction solvent, solvent volume, 200 µL; extraction time, 15 min without addition of sodium chloride at 25 °C; and centrifugation time, 5 min.

The results of recoveries for the fortified distilled water with three different fortification levels (level 1, 0.5 µg L^{-1}; level 2, 2 µg L^{-1}; level 3, 5 µg L^{-1}) were given in Fig. 1. The repeatability of the proposed method, expressed as relative standard deviation (RSD), was found to vary between <1 and 9% for the fortified water samples.

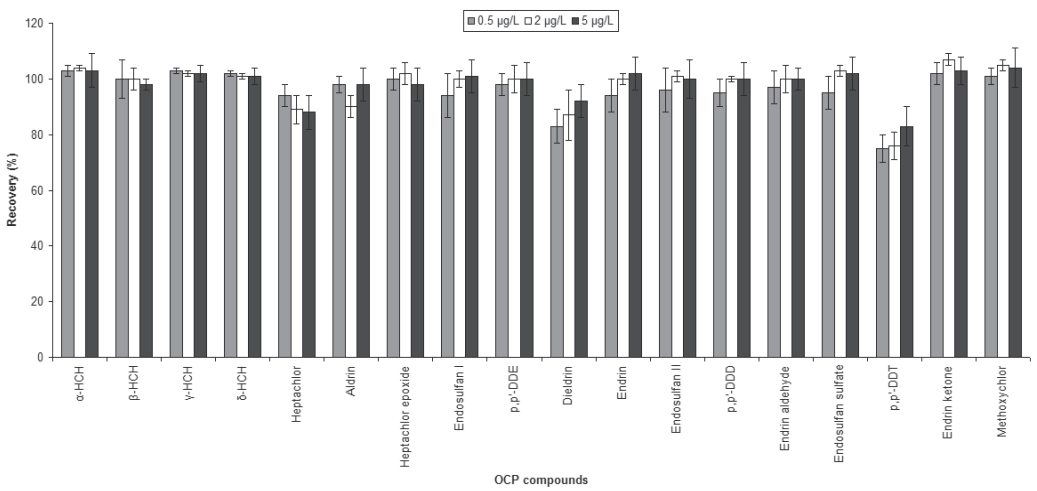

Fig. 1. Recoveries of OCPs in spiked distilled water with three fortification levels using miniaturised ultrasonic extraction method [*n*=8] (Extraction conditions; extraction solvent: chloroform, extraction time: 15 min, sample volume: 10 mL, extraction solvent volume: 200 µL, centrifugation time: 5 min, ionic strength: 0%, ambient temperature: 25 °C)

According to fortification level 1 (0.5 μg L^{-1}), recoveries ranged from 75 ± 5% to 103 ± 2%. Comparable recoveries were also obtained from fortification levels 2 (2 μg L^{-1}) and 3 (5 μg L^{-1}). When statistical evaluation was carried out between recoveries of OCPs from fortification level 1 and level 2, no significant differences ($p>0.05$) were observed. Additionally, no significant differences were observed when the same statistical evaluations were carried out between fortification levels 1–3 and 2–3. This indicates that developed miniaturised ultrasonic extraction method (in other word, USAEME method) was of considerable efficiency in extracting OCPs from water samples.

The validation of the miniaturised ultrasonic extraction procedure was carried out using both fortified distilled water and fortified real water and wastewater samples. In addition, the efficiency of the method was also compared with traditional LLE and SPE techniques on the fortified real water samples. The recoveries were given in Figs. 2 and 3, which indicated that the recoveries of examined OCPs were higher than 78% with R.S.D. below 9%. Analyses of real water samples showed that sample matrices had no adverse effect on the efficiency of the miniaturised ultrasonic solvent extraction procedure.

When recoveries of OCPs were gauged against absolute limits of 70% and 130% (US EPA, 1995) it was seen that method gave satisfactory results. The efficiency of the miniaturised ultrasonic extraction was also compared with those involving traditional LLE and SPE method on the same fortified real samples. As seen in Fig. 2 and Fig. 3, the method gave comparable results with traditional LLE and SPE methods. However, it should be emphasized that the ultrasonic extraction is not time-consuming procedure and it is not necessary a re-concentration step prior to the GC analysis. Furthermore, it needs much lower volumes of solvent and water sample than the traditional LLE and SPE techniques.

4.2 Soil analysis

The recovery experiments were carried out for optimization of an miniaturised ultrasonic extraction of OCPs from soil samples. The factors affecting the performance of ultrasonic extraction (i.e., amount of sample, volume of extraction solvent and number of extraction step) were optimized by using a 2^3 factorial experimental design. The applicability of the ultrasonic extraction was tested by a comparison with conventional soxhlet, shake flask and large-scale ultrasonic extraction of real soil samples with spiked OCPs.

Different solvents with a wide polarity range such as *n*-hexane, ethyl acetate, acetone and a mixture of petroleum ether and acetone (1/1, v/v) were examined for the optimization of large-scale ultrasonic extraction of OCPs from soil (Tor et al., 2006a). The mixture of petroleum ether and acetone (1/1, v/v) gave the highest recoveries followed by acetone, ethyl acetate and *n*-hexane in ultrasonic extraction. Therefore, the mixture of petroleum ether and acetone (1/1, v/v) was used as extraction solvent for the optimization experiments.

After choice of the mixture of petroleum ether and acetone (1/1, v/v) as the optimum extraction solvent, several other factors affecting the ultrasonic extraction procedure, such as amount of sample (denoted as factor 1), volume of extraction solvent (denoted as factor 2) and number of extraction step (denoted as factor 3) were optimized by using a 2^3 factorial experimental design. The corresponding levels (low and high level) for factors 1–3 were 0.5 and 1.5 g, 2 and 5 mL, 1 and 3, respectively. All the experiments were performed in duplicate and randomized. After processing the data by ANOVA, the ANOVA tables were constructed to test the significance of the effect of each factor on the extraction efficiency. At

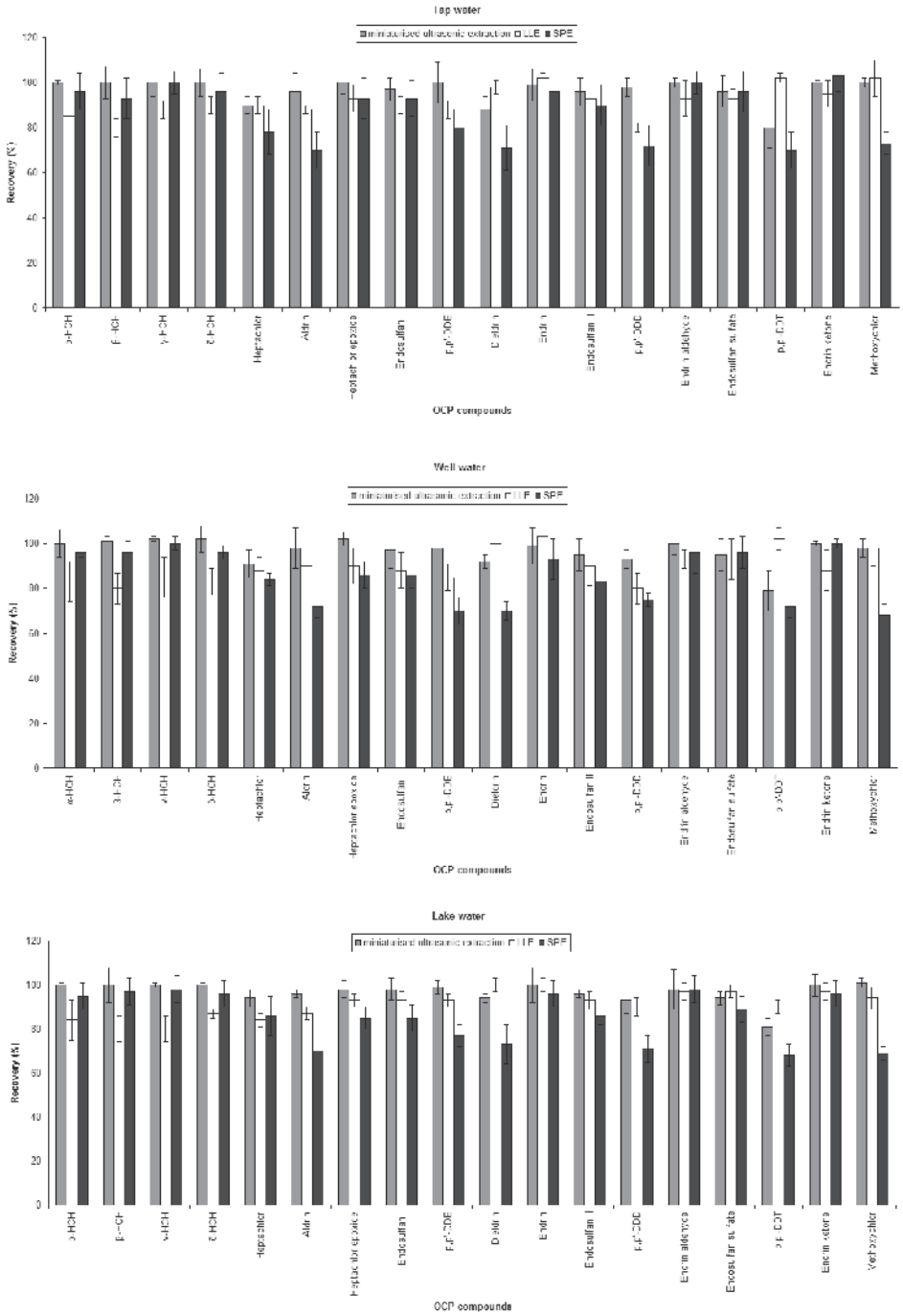

Fig. 2. Comparison of extraction efficiency of the miniaturised ultrasonic extraction method with LLE and SPE for OCPs in fortified real water (tap, well and lake) samples (fortification concentration for each compound: 2 µg L^{-1}), [*n*=4].

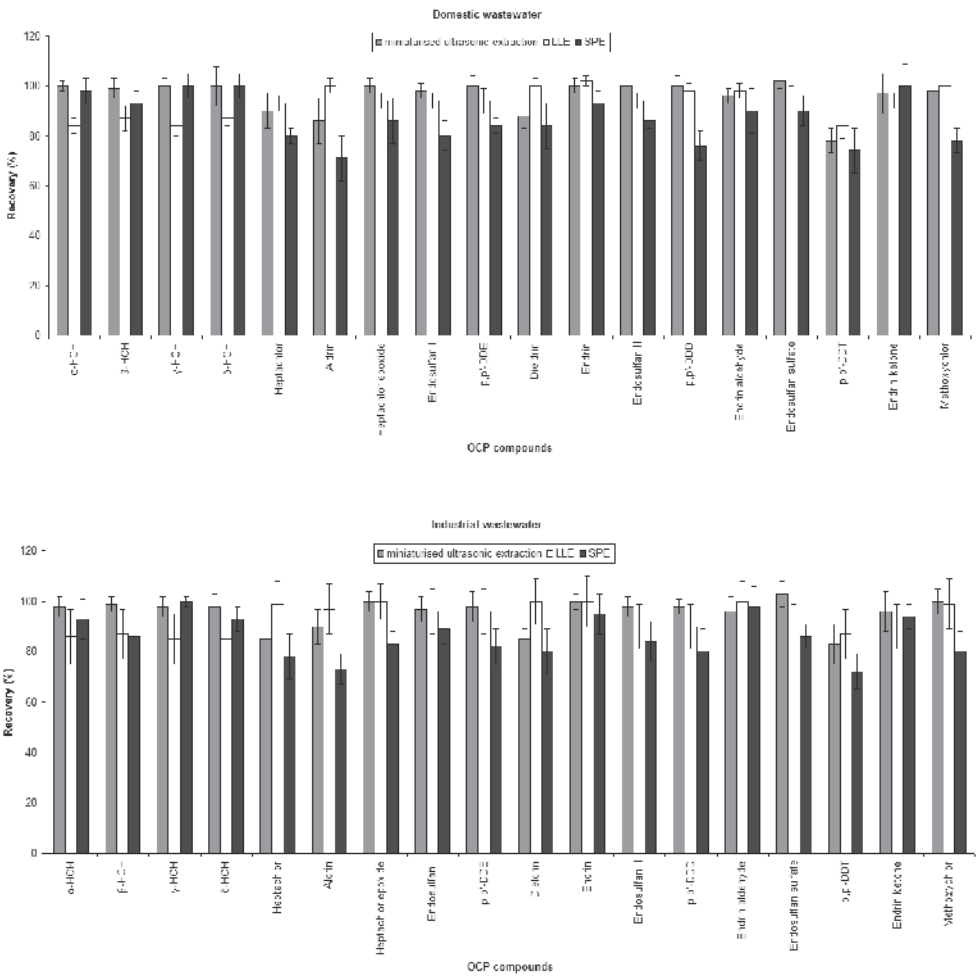

Fig. 3. Comparison of extraction efficiency of the miniaturised ultrasonic extraction method with LLE and SPE for OCPs in fortified real wastewater (domestic and industrial) samples (fortification concentration for each compound: 2 μg L^{-1}), [n=4].

significance level of 5%, the factor with F-value over critical F-value (5.318) has a significant effect on the extraction efficiency.

0.5 g of soil sample was sonicated for 5 min with 5 mL of petroleum ether and acetone mixture (1/1, v/v) in an ultrasonic bath. The extraction was repeated three times. After each extraction, extracts were collected in a pointed flask and reduced in volume to 300 μL by a gentle nitrogen stream. Then clean-up procedure and GC/μ-ECD analysis were performed as described in Sections 3.3. and 3.2., respectively.

As it is seen in Table 2, for all compounds, the significant factors were sample amount, solvent volume and number of extraction. Additionally, interactions between the sample amount and solvent volume and between the sample amount and number of extraction were found to be significant. Lastly, interaction between the solvent volume and number of extraction was also significant.

Sample amount negatively affected the extraction of all OCPs. It is expected that high sample amount may require longer sonication time needed for the extraction of all OCPs from soil. In other words, fixed sonication time (5 min) was insufficient for extraction of 1.5 g of sample. Another reason may be that 2 mL of extraction solvent is not adequate for completely extraction of OCPs from 1.5 g of soil sample. Hence, 0.5 g of sample is better than 1.5 g for the extraction of OCPs with 2 mL of solvent and 5 min of sonication time.

Experiment no	Codified parameters			No codified parameters			Average recovery (%)
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3	
1-9	-	-	-	0.5	2	1	36
2-10	+	-	-	1.5	2	1	20
3-11	-	+	-	0.5	5	1	66
4-12	+	+	-	1.5	5	1	47
5-13	-	-	+	0.5	2	3	82
6-14	+	-	+	1.5	2	3	60
7-15	-	+	+	0.5	5	3	93
8-16	+	+	+	1.5	5	3	59
Factor 1, sample amount, Factor 2, solvent volume, Factor 3, number of extraction step							

Table 2. Design matrix for factorial design and average recoveries of OCPs for the effect of parameters on the miniaturised ultrasonic extraction procedure (Ozcan et al., 2009d).

Solvent volume had positive sign. 5 mL is better than 2 mL for the extraction. In soil and sludges, OCPs are adsorbed on or in aggregates. Namely, 5 mL of extraction solvent is of more capability than 2 mL for disintegration of the soil aggregates and extraction of OCPs from soil. Number of extraction had also positive sign for all studied OCPs. Increasing the number of extraction step also increased the extraction efficiency. Thus, recoveries obtained from three times extraction are higher than those from single step extraction. As a result from experimental factorial design, optimum conditions for ultrasonic extraction of OCPs from soil were as follows: sample amount: 0.5 g; solvent volume: 5 mL mixture of acetone-petroleum ether (1/1, v/v) and number of extraction step: 3, with a 5 min sonication.

The optimum extraction procedure was examined by using of three different fortification levels (levels 1, 25 μg kg^{-1}; level 2, 50 μg kg^{-1}; level 3, 100 μg kg^{-1}). The results of recoveries were given in Fig. 4. According to fortification level 1, recoveries ranged from 86 (±1)% to 104 (±4)%. Comparable recoveries were also obtained from fortification levels 2 and 3 (see Fig. 4). When statistical evaluation was carried out between quantities of OCPs extracted

from fortification levels 1 and 2, no significant differences ($p > 0.05$) were observed. Moreover, the same statistical evaluations were carried out between fortification levels 1–3 and 2–3, no significant differences were observed. This indicated that optimized ultrasonic solvent extraction was of considerable efficiency in order to extract OCPs from soil sample.

The applicability of the miniaturised ultrasonic extraction method to the real soil samples was investigated by comparing with soxhlet, shake flask and large-scale ultrasonic extraction method. The analyses for three different soil samples were carried out. Soil sample A, B and C were spiked with OCPs (spike level for each compound: 50 µg kg^{-1}) and analysis was performed by using miniaturised ultrasonic solvent extraction, soxhlet extraction, shake flask extraction and large-scale ultrasonic extraction. The results are given in Fig. 5.

As seen in Fig. 5, miniaturised ultrasonic extraction gave comparable results with especially soxhlet and large-scale ultrasonic extraction method. In addition, extraction efficiency of the proposed procedure is generally higher than that of shaking flask extraction. As a results, when recoveries of OCPs for the ultrasonic extraction were gauged against absolute limits of 70% and 130% (US EPA, 1995), it was seen that proposed miniaturised method gave satisfactory results.

Soxhlet and shake flask extractions have been the traditional methods used for extraction of OCPs from soils (ISO, 2002). The main disadvantages of these methods are that there are needs for more volume of solvent, long time for extraction, reconcentration and clean-up steps (Bøwadt et al., 1995; Hartonen et al, 1997). Ultrasonication allows an intensive contact between soil particles and solvent and it reduces the extraction time. Therefore, miniaturised ultrasonic extraction can be used to extract OCPs from soil as an alternative to common Soxhlet and shake flask extraction.

Soxtec extraction, based on Soxhlet system, is a two step extraction procedure, involving a boiling and rinsing step, which drastically reduces the total time of extraction. However, reconcentration and clean-up steps are also required for both ultrasonic and soxtec extraction techniques (Pastor et al., 1997; Popp et al., 1997).

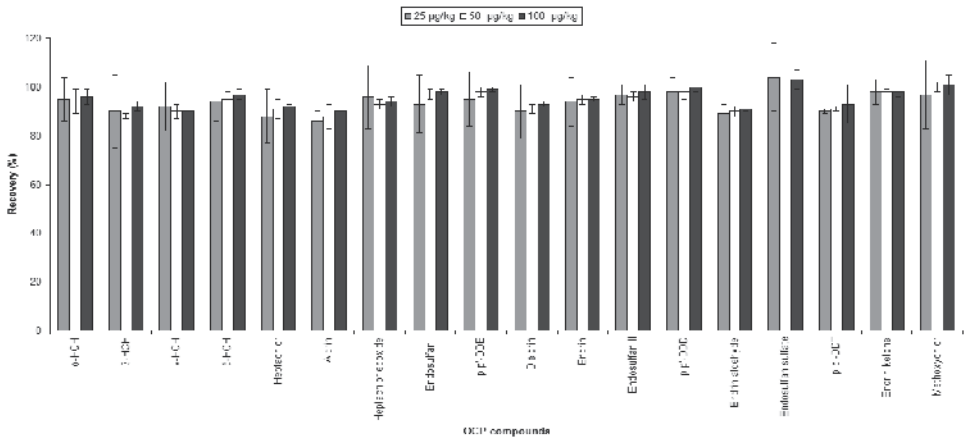

Fig. 4. Recoveries of OCPs from spiked soil with three fortification levels using miniaturised ultrasonic extraction method [n=5]. (Extraction conditions; extraction solvent: acetone/petroleum ether (1/1, v/v), soil sample amount: 0.5 g, extraction solvent volume: 5 mL, number of extraction step: 3, extraction time: 5 minutes, temperature: 25 ^{o}C)

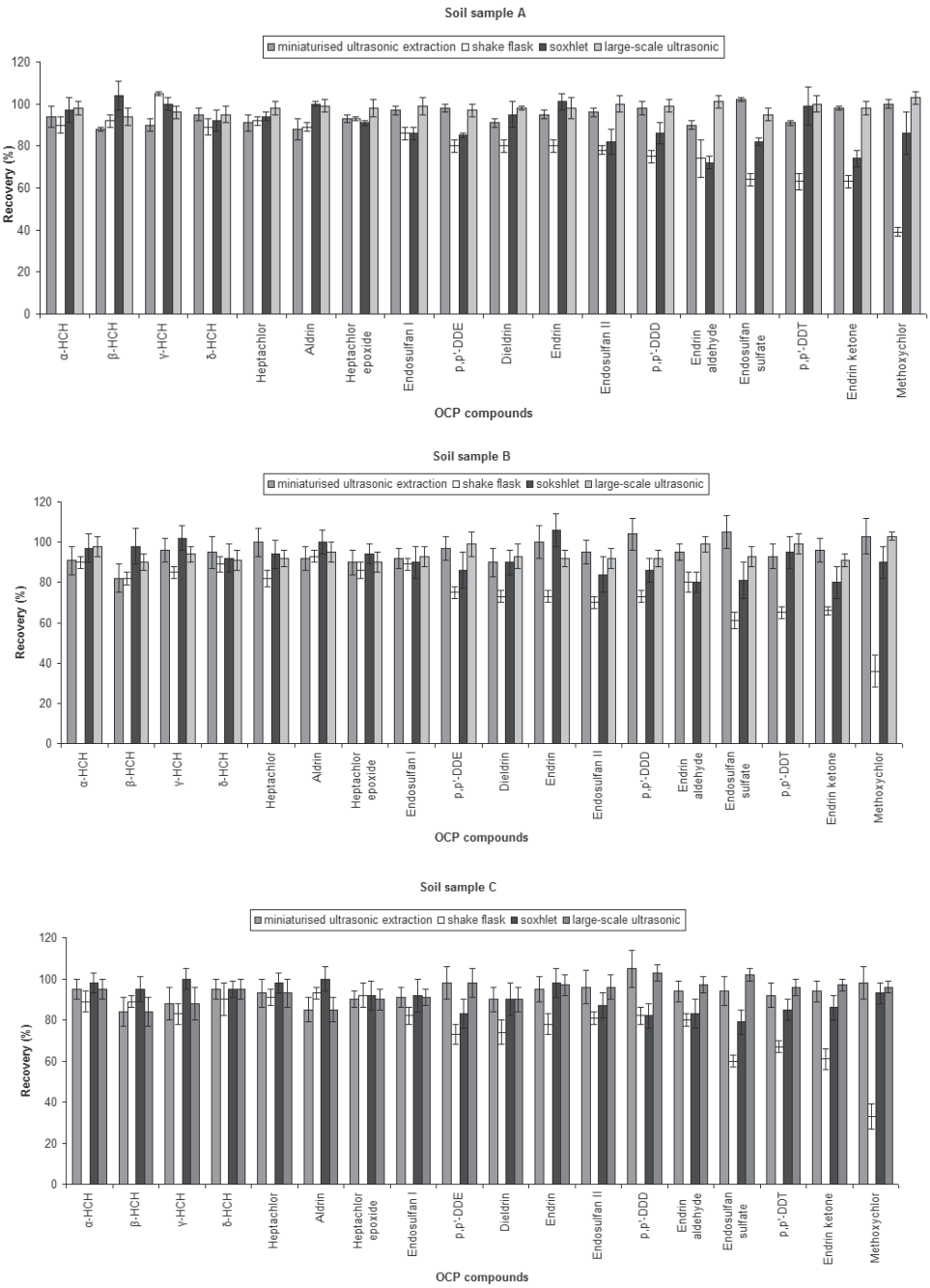

Fig. 5. Comparison of extraction efficiency of the miniaturised ultrasonic method with soxhlet, shake flask and large-scale ultrasonic extraction for OCPs in fortified real soil samples (fortification concentration for each compound: 50 μg kg^{-1}), [*n*=5].

Apart from these methods, three more recent techniques from literature, including supercritical fluid extraction (SFE) (Sun & Lee, 2003), microwave-assisted extraction (MAE) (Eskilsson and Björklund, 2000) and accelerated solvent extraction (ASE) (Björklund et al., 2000), were also compared with for the extraction of OCPs from soil. The main key to shorter extraction times and reduced solvent consumption with these techniques is the possibility of working at elevated temperatures above the boiling point of the solvent. Thereby the extraction process is facilitated due to increased analyte desorption and diffusion from the solid matrix. SFE and ASE techniques need much lower volumes of organic solvents than other extraction techniques. Except for SFE technique, reconcentration and clean-up steps have to be performed for MAE and ASE techniques (Berset et al., 1999). Obviously the contamination risk for those extraction techniques which require reconcentration and clean-up steps are higher than that of SFE. On the other hand method development time and costs for SFE are quite high as well as for ASE (Berset et al., 1999). Compared to the conventional soxhlet and shake flask extraction techniques, the miniaturised ultrasonic solvent extraction in this study has many advantages including minimal solvent use, short extraction and preconcentration time, low cost, simplicity and ease of use. In addition, this method is cheaper and easier than MAE, ASE and SFE techniques.

5. Conclusion

This chapter has outlined the successful development and application of miniaturised ultrasonic extraction procedure for the determination of OCPs in water and soil samples by using GC/μ-ECD. Analyses of real samples showed that sample matrices had no adverse effect on the efficiency of ultrasonic extraction procedure. As a consequence, the proposed miniaturised ultrasonic extraction method is precise, reproducible and rapid and easy for the analyses of OCPs in water and soil samples. It also requires only small volumes of extraction solvent and sample materials. In addition, the miniaturised ultrasonic extraction method has been demonstrated to be viable, rapid and easy to use for the qualitative and quantitative analysis of OCPs in different water and soil samples. Additionally, the miniaturised method uses less solvent than traditional approaches (i.e., liquid-liquid, solid phase, shake flask, soxhlet, large-scale ultrasonic extraction), reducing the costs associated with solvent purchase and waste disposal. The proposed method will reduce laboratory expenses without substantial new equipment and without compromising accuracy and precision. Furthermore, it is cheaper and easier than LLE, SPE, SPME, MAE, ASE and SFE techniques and it can be concluded that most commercial laboratories can efficiently used the proposed method for the extraction of OCPs from water and soil.

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4

Factors Affecting the Accurate Quantification of Pesticide Residues in Non-Fatty Matrices

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1. Introduction

The presence of pesticide residues is regarded as a potential chemical hazard in several foodstuffs, such as fruits and vegetables. Based on the increasing consumers' concern about the residues persistence in their food, a large number of multiresidue extraction methods (MRMs) has been evaluated to ensure accurate residues determination (Greve, 1988; van Zoonen, 1996; Schenck & Wong, 2008). The widely diffused MRMs in the analysis of non-fatty matrices apply extraction with appropriate solvents (e.g., ethyl acetate – EtOAc, acetone, acetonitrile – ACN, methanol) in the first step and gas chromatography (GC) with sensitive and selective detectors (e.g., nitrogen phosphorus – NPD, electron capture – ECD, flame photometric – FPD, mass spectrometry – MS) in the final part of determination (Motohashi et al., 1996; Seiber, 1999; Beyer & Biziuk, 2008; Sannino, 2008; Schenck & Wong, 2008). A large number of modifications in the possible additional clean-up of the organic solvent extract are also included to result in more accurate result of analysis (Tekel & Hatrik, 1996; Schenck & Lehotay, 2000; Lee & Richman, 2002; Schenck et al., 2002). Nowadays, most of the approaches applied are effective in detecting and quantifying several analytes in a large scale of matrices within a relatively short period by minimizing reagents consumption (Lee et al., 1991; Hajšlová et al., 1998; Egea González et al., 2002; Majors, 2007).

However, the MRMs application has sometimes increased the result inaccuracy caused by several parameters, such as matrix analysed, concentration level of pesticide identified, extraction solvent and/or determination technique applied and phenomena like "matrix-induced enhancement effect" (Erney et al., 1993; Cai et al., 1995; Hajšlová et al., 1998; Schenck & Lehotay, 2000; Anastassiades et al., 2003a; Maštovská & Lehotay, 2004; Menkissoglu-Spiroudi & Fotopoulou, 2004; Georgakopoulos et al., 2007). These factors, individually or combined, are able to lead in several adverse effects by under- or over-estimation analysis result, detection of unknown peaks, masking of analysed residue peak by co-extract components etc. (Hajšlová et al., 1998; Hajšlová & Zrostlíková, 2003; Poole, 2007). Concerning the above, a lot of studies involving the factors affecting the quantification of the residue(s) have been applied aiming to: (a) determine the parameter(s) introducing the inaccuracy of the result, (b) evaluate and correct the effect of factor(s) influencing the results of the determination, (c) suggest more optimal analytical conditions in cost-effective MRMs and (d) evaluate some critical parameters for possible method validation.

The objective of this chapter is to review some important findings from the evaluation of the critical factors affecting the accurate residues quantification in non-fatty matrices,

incorporating recent results of our laboratory dealing with the validation of an MRM. More specifically, there were efforts to investigate whether the collection of validation data from a single product of a botanical category and then validate the method for lots of commodities of the same botanical group is or not an erroneous practice. Finally, some future perspectives are also referred with purpose both to generalize the findings and validate the same MRM in the same laboratory for lots of commodities with limited error occurrence.

2. Critical factors introducing uncertainty of the residue analysis result

As already mentioned, there are a lot of parameters influencing the accuracy/precision of an analytical measurement. Provided the fact that analytical GC instrumental parameters, such as capillary analytical column, injector and detector are suitable for the separation and the identification/quantification of residues, these agents are able to significantly affect the final result. The factors with their possible effects, not always be predicted or corrected, are analysed in the following paragraphs.

2.1 Extraction solvent suitability for pesticide residues determination

The extraction's step objective is to separate most of the non-ionic residue(s) quantity from the plant matrix components by the application of organic solvent(s). Several solvents have been used for the extraction techniques with acetone, dichloromethane, methanol, EtOAc, petroleum ether and ACN to be the most popular (Luke et al., 1975; Ambrus et al., 1981; Greve, 1988; Hernández et al., 1990; Andersson & Pålsheden, 1991; Cai et al., 1995; van Zoonen, 1996; Anastassiades et al., 2003b; Maštovská & Lehotay, 2004; Schenck & Wong, 2008). EtOAc plus aliquots of salt (e.g., anhydrous sodium sulfate) to bind the water content of the plant product from the organic phase (Greve, 1988; Cai et al., 1995; Dorea et al., 1996; Pugliese et al., 2004; Berrada et al., 2006; Georgakopoulos et al., 2007), acetone with the addition of non-polar solvents, such as mixtures of dichloromethane-petroleum ether (van Zoonen, 1996; Bempelou & Liapis, 2006; Cengiz et al., 2006; Georgakopoulos et al., 2009), hexane-methylene chloride (Andersson & Pålsheden, 1991), dichloromethane-hexane (Lacassie et al., 1997) etc. and ACN combined with salts addition (anhydrous magnesium sulfate and sodium chloride) and dispersive solid phase extraction (dSPE) techniques (Anastassiades et al., 2003a; Schenck & Hobbs, 2004; Leandro et al., 2005; Lehotay et al., 2005; Hernández-Borges et al., 2009) represent the most commonly extraction procedures.

The efficiency of those mentioned MRMs to determine residues of different physicochemical properties has been compared in a lot of researches. Although EtOAc and acetone partition of several fruit extracts generally gave acceptable organophosphorus pesticides (OPs) recoveries (%R) of 70 to 110%, the EtOAc procedure resulted in better values for polar molecules (e.g., methamidophos, omethoate, acephate); a higher co-extracts number was also observed in EtOAc extracts (Andersson & Pålsheden, 1991). For instance non-acceptable low mean recovery of 58% was observed in the extremely polar methamidophos with acetone, plus hexane-methylene chloride, method compared to the respective 96% with the EtOAc method. From different solvents evaluated (methanol, acetone with and without partition in dichloromethane-petroleum ether and EtOAc), EtOAc was the most preferable for the extraction of polar OPs (acephate, methamidophos, oxydemeton-methyl etc.) from grape and cabbage matrices (Mol et al., 2003). It is notable that acetone partition resulted in recoveries of 12 to 76% for such OPs. Although the majority of 90 pesticide recoveries for various fruits and vegetables were higher than 80% in concentration ranges from 0.01 to 0.5

mg/kg with the rapid extraction of acetone using vortex mixing and solid phase extraction (SPE), the most polar OPs could not be determined (Štajnbaher & Zupančič-Kralj, 2003). EtOAc extraction provided better average %R, with satisfactory validation parameters, than dichloromethane extraction for 16 organochlorine pesticides (OCs) (Yenisoy-Karakaş, 2006). EtOAc non-fatty, fruit-based baby food extracts provided (a) higher recoveries for polar dimethoate, (b) lower recoveries for semi- and non-polar chlorpyrifos, methidathion, diazinon and phosalone and (c) higher amount of lipophilic compounds affecting the measurement than the relevant acetone partition extracts (Georgakopoulos et al., 2009). Among different extraction solvents, known to result in acceptable %R for a wide range of pesticides, ACN was chosen to the modern method named QuEChERS, as an acronym of quick, easy, cheap, effective, rugged and safe (Anastassiades et al., 2003a). This was due to the lower degree of matrix co-extracts in fatty matrices and higher %R of certain pH-dependent pesticides compared to the "dirtier" EtOAc extracts. The larger amount of remained co-extracts seems to be the major disadvantage of the EtOAc method (Ambrus & Thier, 1986; Greve, 1988); the amount of lipophilic co-extracts decreases in the order EtOAc>acetone>>ACN compared with the respective amount of sugar interferences (decreasing order of acetone>ACN>EtOAc) (Maštovská & Lehotay, 2004).

The slightly water-miscible EtOAc with the addition of anhydrous sodium sulfate aliquots to remove co-extracted water and force the polar pesticides into the organic phase (Schenck & Wong, 2008) is proved to be the favourable MRM for the analysis of polar and semi-polar analytes from non-fatty matrices, containing zero or minimum amounts of non-volatile compounds (Georgakopoulos et al., 2007). The acceptable validation parameters combined with the properties of easy and quick to handle and cost-effective (Andersson & Pålsheden, 1991; Fernandez-Alba et al., 1994) have made this MRM as one of the most favourable in the residue analysis. The more complex the matrix (containing more lipophilic co-extracts), the more the need for an extra clean-up step, such as gel permeation chromatography (Hajšlová et al., 1998) or Florisil column (Dorea et al., 1996). The polar, miscible with water acetone, requiring a series of liquid-liquid partition steps with non-polar solvents, seems to give accurate analysis results for a more wide range of pesticides, except for extremely polar OPs (Majors, 2007), in the analysis of non-fatty matrices containing or not non-volatile components. Thus, there is elimination of undesirable interference effects in the final residue result even if no further clean-up is applied (van Zoonen, 1996; Georgakopoulos et al., 2009). The new approach of QuEChERS, employing shaking of the matrix with ACN, followed by the addition of salts and dSPE with appropriate sorbent amounts (Anastassiades et al., 2003a; Majors, 2007; Schenck & Wong, 2008) represents the most suitable MRM for the analysis of polar, semi- and non-polar analytes in non-fatty and low-fatty matrices (e.g., containing 2 to 20% of fat) with large amounts of non-volatile compounds.

2.2 Pesticide residue physicochemical properties

The use of pesticides has rapidly increased over the last 60 years; nowadays over 1100 substances are registered as pesticides (Anonymous, 2006) and around 2.5 million tones of their formulations per year are applied (Tadeo et al., 2008). These compounds belong to different chemical groups (e.g., OCs, OPs, carbamates, pyrethroids, benzoylureas) (van der Hoff & van Zoonen, 1999; Sannino, 2008), presenting much different physicochemical properties, such as water solubility (w.s.), polarity, vapor pressure (v.p.), melting point etc. Since great differences among molecules even belonging to the same group are observed (e.g., extremely polars methamidophos and acephate contrary to non-polars chlorpyrifos

and parathion of Ops; Noble, 1993), the selection of an MRM to accurate determine a large variety of pesticides seems very difficult. The most significant properties, apart from volatility indicating the effective detection by GC, are polarity and resistance to different pH ranges determining parameters such as the extraction solvent selection and the type of possible clean-up step (Anastassiades et al., 2003a; Schenck & Wong, 2008).
The type of the analysed pesticides is proved to influence both its %R and occurrence of matrix effect ($p<0.05$), as the recoveries for the same matrix GC-extracts vary with the different pesticides (Erney et al., 1993; Hajšlová et al., 1998; Georgakopoulos et al., 2007). Acetone partition and ACN, both plus clean-up using SPE cartridges, fruit extracts gave different OPs recoveries (Schenck & Lehotay, 2000). Specifically, pesticides containing amides and/or multiple polar P=O bonds, such as omethoate, monocrotophos and dicrotophos, presented excessively high recoveries ranged from >110% to >200%. Furthermore, compounds containing single P=O bonds, such as acephate and methamidophos rather than non-polar P=S bonds, such as chlorpyrifos and malathion, were identified as tending to give particularly high %R. Regarding the physicochemical properties, especially values of w.s. and logarithm of *n*-octanol partition coefficient ($\log k_{ow}$) which are a degree of polarity (Hajšlová et al., 1998), methamidophos and dimethoate are more polar than methidathion and chlorpyrifos and for this reason their recoveries were higher in EtOAc extracts (Georgakopoulos et al., 2007). In this study, methamidophos recoveries were of poorer precision in the independent replicates; similar behavior with higher recoveries in combination with high relative standard deviation (RSD) values has been reported for captan and other polar analytes (Cai et al., 1995; Hajšlová et al., 1998). Moreover, many commonly applied pesticides are sensitive to specific pH values ($p<0.05$) (Lehotay et al., 2005; Payá et al., 2007). For instance, base-sensitive compounds (e.g., tolylfluanid, captan and folpet) degrade rapidly at high pH-extracts (Lehotay & Maštovská, 2009); an adjustment of acidic pH should be performed to avoid partial loss of those residues (Anastassiades et al., 2003a; Lehotay et al., 2005). Similarly, basic compounds, such as thiabendazole and imazalil, are generally poorly recovered from matrix extracts of low pH (Anastassiades, 2003a; Anastassiades et al., 2006). ACN apple juice extracts of pH values ranging from 2.5 to 7.0 gave negligible loss (recoveries of 90 to 100%) of such analytes in acidic solutions compared with the significant losses (recoveries of 50 to 70%) in the respective EtOAc extracts (Anastassiades et al., 2003a).
Therefore, the largest chemical group of OPs, covering a wide range of polarity from e.g. the extreme polar methamidophos of negative $\log k_{ow}$ (-0.8) to non-polar ethion of high $\log k_{ow}$ (5.1) may be successfully analysed by simple, cost-effective MRMs, such as EtOAc and acetone partition. It should be reminded that polars are better extracted by EtOAc in comparison with medium- and non-polars better extracted by the acetone partition method (van Zoonen, 1996; Mol et al., 2003; Maštovská & Lehotay, 2004; Georgakopoulos et al., 2009). The selection of the more appropriate MRM should be based on the physicochemical properties of target compounds. To cover the analysis of more compounds, including troublesome analytes in terms of polarity and/or acidity, QuEChERS application with ACN as the extraction solvent (Anastassiades et al., 2003a; Lehotay et al., 2005; Majors, 2007) with or without slight modifications seems to be one of the best modern MRM approaches.

2.3 Concentration level of the analysed residue(s)

The ratio of analyte and matrix concentration in the GC-extract seems to be a crucial point in the accuracy of the final residue result, since differences in the recovery portions among the

fortification levels of the same pesticide are commonly observed ($p<0.05$). Hajšlová et al. (1998), Jiménez et al. (2001) and Anastassiades et al. (2003b) noticed unacceptable %R and matrix-induced enhancement effects at lower concentration levels of target pesticides and/or at higher matrix components. Higher apparent recoveries of >200% were obtained for certain susceptible to matrix enhancement effect analytes (e.g. captan, iprodione) with solvent standard quantification at the low concentrations of ≤0.02 mg/kg in vegetable matrices (Menkissoglu-Spiroudi & Fotopoulou, 2004). From recent results presented (Georgakopoulos et al., 2007), it was concluded that the lower the fortification level, the higher the %R. The phenomenon was more evident in the Maximum Residue Levels (MRLs) of 0.01 and 0.02 mg/kg especially for the polars methamidophos and dimethoate in almost all the examined fruits and vegetables with solvent standards quantification. It should also be addressed that significant matrix effects were obtained for five pesticides tested (dimethoate, parathion methyl, chlorothalonil, diazinon and fenitrothion) in all fruit extracts of the low concentration equal to 0.05 mg/kg (Freitas & Lanças, 2009). This repeatable behavior can be attributed to the lower competitive effect of the pesticide standards, when they are found in trace fortification levels, for covering the active sites of the injection liner (Hajšlová & Zrostlíková, 2003); a phenomenon connected with the presence of matrix effects extensively analysed in a following paragraph.

2.4 Chemical composition and co-extracts of analysed matrix

MRMs should be able to effectively quantify lots of residues in several matrices presenting a large variety of components and remained co-extracts. The water, protein, fat and sugar content of commonly commodities analysed is much different, as shown in Dorea et al. (1996), Hajšlová et al. (1998), Egea González et al. (2002), Lesueur et al. (2008) etc. The choice of the appropriate MRM is strongly associated with the composition of the matrix, and especially the fat content (Motohashi et al., 1996). According to Greve (1986) non-fatty samples contain less than 5% total fat contrary to fatty samples. Food and Drug Administration (FDA) extraction methods are designed for fatty, containing ≥2% fat, and non-fatty, containing <2% fat, matrices (Sannino, 2008). Lehotay et al. (2005) presented a more suitable matrix taxonomy for MRMs; non-fatty samples contain <2%, low fatty contain 2 to 20% and fatty ≥20% total fat. Non-fatty products have been divided according to the water percentage as moist (containing >80% water), medium water content (containing and samples presenting sugars of 5 to 30%) and dry (Greve, 1988; Tekel & Hatrik, 1996). Furthermore, with purpose to choose the more suitable MRM for several matrices, plant products have been categorized according to their chemical composition (Ambrus et al., 1981) or botanical characteristics (Bates & Gorbach, 1982). Thus, it has been proposed that collecting validation parameters deriving from only one representative commodity (e.g., orange from citrus fruits) should provide validation ability for lots of products belonging to the same botanical category (e.g., lemon, mandarin, kiwi fruit).

The recovery portion of pesticides depends greatly on the chemical composition of the examined matrix ($p<0.05$). Lemon and onion, recognized as high acid and high sulfur content respectively, gave much lower %R for ≈150 pesticides by QuEChERS plus GC-MS compared with tomato and grape extracts (Lesueur et al., 2008). Unacceptably high %R were observed to non-fatty extracts containing more non-volatile compounds, such as chlorophylls in leafy vegetables, carotenoids in fruiting vegetables (e.g., lycopene in tomatoes), essential oils in citrus peels, waxes in grapes (Georgakopoulos et al., 2007). Freitas & Lanças (2009) indicated that the enhancement or decrease of the response and %R

significantly differed from matrix to matrix among 6 fruits tested having variable chemical composition. Furthermore, the type of co-extracts that remains in the final sample leads to a markedly different detector response and causes false positive results (Erney et al., 1993; Hajšlová et al., 1998; Poole, 2007). Scientific evidence suggests that more distinct matrix effects have been reported for matrix extracts rich in pigments and lipids (Hajšlová et al., 1998; Godula et al., 1999; Anastassiades et al., 2003b). Organosulfur compounds, not removed by the SPE columns evaluated, of cabbage interfered with the detection of early eluting OPs in the GC-FPD analysis (Schenck et al., 2002). Among 21 kinds of vegetables tested, only garlic, onion and leek extracts gave "unknown" peaks in GC-FPD due to large amounts of sulfur constituents (Cai et al., 1995). A peak in orange extracts appeared in every fruit (by GC-NPD), either of organic produce or of conventional crops, and detected in the peel orange extract analysed itself compared with the analysis of orange juice (Georgakopoulos et al., 2007) may be the reason why citrus peels require an extract clean-up, described by Dorea et al. (1996). EtOAc fruit purée and cocktail extracts, presenting more complicated components and higher co-extract amounts, influenced negatively both the NPD response and the accurate determination in contrast to the respective fruit juice extract (Georgakopoulos et al., 2009). To overcome these co-extracts effects, additional clean-up steps, compatible with pesticides analysed and solvent(s) applied, have been proposed (Tekel & Hatrik, 1996; Schenck & Lehotay, 2000; Schenck & Wong, 2008). Their application represents a compromise between the time and cost required on the one hand and the "cleaner" (containing less constituents affecting the %R and detection limit) extract on the other hand (Seiber, 1999; Lee & Richman, 2002).

2.5 Matrix-induced enhancement effects

The quantification of certain analytes by GC is strongly affected by a phenomenon known as matrix-induced chromatographic response enhancement, which was first described by Erney et al. (1993) and causes excessively high recovery results. The phenomenon takes place during the analysis of samples containing a wide range of components (e.g., pigments, lipids, waxes) that may remain after the preparation of the extract and its possible clean-up (Godula et al., 1999; Hajšlová & Zrostlíková, 2003). Such non-volatile constituents accumulate in the GC inlet and/or in the front part of a capillary column, resulting in the reduction of the loss and protection of the analyte(s) from adsorption and thermal degradation (Erney et al., 1993; Poole, 2007). Particularly, during analysis of a pesticide(s) standard solution, more active sites, especially in the injection liner, are available for the analyte(s) molecules compared with those available during analysis of an extract also containing matrix components (Schenck & Lehotay, 2000). This is because the latter components block the active sites both presenting in the (a) liner and (b) connection of the injector with the capillary column (Erney et al., 1993), increasing the transfer of analyte(s) to the separation column and detector (Poole, 2007). Therefore, when free-matrix standard solutions are injected, poor peak shapes combined with peak tailing and low response results for some affected compounds, such as those presented in Poole (2007), are observed contrary to the respectives of matrix extract solutions (Anastassiades et al., 2003b).

Nowadays, the matrix effect is considered as one of the most persistent sources of uncertainty in pesticide residue analysis (Egea González et al., 2002) by increasing the level of random errors and/or introducing a systematic effect on the result (Cuadros-Rodríguez et al., 2002). Available studies involving the analysis of various residues in different matrices (Erney et al., 1993; Erney et al., 1997; Johnson et al., 1997; Jimenez et al., 2001; Menkissoglu-

Spiroudi & Fotopoulou, 2004) prove that its presence and extent depends on several parameters, most of which were previously reported. More specifically, many thermolabile compounds, containing polar structure/functional groups, quantified in low concentration (e.g., <0.1 mg/kg), are referred as "troublesome analytes" (e.g., methamidophos, acephate, captan, chlorothalonil, monocrotophos, folpet) since they are susceptible to matrix enhancement (Lee et al., 1991; Bernal et al., 1997; Hajšlová et al., 1998; Godula et al., 1999; Hajšlová & Zrostlíková, 2003; Poole, 2007). Moreover, many non-fatty matrices are identified as tending to give matrix effects, such as apple, tomato, banana, orange peel, stone fruits, carrot, leafy vegetables, wheat, wine etc. (Miyahara et al., 1994; Egea González et al., 2002; Navarro et al., 2002; Patel et al., 2004; Georgakopoulos et al., 2007; Freitas & Lanças, 2009), due to the high co-extracts amount persisting in the GC analytical sample, necessitating the application of clean-up step(s) (Dorea et al., 1996; Hajšlová et al., 1998; Schenck & Lehotay, 2000; Li et al., 2008). It should also be addressed that matrix effects are difficult to study because of the different analysis conditions for the samples, since the effects of simple maintenance application (e.g. changing the injection liner, cutting the front part of capillary column) are unpredictable (Godula et al., 1999; Schenck & Lehotay, 2000). Thus as Hajšlová et al. (1998) indicated the history of the GC system, especially changes in the injection port, plays an important role in the occurrence of such phenomena. As a consequence, recoveries of several pesticides are not reproducible and the effects of co-extracts cannot be considered as stable and foreseeable (Georgakopoulos et al., 2007).

Several injection techniques have been proposed to compensate for matrix effects and eliminate the uncertainty of the final result; these are not always available for analytical laboratories due to the increasing cost required (Schenck & Wong, 2008). For instance, the use of cold on-column injection is considered as one of the most practical approaches by which pesticides thermolysis and decomposition or adsorption inside the inlet could be avoided (Wylie & Uchiyama, 1996; Godula et al., 1999). Furthermore, polar pesticides and matrices containing non-volatile constituents could be analysed by on-column injection with the parallel use of a packed column or a deactivated pre-column (to keep most of matrix components) connected to the injector site. However, the main disadvantage of on-column injection is related with the much increased maintenance necessity of the column, being impractical for complex or relatively un-cleaned matrices compared with the conventional hot splitless injection (Anastassiades et al., 2003b). Programmable temperature vaporization (PTV) may result both in decreased analyte discrimination during injection and limited adverse effects of non-volatiles by introducing large volumes of sample (Grolimund et al., 1998; Godula et al., 2001; Poole, 2007). Pulsed splitless injection, involving an increasing of column head pressure for 1 to 2 min during the injection, reduces the residence time of analyte(s) in the inlet and minimizes solvent expansion volumes (Wylie & Uchiyama, 1996; Godula et al., 1999). The main drawbacks of these techniques are related with the (a) increasing amount of non-volatile components into the column more than the desirable and (b) reducing but not eliminating the occurrence of matrix effects (Godula et al., 1999; Anastassiades et al., 2003b).

An alternative approach dealing with the preparation of the analytical sample is the application of an extensive clean-up step after the extraction. Its use may result in several benefits, such as elimination of matrix interferences causing such phenomena, high recoveries, detection and quantification limits (LODs and LOQs, respectively), reduction of maintenance needs for the GC instrument due to the relatively clean extract (e.g., lower changes of liners and capillary columns, smaller detector contamination by the impurities)

and restriction of enhancement effects (Hajšlová et al., 1998; Schenck & Lehotay, 2000; García-Reyes et al., 2007). The major disadvantage is the demanding for extra labor time and cost (Greve, 1988; Egea González et al., 2002; Beyer & Biziuk, 2008; Schenck & Wong, 2008) because of the increasing needs for additional solvent amounts, columns (Florisil, SPE) and sorbents (primary secondary amine-PSA, octadecyl-C_{18}, graphitized carbon black-GCB) (Dorea et al., 1996; Schenk et al., 2002; Anastassiades et al., 2003a; Li et al., 2008). It should also be noticed that the more the steps of an MRM, the higher the possibility for analytes partial loss and the increase of the combined uncertainty during the procedure (Hajšlová et al., 1998; Menkissoglu-Spiroudi & Fotopoulou, 2004; Poole, 2007).
Among the mentioned and other suggested approaches, such as the use of correction functions (Egea González et al., 2002; Cuadros-Rodríguez et al., 2002) or analyte protectants (Anastassiades et al., 2003b; Poole, 2007), the most practical solution to eliminate matrix effects seems to be the application of matrix-matched standard solutions (Erney et al., 1993; Erney et al., 1997; Poole, 2007). These standards are prepared by adding appropriate aliquots from solvent standard solutions in blank matrix extracts (Erney et al., 1997; Štajnbaher & Zupančič-Kralj, 2003; Lesueur et al., 2008; Freitas & Lanças, 2009; Georgakopoulos et al., 2009). Their application has nowadays been included in the calibration step for pesticides quantification (Erney et al., 1993; Bernal et al., 1997; Egea González et al., 2002; Martínez Vidal et al., 2004). For calibration by comparing the quantity of a fortified extract with the respective of a matrix-matched standard, the concentration of the standard should be equal to the final concentration of the extract; otherwise the result is incorrect (Erney et al., 1997; Georgakopoulos et al., 2009). When applied as reference materials, matrix standards have provided acceptable %R and overcome enhancement in detector response, since the interferences effects were approximately similar to the fortified extracts analysed (Erney et al., 1997). Available studies prove their effectiveness in hundreds of residues analysis for various product extracts. Indicatively, excessively high OP recoveries of >120 to 240% were reduced to 81 to 97% with matrix standard calibration for potato extracts (Lehotay & Eller, 1995), extremely high recoveries of >200 to 1000% for lots of analytes in honey extracts were also corrected to the acceptable range with controlled spiked blank extracts (Jiménez et al., 1998), recoveries approaching the 300% were reduced to 70 to 110% in white wine (Holland et al., 1994), recoveries much higher than 110% for some pesticides (e.g., parathion methyl) as a result of non-matrix calibration plots were significantly reduced by matrix-matched vegetable calibration curves (Johnson et al., 1997), standard solutions of blank fruiting vegetables were found to correct the high recoveries of >200% of most pesticides to the acceptable 70 to 110% (Menkissoglu-Spiroudi & Fotopoulou, 2004) etc. The disadvantages of this technique have mainly to do with the increasing demands for more blank extracts (larger quantities of matrix and extraction solvent), more labor time for preparation and larger needing for GC maintenance.

2.6 Confirmatory results of the factors affecting residues quantification

The presence and the extent of matrix effects in pesticide residue analysis were assessed by the application of an official MRM (acetone partition with dichloromethane-petroleum ether) and GC-NPD. Recoveries of 5 OPs presenting different polarity were evaluated in non-fatty matrix and fortification level (MRL and one multiple of it) combinations by standards prepared both in solvent and matrix-matched solutions (Tables 1 to 5). Unacceptably high %R combined with pronounced matrix effects were observed to the more polar dimethoate (Table 1) and to the lower fortification levels of <0.1 mg/kg (Tables 1 to 3).

The matrix standards single point determination resulted in recoveries of the acceptable 70 to 110% contrary to the relevant of solvent standards determination in lots of the examined combinations ($p<0.05$). This is more evident in the analytes dimethoate (Table 1) and phosalone (Table 5), in specific matrix extracts (especially those of lettuce) and in various low fortification level – analyte – matrix combinations (Tables 1 to 5). It is notable that all calculated by matrix-matched standards recoveries of chlorpyrifos (Table 2) and fenitrothion (Table 3) and almost all of diazinon (Table 4) and phosalone (Table 5) were found in the acceptable range. The results also proved that when the purpose is the identification and the monitoring of MRLs, conventional, cost-effective quantification by solvent standards could be successfully utilized if the triptych non-polar analyte (e.g., chlorpyrifos, fenitrothion, diazinon) – concentration (higher MRL values of >0.1 mg/kg) – plant product analysed tends to give no matrix effects and results overestimation (Tables 1 to 5). In those conditions described the effects of factors are limited; thus the analysis may be accurate both without spending more laborious time and cost for matrix standards or clean-up step(s) and without demanding the application of mass spectrometry techniques not available by many laboratories.

Matrix	Recoveries ± RSDs (%) (n=3) of dimethoate					
	C_1 (mg/kg)	Solvent standards	Matrix standards	C_2 (mg/kg)	Solvent standards	Matrix standards
Pear	0.02	nd*	nd*	0.2	**93.5 ± 1.4a**	**76.7 ± 3.0b**
Orange	0.02	158.3 ± 3.3a	**103.8 ± 5.1b**	0.2	**72.0 ± 0.8a**	63.2 ± 3.6b
Tomato	0.02	320.4 ± 0.9a	118.7 ± 3.0b	0.2	**75.9 ± 2.1a**	**82.7 ± 3.2b**
Lettuce	0.5	**100.4 ± 2.7a**	**71.5 ± 1.5b**	0.05	**77.8 ± 6.3a**	**109.6 ± 6.6b**
Peach	0.02	164.5 ± 3.5a	**102.1 ± 4.2b**	0.2	115.7 ± 6.2a	57.9 ± 10.0b

nd*: non detectable

a, b: within a specific matrix and fortification level, those values lacking a common letter are different ($p<0.05$)

Table 1. Recoveries ± RSDs (%) of dimethoate in matrix – fortification level (C_1 equal to MRL established by European legislation, C_2 a multiple of it) combinations using both solvent and matrix-matched standard solutions (n=3)

Matrix	Recoveries ± RSDs (%) (n=3) of chlorpyrifos					
	C_1 (mg/kg)	Solvent standards	Matrix standards	C_2 (mg/kg)	Solvent standards	Matrix standards
Pear	0.5	**88.9 ± 1.9a**	**86.8 ± 0.8a**	0.05	**85.9 ± 4.6a**	**95.7 ± 0.3b**
Orange	0.3	**100.1 ± 3.2a**	**100.4 ± 1.2a**	0.03	141.2 ± 2.3a	**79.0 ± 1.1b**
Tomato	0.5	**100.1 ± 2.9a**	**105.7 ± 0.6a**	0.05	117.0 ± 2.0a	**76.4 ± 5.6b**
Lettuce	0.05	205.5 ± 5.0a	**76.3 ± 0.6b**	0.5	**85.4 ± 1.6a**	**96.4 ± 5.7b**
Peach	0.2	**72.2 ± 1.4a**	**82.3 ± 2.7b**	0.02	235.1 ± 2.0a	**103.3 ± 4.3b**

a, b: within a specific matrix and fortification level, those values lacking a common letter are different ($p<0.05$)

Table 2. Recoveries ± RSDs (%) of chlorpyrifos in matrix – fortification level (C_1 equal to MRL established by European legislation, C_2 a multiple of it) combinations using both solvent and matrix-matched standard solutions (n=3)

Matrix	Recoveries ± RSDs (%) (*n*=3) of fenitrothion					
	C_1 (mg/kg)	Solvent standards	Matrix standards	C_2 (mg/kg)	Solvent standards	Matrix standards
Pear	0.5	**87.6 ± 2.8a**	**79.1 ± 2.5b**	0.05	**99.7 ± 6.7a**	**90.2 ± 3.6a**
Orange	2.0	65.2 ± 1.1a	**71.9 ± 4.0b**	0.02	**104.4 ± 1.5a**	**111.0 ± 1.1b**
Tomato	0.5	**99.4 ± 1.1a**	**104.6 ± 1.6a**	0.05	149.3 ± 2.2a	**105.4 ± 4.7b**
Lettuce	0.5	**105.7 ± 0.5a**	**91.7 ± 0.5b**	0.05	173.7 ± 1.4a	**110.5 ± 7.1b**
Peach	0.5	**84.1 ± 3.5a**	**75.8 ± 2.6b**	0.05	180.5 ± 3.8a	**97.9 ± 6.0b**

a, b: within a specific matrix and fortification level, those values lacking a common letter are different ($p<0.05$)

Table 3. Recoveries ± RSDs (%) of fenitrothion in matrix – fortification level (C_1 equal to MRL established by European legislation, C_2 a multiple of it) combinations using both solvent and matrix-matched standard solutions (*n*=3)

Matrix	Recoveries ± RSDs (%) (*n*=3) of diazinon					
	C_1 (mg/kg)	Solvent standards	Matrix standards	C_2 (mg/kg)	Solvent standards	Matrix standards
Pear	0.3	**88.5 ± 0.8a**	**107.2 ± 5.9b**	0.03	180.4 ± 3.6a	**102.4 ± 1.6b**
Orange	1.0	**85.2 ± 4.0a**	**80.3 ± 3.2b**	0.01	**102.5 ± 1.5a**	**102.9 ± 1.9b**
Tomato	0.5	**74.0 ± 3.2a**	**92.1 ± 3.7b**	0.05	**111.5 ± 3.1a**	**97.0 ± 5.2b**
Lettuce	0.02	56.7 ± 4.9a	**87.5 ± 1.9b**	0.2	**99.0 ± 1.0a**	**105.0 ± 5.3b**
Peach	0.02	**97.0 ± 2.0a**	122.9 ± 4.3b	0.2	**85.7 ± 1.7a**	119.7 ± 3.4b

a, b: within a specific matrix and fortification level, those values lacking a common letter are different ($p<0.05$)

Table 4. Recoveries ± RSDs (%) of diazinon in matrix – fortification level (C_1 equal to MRL established by European legislation, C_2 a multiple of it) combinations using both solvent and matrix-matched standard solutions (*n*=3)

Matrix	Recoveries ± RSDs (%) (*n*=3) of phosalone					
	C_1 (mg/kg)	Solvent standards	Matrix standards	C_2 (mg/kg)	Solvent standards	Matrix standards
Pear	2.0	43.0 ± 1.4a	**107.5 ± 4.0b**	0.2	53.1 ± 3.2a	119.7 ± 7.2b
Orange	1.0	44.8 ± 2.0a	**90.0 ± 1.1b**	0.1	64.8 ± 2.7a	**107.0 ± 1.8b**
Tomato	1.0	46.5 ± 3.1a	**109.9 ± 1.1b**	0.1	57.8 ± 1.5a	**108.7 ± 3.8b**
Lettuce	1.0	41.9 ± 4.4a	65.8 ± 2.4b	0.1	**87.2 ± 1.0a**	**75.5 ± 1.9b**
Peach	2.0	37.9 ± 4.3a	**70.9 ± 9.4b**	0.2	62.4 ± 2.2a	**110.8 ± 2.4b**

a, b: within a specific matrix and fortification level, those values lacking a common letter are different ($p<0.05$)

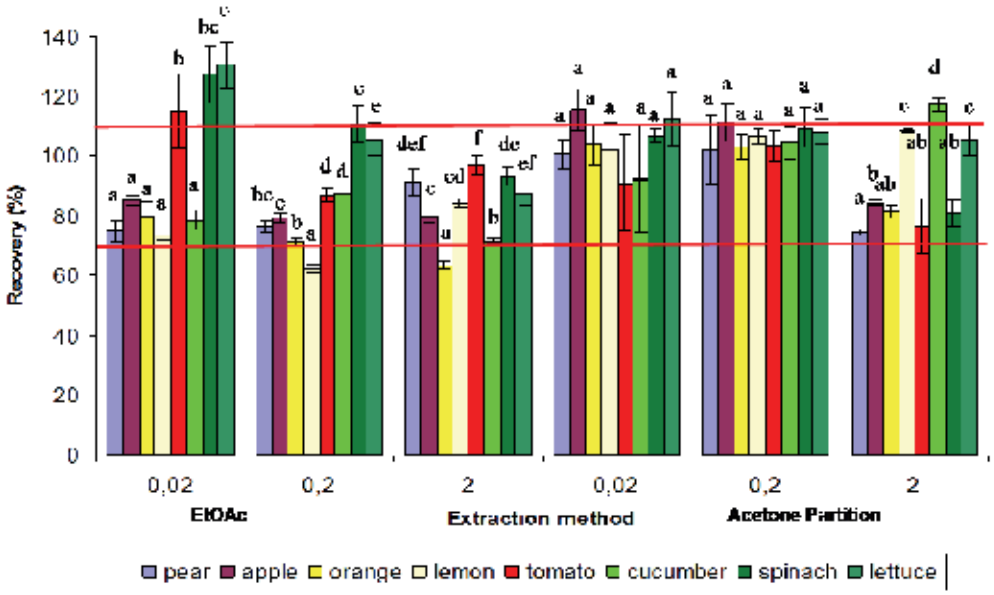
Table 5. Recoveries ± RSDs (%) of phosalone in matrix – fortification level (C_1 equal to MRL established by European legislation, C_2 a multiple of it) combinations using both solvent and matrix-matched standard solutions (*n*=3)

3. Validation of an MRM in plant products belonging to the same botanical group: Is a correct or an erroneous practice?

The validation of an MRM is a continuous procedure including the performance verification of critical parameters, such as accuracy, precision, sensitivity, repeatability and reproducibility during its application (Jensen, 1988; Hill & Reynolds, 1999; Ambrus, 2004). Basic concepts of the validation criteria have been developed by EURACHEM, AOAC International and national organizations (Ambrus, 2008). The general guidelines ensure the method reliability under the prescribed conditions and requirements of the validation protocol being available in the records of every validated laboratory (Huber, 1998; Fong, 1999). It should also be noticed that validation is a complicated procedure, demanding the accurate evaluation of some analytical parameters, such as %R, LOD, LOQ, %RSD, linearity, repeatability, reproducibility, matrix effects, combined uncertainty, random and systematic errors, referred in European standards (ISO 17025;2005), being recorded for the analytes – matrix combinations in fixed periods of time (Huber, 1998; Fong, 1999; Wood, 2006).

The appropriate MRM choice depends on all mentioned parameters, the main of which seem to be the effects of matrix. This is due to the significantly different %R and LODs-LOQs of specific analytes among the different examined commodities because of the co-extracts remained even after the application of clean-up step(s). However, the application of different approaches in the MRMs within the same laboratory among the matrices analysed is practically impossible. Therefore, there are increasing demands to extend a specific procedure to variable matrices with parallel acceptable analytical parameters. For this reason, several commodities have been categorized according to different criteria of taxonomy. Ambrus et al. (1981) suggested six major groups of plant products according to their chemical composition (e.g., group I including tuberous and root vegetables such as carrot, potato, garlic, onion, group II containing products with absence or low chlorophyll and fat content such as stone fruits, fruiting vegetables, banana, radish etc.) (Tekel & Hatrik, 1996). Bates & Gorbach (1982) applied the differences of botanical characteristics in the plant products, as adopted by Codex Alimentarius (e.g., lettuce, spinach, radish etc belong to leafy vegetables, orange, mandarin, lemon etc are in the group of citrus fruits), to classify plant products for the appropriate MRM selection. Some extensions or limitations of MRM validation to more matrices are reported to Hill & Reynolds (1999); for example analytical data for grains are not enough to prove its effectiveness for beer or data for one brassica vegetable may be applicable to similar products of this group. In our previous study, there were significant differences in the recoveries of pesticides in extracts derived from matrices belonging to the same botanical group (especially in the categories of pome fruits and citrus) under the examined conditions (Georgakopoulos et al., 2007). Therefore, obtaining analytical data, by EtOAc method without additional clean-up and GC-NPD solvent standard single point determination, from only one representative matrix with the purpose to validate the procedure in its botanical category was proved an erroneous practice.

Based on the information described and taking into consideration that the influence of each matrix on the chromatographic response should not always be correlated with its botanical characteristics, there were evaluations of when this practice is or not efficient while still using cost-effective MRMs and GC-NPD. Analytical parameters, such as recovery data, LOD, LOQ, repeatability and combined uncertainty were generally within the acceptable



In all Figs: (a) error bars designate %RSDs for the %R in the three independent replicates (n=3) and (b) a, b, c, d, e, f: within a specific MRM and fortification level, those values lacking a common letter are different ($p<0.05$)

Fig. 1. Recovery ± RSD (%) of dimethoate among the different matrix – fortification level – extraction method combinations.

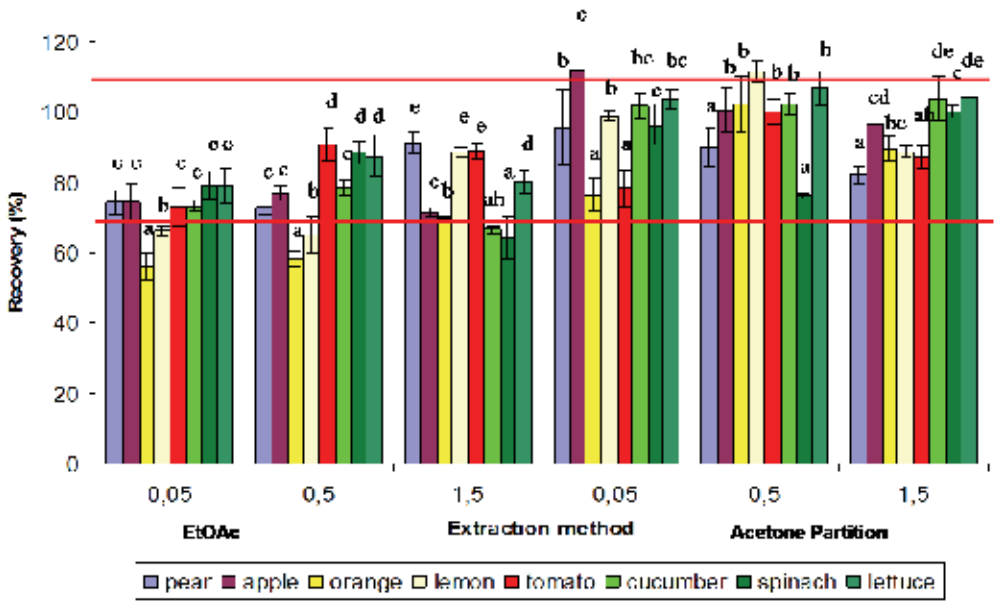


Fig. 2. Recovery ± RSD (%) of chlorpyrifos among the different matrix – fortification level – extraction method combinations.

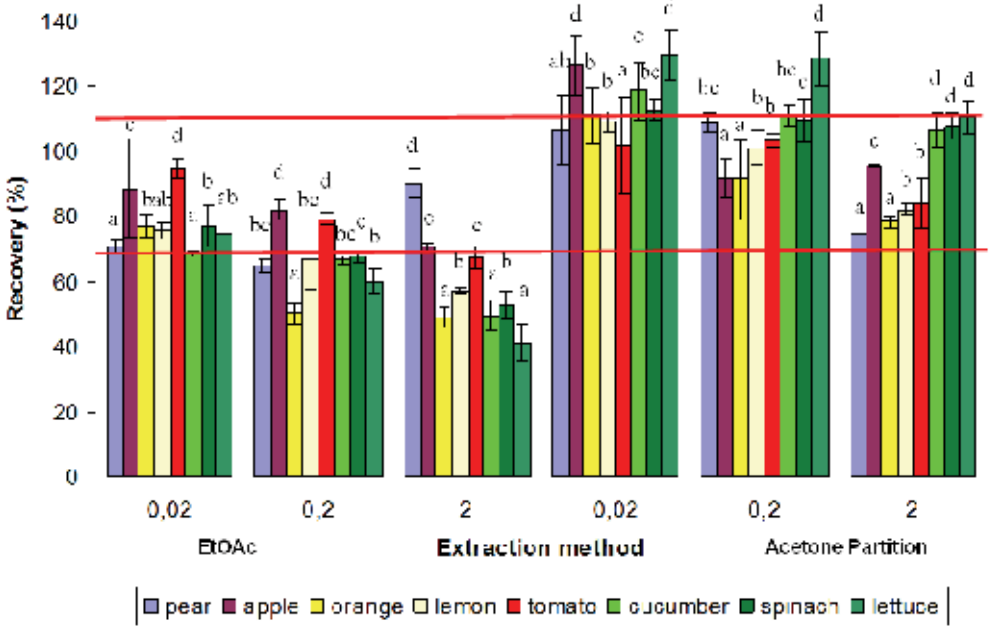


Fig. 3. Recovery ± RSD (%) of methidathion among the different matrix – fortification level – extraction method combinations.

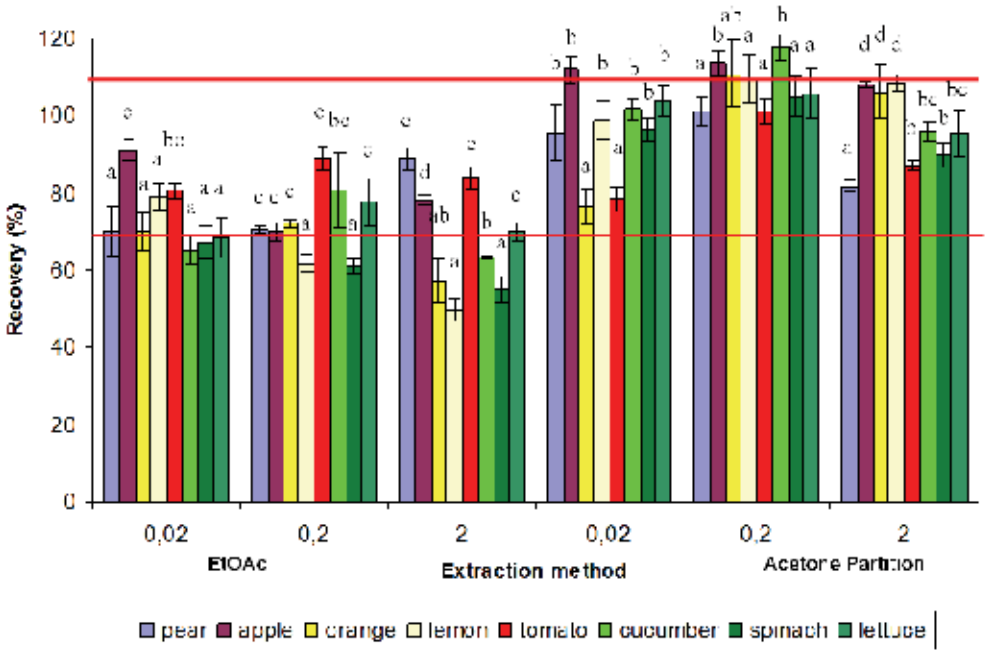
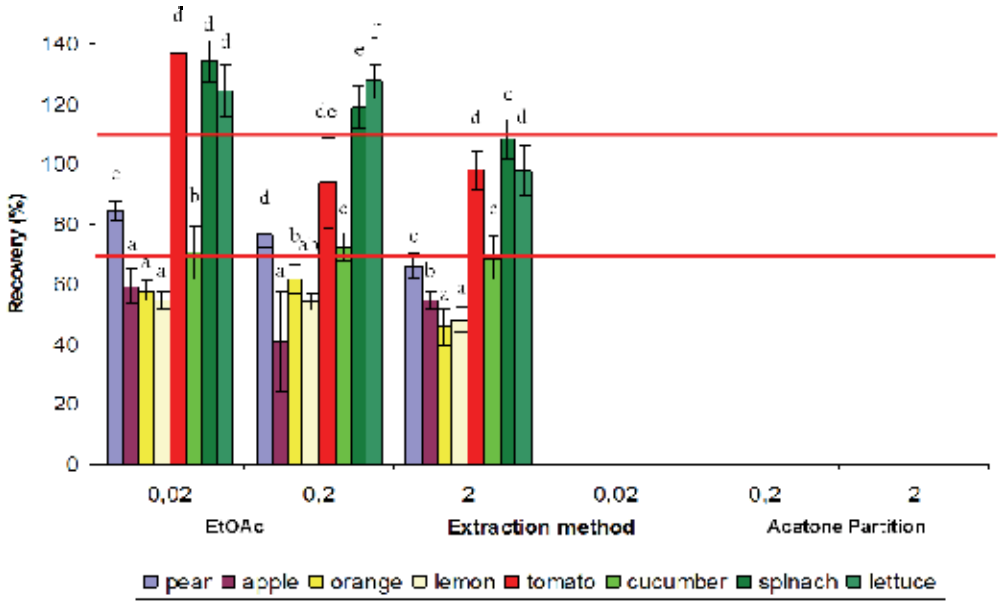


Fig. 4. Recovery ± RSD (%) of diazinon among the different matrix – fortification level – extraction method combinations.



Bars are not presented in acetone partition for methamidophos since it was not even detected in the relevant extracts.

Fig. 5. Recovery ± RSD (%) of methamidophos among the different matrix – fortification level – extraction method combinations.

ranges by the application of matrix-matched standard solution determination for EtOAc and acetone partition method (Figs 1 to 5 and Table 6). Thus, the effects of factors suspected for further uncertainty of the final result seem to be limited without much increasing demands for additional costs and techniques. However, the method of acetone resulted in higher recoveries than the EtOAc ($p<0.05$); this was more intense in semi- and non-polar analytes (acetone partition extracts generally gave %R of 90 to 110%) contrary to dimethoate and especially methamidophos (not detected in any acetone partition extract) (Figs 1 to 5). Furthermore, chromatograms of acetone partition extracts were free of unknown peaks in contrast to the respective of EtOAc leafy vegetable and citrus extracts (Fig. 6).

Recoveries derived from the matrices of the same botanical group did not appear differences ($p\geq0.05$) in acetone partition contrary to the respective of EtOAc ($p<0.05$) (Figs 1 to 4). For instance, concentrations of 0.02 and 0.2 mg/kg did not present any %R difference in among the eight products examined (Fig. 1). Similar behavior was observed to the majority of the examined combinations, such as those of diazinon and acetone partition extracts. Therefore, this MRM may be successfully applied to collect data from a single product and extend the validation for lots of commodities of the same botanical group.

4. Conclusions and future perspectives

The accurate determination of analytes is significantly affected by several factors, which may induce false results about the quantity of residues. The most important factors leading in various adverse effects are summarized as follows: (a) solvent and other materials (e.g., type of sorbents for clean-up) applied for the residues extraction from the matrix analysed,

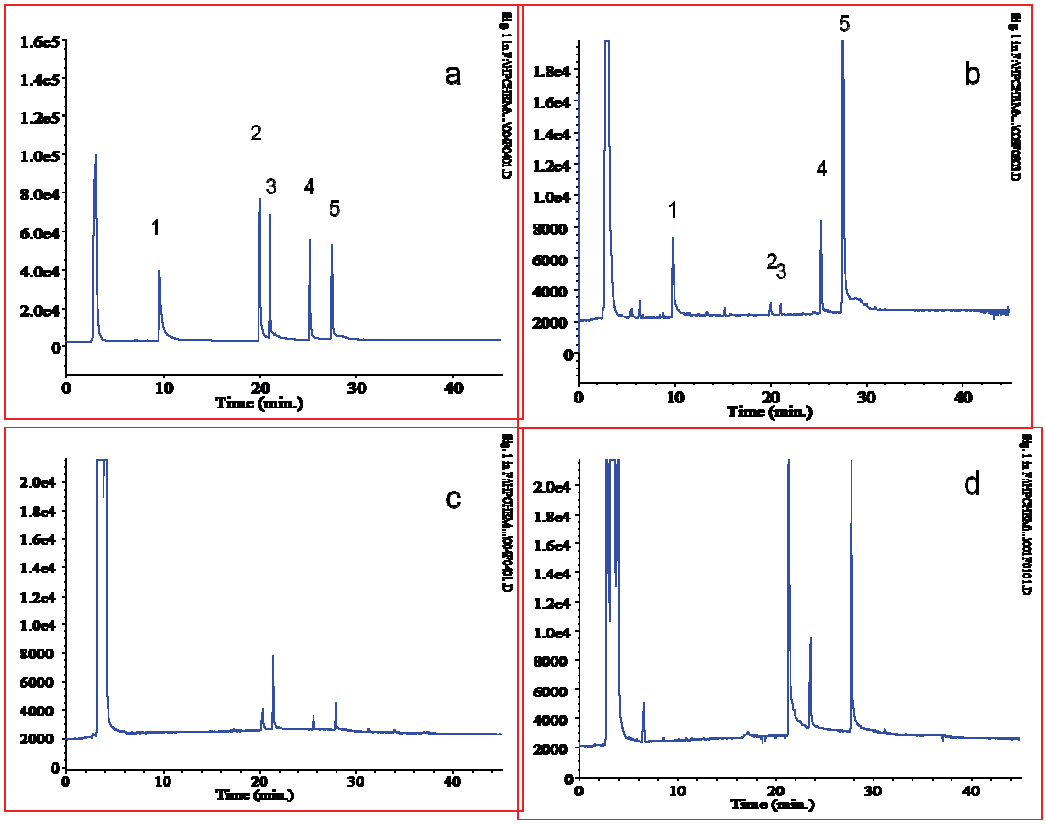


Fig. 6. GC-NPD chromatograms of fortified with the pesticides mixture (a) tomato – EtOAc, (b) orange – EtOAc, (c) tomato – acetone partition and (d) orange – acetone partition extracts. Peaks identification: 1. methamidophos, 2. dimethoate, 3. diazinon, 4. chlorpyrifos and 5. methidathion.

(b) detected molecule polarity and its determined concentration level, (c) matrix chemical composition and remained co-extracts in the final GC-extracts leading in enhancement effects, (d) GC system history related with the appropriate maintenance application. Even after the application of alternative approaches, such as additional clean-up step, on-column injection, GC-MS/MS, the phenomena of matrix-induced effect may not always be predicted or minimized; matrix-matched standard solutions are likely proved to reduce the extents of such effects. Some efforts should also be applied to the cost-effective MRMs, since they are able to provide adequate validation data without the extra needing for modern expensive techniques not always being available by many analytical laboratories. Moreover, with the purpose to validate an MRM to several commodities of the same botanical characteristics, the application of acetone partition plus GC-NPD with matrix standard single point determination was proved as an encouraging practice contrary to the failure techniques of previous studies. However, in order to generalize the findings, a higher number of pesticides, including much more analytes from the different polarity categories, should be utilized in the fortification procedures of the plant products belonging in different groups of botanical categories.

Pesticide	Matrix-MRM	LOD (mg/kg)*	LOQ (mg/kg)*	Repeatability (%RSD)**	Combined uncertainty (%RSD)**
Chlorpyrifos	Pear-Ac. Part.	0.002	0.023	0.8 (0.5)	6.5
	Apple-Ac. Part.	0.003	0.032	6.7 (0.5)	9.4
	Orange-Ac. Part.	0.004	0.027	3.8 (0.3)	10.8
	Lemon-Ac. Part.	0.004	0.031	2.7 (0.2)	5.3
	Tomato-Ac. Part.	0.003	0.030	4.1 (0.5)	6.2
	Cucumber-Ac. Part.	0.003	0.028	3.1 (0.05)	4.2
	Spinach-Ac. Part.	0.004	0.033	8.5 (0.05)	11.4
	Lettuce-Ac. Part.	0.004	0.032	9.1 (0.05)	12.7
	Pear-EtOAc	0.002	0.020	1.8 (0.5)	3.3
	Apple-EtOAc	0.003	0.027	2.2 (0.5)	3.7
	Orange-EtOAc	0.001	0.015	1.8 (0.3)	3.5
	Lemon-EtOAc	0.001	0.015	3.7 (0.2)	6.5
	Tomato-EtOAc	0.001	0.021	4.3 (0.5)	6.6
	Cucumber-EtOAc	0.001	0.020	2.5 (0.05)	3.8
	Spinach-EtOAc	0.003	0.022	6.6 (0.05)	8.9
	Lettuce-EtOAc	0.003	0.025	7.9 (0.05)	9.3
Diazinon	Pear-Ac. Part.	0.001	0.024	1.5 (0.3)	4.6
	Apple-Ac. Part.	0.001	0.019	3.4 (0.3)	8.1
	Orange-Ac. Part.	0.002	0.018	2.6 (1.0)	4.4
	Lemon-Ac. Part.	0.002	0.027	3.1 (LOQ)	5.1
	Tomato-Ac. Part.	0.001	0.024	5.2 (0.5)	5.5
	Cucumber-Ac. Part.	0.001	0.024	9.7 (LOQ)	10.5
	Spinach-Ac. Part.	0.002	0.025	10.4 (LOQ)	13.4
	Lettuce-Ac. Part.	0.002	0.028	9.9 (LOQ)	12.8
	Pear-EtOAc	0.003	0.023	1.2 (0.3)	2.6
	Apple-EtOAc	0.001	0.022	2.3 (0.3)	3.9
	Orange-EtOAc	0.001	0.009	0.9 (1.0)	2.5
	Lemon-EtOAc	0.001	0.015	4.9 (0.02)	5.8
	Tomato-EtOAc	0.001	0.024	3.4 (0.5)	6.1
	Cucumber-EtOAc	0.005	0.032	8.0 (LOQ)	12.8
	Spinach-EtOAc	0.003	0.026	8.3 (LOQ)	13.1
	Lettuce-EtOAc	0.003	0.025	7.7 (LOQ)	12.6
Methidathion	Pear-Ac. Part.	0.007	0.044	3.3 (0.3)	4.7
	Apple-Ac. Part.	0.003	0.033	4.2 (0.3)	7.4
	Orange-Ac. Part.	0.007	0.041	6.2 (2.0)	10.7
	Lemon-Ac. Part.	0.003	0.031	2.7 (2.0)	4.4
	Tomato-Ac. Part.	0.009	0.033	9.3 (LOQ)	17.4
	Cucumber-Ac. Part.	0.004	0.030	9.4 (LOQ)	11.9
	Spinach-Ac. Part.	0.009	0.037	11.0 (LOQ)	15.6
	Lettuce-Ac. Part.	0.008	0.040	13.4 (LOQ)	16.8
	Pear-EtOAc	0.001	0.021	2.2 (0.3)	3.6
	Apple-EtOAc	0.002	0.025	3.9 (0.3)	5.4
	Orange-EtOAc	0.001	0.013	2.7 (2.0)	4.5
	Lemon-EtOAc	0.004	0.026	2.2 (2.0)	3.2
	Tomato-EtOAc	0.004	0.024	16.8 (LOQ)	21.9
	Cucumber-EtOAc	0.001	0.017	3.6 (0.02)	4.7
	Spinach-EtOAc	0.003	0.026	10.0 (LOQ)	14.4
	Lettuce-EtOAc	0.004	0.028	9.4 (LOQ)	14.1
Dimethoate	Pear-Ac. Part.	0.003	0.030	5.8 (LOQ)	7.8

Pesticide	Matrix-MRM	LOD (mg/kg)*	LOQ (mg/kg)*	Repeatability (%RSD)**	Combined uncertainty (%RSD)**
	Apple-Ac. Part.	0.002	0.024	8.7 (LOQ)	9.6
	Orange-Ac. Part.	0.004	0.035	7.3 (LOQ)	10.1
	Lemon-Ac. Part.	0.006	0.039	7.4 (LOQ)	11.8
	Tomato-Ac. Part.	0.009	0.047	13.3 (LOQ)	20.7
	Cucumber-Ac. Part.	0.010	0.052	14.2 (LOQ)	23.0
	Spinach-Ac. Part.	0.010	0.050	12.6 (LOQ)	18.2
	Lettuce-Ac. Part.	0.009	0.048	11.7 (LOQ)	17.4
	Pear-EtOAc	0.002	0.021	5.9 (LOQ)	7.3
	Apple-EtOAc	0.001	0.019	3.3 (LOQ)	5.3
	Orange-EtOAc	0.002	0.023	5.0 (LOQ)	7.2
	Lemon-EtOAc	0.001	0.017	7.4 (LOQ)	7.9
	Tomato-EtOAc	0.008	0.051	15.1 (LOQ)	19.6
	Cucumber-EtOAc	0.003	0.030	10.4 (LOQ)	11.0
	Spinach-EtOAc	0.008	0.047	13.1 (LOQ)	15.8
	Lettuce-EtOAc	0.008	0.050	11.6 (LOQ)	15.4
Methamidophos***	Pear-EtOAc	0.006	0.031	13.0 (LOQ)	20.0
	Apple-EtOAc	0.004	0.022	14.7 (0.05)	20.2
	Orange-EtOAc	0.002	0.016	7.0 (0.02)	8.7
	Lemon-EtOAc	0.002	0.015	4.2 (0.2)	5.3
	Tomato-EtOAc	0.003	0.030	10.9 (0.5)	18.6
	Cucumber-EtOAc	0.002	0.015	2.8 (1.0)	7.0
	Spinach-EtOAc	0.006	0.034	13.4 (LOQ)	19.6
	Lettuce-EtOAc	0.007	0.039	8.9 (0.2)	12.4

*: LOD was estimated as the analyte concentration resulted in signal (S) to noise (N) ratio of 3 (S/N=3) (Huber, 1998) and verified by the analysis of the pesticide mixture fortified at 0.01 mg/kg (six independent replicates) as three times the standard deviation (LOQ=3*SD) (Yenisoy-Karakaş, 2006; Barriada-Pereira et al., 2007; Georgakopoulos et al., 2009); LOQ was defined as the analyte concentration resulting in S/N of 10 (Huber, 1998) and verified by the afore-mentioned procedure applied for LOD. LOQ equals "mean+10*SD", where mean is the average of concentration levels determined in the six independent replicates by the analysis procedures (Yenisoy-Karakaş, 2006; Barriada-Pereira et al., 2007; Georgakopoulos et al., 2009).

**: Repeatability determination was based on the %RSDs derived from six independent replicates prepared by the same analyst, analytical method and analysis day (Ambrus, 2004) of matrix extracts fortified with the MRL level (in mg/kg) as established by the European legislation shown in parenthesis of every combination; LOQ level was used in the cases that the MRL was lower than the LOQ; the same levels were used for the determination of combined uncertainty the calculation of which included the major sources of uncertainty, such as (Huber, 1998; Cuadros-Rodríguez et al., 2002; Ambrus, 2004).

***: Methamidophos validation data are not presented for acetone partition, since this analyte was not even detected in any extract of this MRM.

Table 6. Critical analytical parameters for MRM validation among different pesticide – matrix – extraction method combinations

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5

Chemical Analysis of Pesticides Using GC/MS, GC/MS/MS, and LC/MS/MS

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1. Introduction

There are well over 500 registered pesticides worldwide for use in agricultural regions and new agrochemicals are introduced to the marketplace continuously. This chapter deals with the chemical analysis methods for the main pesticide chemical classes that are most frequently analyzed with gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS). GC amenable pesticide chemical classes which do not require derivatization include organochlorines (OCs), pyrethroids, organophosphorus pesticides (OPs), triazines, and chloroacetanilides. In addition some transformation products of organochlorines, triazines, and phenylureas are GC amenable and when derivatized some transformation products of OPs, pyrethroids, and phenoxyacid herbicides are also GC amenable. Specific methods have been developed with other injector choices than the standard splitless injection for more thermally labile chemical classes such as trihalomethylthio fungicides to extend the range of GC amenable pesticides. Some chemical classes which are more polar such as phenoxy acid herbicides and carbamates can still be analyzed by GC/MS methods but require derivatization to make them GC amenable. For some other chemical classes a few pesticides have been analyzed by GC/MS usually included in multiresidue methods but these methods have not tackled the entire range of compounds within the chemical class. These include chemical classes such as dicarboximides (vinclozin, iprodione), dinitroaniline (trifluralin, ethalfluralin), dinitrophenol (dinoseb), and dithiocarbamate (triallate). A large number of pesticide classes generally of higher polarity suffer from poor chromatographic performance, poor MS source ionization or stability in GC/MS injectors, on-column, or in MS. For these chemical classes and also to minimize the need for derivatization prior to GC there has been a gradual shift to the development of new methods utilizing LC coupled with tandem mass spectrometry (MS/MS). Tandem mass spectrometry in selected reaction monitoring (SRM) mode is generally now more frequently used for LC rather than selected ion monitoring (SIM) with LC/MS as the ionization process for LC/MS is a softer process (change processes to process) than that of GC/MS ion sources such as EI and CI. For atmospheric pressure ionization (API) sources most frequently used in LC/MS/MS most pesticides have only one ion formed during ionization (the protonated or deprotonated molecular ion or sometimes an adduct ion (eg. sodium or ammonium adduct)) and consequently there is little confirmation ability. Tandem mass spectrometry allows for the controlled collision induced dissociation

(CID) of the parent ion making discrimination possible from co-eluting matrix components. No additions (LC/MS/MS or LC/MS methods include phenoxyacid herbicides other pesticides of interest. The main chemical classes of pesticides that have been more recently analyzed by LC/MS/MS or LC/MS methods and include phenoxyacid herbicides and a related nitrile herbicide (bromoxynil) often used in formulations with phenoxyacid herbicides, phenylureas, sulfonyl ureas, carbamates, pyrethroids, azoles, and a more extensive list of dithiocarbamates. Phenylureas, sulfonylureas, and most dithiocarbamates are not GC amenable and many azoles have significantly lower detection limits with LC/MS/MS. Some chemical classes including OPs, pyrethroids, carbamates, phenoxyacid herbicides, and azoles have both GC and LC methods coupled to mass spectrometry that have been developed and will be discussed in more detail in this chapter.

There are a large number of factors that require consideration for the selection of the method for analysis whether that is for an individual pesticide, a chemical class of pesticides, a large number of pesticides of different chemical classes, or for inclusion of their transformation products. These factors include: boiling point or polarity; solubility in desired solvent or mobile phases; stability of pesticides in injector ports, on-column, or in mass spectrometer ion sources; selectivity of columns and chromatographic behaviour; interferences in detection; molecular structure or other chemical properties important for both ionization and fragmentation; method detection limit or regulatory requirements; and confirmation ability over linear dynamic range. This chapter does not include a discussion of the sample preparation (pre-concentration or sample clean-up) procedures and does not distinguish methods developed for fruit and vegetables, biological tissues, soil, water, air or other sample matrices. The focus is on issues related to the chromatography-mass spectrometry and instrumental approaches that may be taken advantage of to improve selectivity or sensitivity of analysis.

2. Identification of the problem

Due to the large number of pesticides under investigation users must firstly decide on whether to choose a GC or LC method coupled to mass spectrometry and if the method can achieve the desired quantitative analysis and confirmation needs. Some laboratories may also be more limited in their choice of instruments or skill of analysts so need to be aware of methods that may be equivalent for those chemical classes that can be analyzed by both GC and LC. Many laboratories are looking towards streamlining sample preparation and analysis needs such as with the Quick Easy Cheap Effective Rugged and Safe (QuEChERSA) pesticide multiresidue methods in combination with GC and LC mass spectrometry methods (Cunha et al., 2007; Payá et al., 2007; Pihlström et al., 2007). Due to the large diversity in sample types and pesticides used or of concern in different regions, multiresidue analysis methods can vary significantly in their choice of target pesticides and transformation products and this makes it challenging for an analyst to select a method for analysis as they may not fully understand the factors that went into the selection of the instrumental parameters and the compromises that were made to resolve matrix effects, chromatographic needs, and detection requirements. This chapter takes a chemical class approach which users can then utilize to select methods with their target pesticide list and can be further built on to include compounds not in these major chemical classes. A main goal is to highlight by chemical class some of the preferences for these methods and the demands or options for improvements. Some of the advances in instrumental approaches to

either improve the range of compounds for analysis, reduce background signal, or improve selectivity or sensitivity of the analysis will be highlighted. Due to the increasing need for analysis of transformation products they will be discussed along with their chemical class of parent compounds. Simultaneous analysis of parent pesticides and transformation products is desirable but because of the large diversity in polarity, volatility, stability, and ionization in MS ion sources this is not always feasible. Issues with co-elution of other complex interfering matrix components or other pesticides of interest and their impact on detection and confirmation will also be discussed.

3. GC/MS, GC/MS/MS, and LC/MS/MS for pesticides and their transformation products

3.1 GC/MS and GC/MS/MS methods

One of the most important parameters when considering GC/MS methods of analysis particularly when added selectivity or sensitivity are required is the choice of the ionization mode. Sample matrix and sample preparation procedures including clean-up also dictate selection of the ionization method due to presence of co-eluting pesticides or matrix components which can interfere in analysis if they can not be distinguish in the mass spectra. If pesticides are electron-capturing such as those pesticides which contain halogen, NO_2, or P ester groups then they will generally give an enhanced response (up to two or three orders of magnitude) with negative chemical ionization (NCI) in comparison to electron impact (EI) or positive chemical ionization (PCI) (Raina and Hall, 2009; Liapis et al., 2003; Bailey and Belzer, 2007; Húšková et al., 2009). The selection of ionization mode often depends upon whether the analysis is targeted for specific chemical classes or is a multiresidue analysis methods for determination of hundreds of pesticides in a sample extract. A comparison of GC/MS or GC/MS/MS with EI to LC/MS/MS has been reviewed for a large number of compounds and suggests for most pesticides other than organochlorines that LC/MS/MS can provide lower detection limits (Alder et al., 2006; Pihlström et al., 2007; Paya et al., 2007; Lambropoulou et al ., 2007). However, lower or comparable detection limits have also been found for chloracetanilides (metolachlor, acetochlor, alachlor) and selected triazines by GC/MS or GC/MS/MS with EI relative to LC/APCI-MS/MS (Dagnac et al., 2005) or LC/ESI-MS/MS (Gomides Freitas et al., 2004). GC/MS of a wider range of triazines has also been done by GC-EI/MS (Nagaraju and Huang, 2007; Zambonin and Palmisano, 2000; Jiang et al., 2005; Gonçalves et al., 2006; Albanis et al., 1998). Chemical ionization is often not considered in comparisons of GC and LC mass spectrometry methods. Reduction of matrix interferences particularly for masses <50 (Bailey and Belzer, 2007; Bailey, 2005) is often an important consideration as well as the need for molecular structure information from the MS spectra. Due to the large diversity in properties of pesticides analyzed by multiresidue analysis methods EI is more frequently used however particularly for many halogenated pesticides (excluding chloracetanilides) it does not often give the best sensitivity or selectivity. The clear advantage of EI is the availability of extensive libraries in full scan mode for confirmation of compound identify by library search matching, however sufficient sample concentration must be available. Most quantitative analysis is completed in selected ion monitoring (SIM) mode with peak area of the most abundant ion in the MS spectra used for the quantitative analysis, and the peak area obtained from an additional one or two ions used for confirmation along with the ratio of ion responses and retention time match (Raina and Hall, 2009). At the concentration

levels of routine analysis particularly for environmental sample analysis there is insufficient concentration to obtain full scan MS spectra of sufficient abundance for library matching when quadrupole or ion trap systems are used.

There is another unique feature of pesticide analysis with mass spectrometry that is often not discussed in detail. Relative to other contaminants, many pesticides including OCs, OPs, pyrethroids, and chloroacetanilides exhibit low intensity for the molecular ion regardless of whether EI or CI is used (Raina and Hall, 2009; Húšková et al., 2009; Yoshida, 2009; Feo et al., 2010; Dagnac et al., 2005). Consequently in SIM mode the quantitative or qualifier ion is rarely selected as the molecular ion. In general >90% of pesticides do not monitor the molecular ion by EI or CI methods as at the working concentration ranges of trace analysis generally the molecular ion is too low in abundance to be observed. The exception are the triazines where the molecular ion is one of the ions monitored but may not be the base peak in the EI mass spectra (Nagaraju et al., 2007; Jiang et al., 2005; Zabonin and Palmisano, 2000). The selection of EI versus NCI or PCI may also be based on instrument design and cost and basic GC/MS instruments often do not include CI capability.

In this section the focus will first be on chemical classes of pesticides where GC/MS methods are superior or equivalent to LC/MS/MS methods and derivatization is not required. The chemical classes that will be discussed include organochlorines (OCs), organophosphorus pesticides (OPs), trihalomethylthio fungicides, pyrethroids, triazines, and chloracetanilides. The ion sources used in LC/MS/MS are not suitable for some of these pesticides including many of the OCs and trihalomethylthio fungicides. OC degradation products have been routinely included in GC/MS methods either with EI or NCI and include OCs such as endosulfan sulphate, DDD, DDE, HCH isomers, endrin ketone, endrin aldehyde, heptachlor epoxide, methoxychlor. Chloroacetanilides are more sensitive with EI than CI modes with GC/MS (Raina and Hall, 2009; Dagnac et al., 2005; Gabaldon et al., 2002) but can be done with similar detection limits with LC/ ESI+ or APCI+ MS/MS (Dagnac et al., 2005). The transformation products of chloroacetanilides are not analyzed by GC/MS, however chloroacetanilide (eg alachlor, propachlor, metalochlor) analysis is frequently included with analysis of OCs by GC/MS. Triazines can be analyzed with comparable GC/EI-MS or LC/MS/MS methods and it depends upon the application needs and availability of instrumentation as to which method is choosen. Transformation products of atrazine: deisopropylatrazine (DIA), desethylatrazine (DEA), didealkylatraizine (DDA) and 3,4-chloroaniline which is a transformation product of phenylureas (linuron and diuron) have also been analyzed by GC/EI-MS or GC/EI-MS/MS methods (Planas et al., 2006; Jiang et al., 2005; Dagnac et al., 2005). GC/MS methods are more suitable to a wider range of OPs than LC/MS/MS as not all OPs are ionized efficiently by API sources (eg. parathion). However, a significant number of OPs which are widely used give significantly lower detection limits with LC/MS/MS (Table 1). In addition GC/MS methods suffer from poor chromatographic performance, low sensitivity, and required derivatization for OP transformation products, whereas LC/MS/MS can be used to simultaneous analyze the OP transformation products including OP oxons with detection limits of 0.06-0.38 µg/L (Raina and Sun, 2008) and OP sulfones and sulfoxides (Chung and Chan, 2010; Jansson et al., 2004; Hiemstra et al., 2007; Economou et al., 2009). For some pyrethroids GC/EI-MS has approximately 100 times higher detection limits than LC/MS/MS (Alder et al., 2006) while for many they are comparable (Yoshida et al., 2009). When NCI is used detection limits for some pyrethroids can be 10-100 times lower than EI (Feo et al., 2010) making GC/MS comparable or better than LC/MS/MS methods. Coupling this with large volume injections

can further improve these GC/MS methods if required. In addition, pyrethroid transformation products can be analyzed with GC/EI-MS/MS following 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) derivatization (Arrebola et al., 1999) and there has been no reported LC/MS/MS method.

OP Pesticide	GC/MS or GC/MS/MS Limit of Detection (µg/L)Raina and Hall, 2009	LC/ESI+MS/MS Limit of Detection (µg/L)Raina and Sun, 2008
Chlorpyrifos	4.5	0.19
Chlorpyrifos methyl	7.6	0.27
Diazinon	*0.70*	0.08
Malathion	9.5	0.23
Azinphos methyl	>50*	0.32
Azinphos ethyl	>50*	0.47
Dimethoate	NA	0.05
Phorate	7.8	0.37
Fenchlorphos	7.5	16

Table 1. Comparison of GC/MS and LC/MS/MS Limits of Detection for Selected Organophosphorus Pesticides. *note calculated under GC separation conditions for OCs and OPs retention times 25-27 min (higher than most compounds); NA not available. Italics GC-EI/MS lowest detection limit otherwise NCI was used.

A comparison of 47 chlorinated organics (including OCs and several chloroacetanilides) and OPs analyzed by GC/MS showed that no one ionization mode could be used to analyze all the pesticides at concentrations <100 ng mL^{-1} for a standard splitless 1 µL injection. In general NCI-SIM provided the lowest method detection limits (MDLs) for the largest number of pesticides along with confirmation at these low levels. When confirmation by NCI-SIM was not sufficient, NCI-SRM could be used and gave additional sensitivity and confirmation ability to ~14% of pesticides studied (Raina and Hall, 2009). Others have also found that GC-MS/MS can provide added selectivity (Zhang and Lee, 2006). Although EI-SIM is often used for multiresidue GC analysis methods we found that EI-SIM only provided better sensitivity than NCI-SIM or NCI-SRM for 3 of the 19 OPs (aspon, diazinon, sulfotep), and 9 of the 28 OCs or chloroacetanilides studied (alachlor, aldrin, p,p'-DDD, o,p'-DDE. p,p'-DDE, dieldrin, heptachlor, perthane, propachlor) and for other OCs and OPs an additional confirmation approach would be required at these concentrations if EI was used due to low abundance of the confirmation ion (Raina and Hall, 2009). Chloroacetanilides have been previously identified as best analyzed by GC-EI/MS or MS/MS (Galaldon et al., 2002; Dagnac et al., 2005). Others have also found for a range of OCs, OPs, and some pyrethroids that NCI-SIM is up to 100 times more sensitive than EI-SIM (Húšková et al., 2009; Feo et al., 2010). Better S/N ratio with NCI or reduced matrix background interference response was observed particularly at low masses ($m/z < 50$). NCI provides added selectivity as many interfering matrix components are expected to be hydrocarbons, humic or fulvic acids, or nonhalogenated in nature and thus do not produce a signal with NCI (Bailey, 2005; Bailey and Belzer, 2007). Positive chemical ionization like EI suffers more than NCI from matrix interferences and for these chemical classes of pesticides it is generally less sensitive so it is seldom used for quantitative analysis.

EI full scan mode provides the ability for confirmation with library search matching, however in quantitative analysis generally selected ion monitoring (SIM) is accomplished only with confirmation using an additional one or two ions and the ratio of response of these ions within a specified % relative standard deviation usually determined from standard injections on the day of analysis rather than from libraries. One advantage of GC/MS over LC/MS/MS methods is the lower instrument cost and that pesticides fragment in the EI or CI ion source easily and consequently structural information is available for the pesticide for its confirmation. Fragmentation with EI sources is distinctly different from electrospray ionization used in LC/MS/MS with odd-electron (OE) fragment ions more frequently produced with EI (35% OE ions, 65% EE ions) as compared to 93% even-electron (EE) ions with positive electrospray ionization (Thurman et al., 2007). Chemical ionization is a softer ionization process than EI and the MS spectra generally produce less fragment ions, however for > 90% of pesticides analyzed by GC/MS the two most abundant ions in any ionization mode still generally do not include the molecular ion even when PCI or NCI are used (Raina and Hall, 2009; Húšková et al., 2009; Feo et al., 2010). In addition there may be relatively few fragments available of sufficient abundance for confirmation and consequently often isotope masses of fragment ions are used for confirmation. This has implications on the applicability of GC/MS/MS with our results showing that EI is not suitable for the analysis of OCs or OPs < 100 ng mL^{-1} and NCI-SRM is generally less sensitive than NCI-SIM even though there is reduced background noise (Raina and Hall, 2009). For fruit and vegetable analysis where higher levels of pesticides can be achieved in sample extracts, GC/MS analysis with SRM in EI mode has been used for a similar range of OPs and OCs with preference for these pesticides analyzed by GC/MS/MS over LC/MS/MS (Pihlström et al, 2007). As pesticides easily fragment in the ion sources of GC/EI or NCI-MS, the parent ion selected for collision induced dissociation (CID) is often a fragment ion and this ion must be capable of further fragmentation. In a number of cases for these chemical classes with NCI the presence of higher mass parent ions or the molecular ion improved the potential for lower MDLs with NCI-SRM as compared to EI-SRM. However, most pesticides did not have an abundant molecular ion. Even in NCI-SRM for OCs the SRM transition selected were often $f_1^+ > Cl^-$ (m/z=35) with the confirmation SRM utilizing an isotope peak mass (eg $f_1^+ > Cl^-$ (m/z=37)) (Raina and Hall, 2009). The fact that the parent ions with GC/MS/MS are often fragment ions makes finding suitable product ions more challenging than with LC/MS/MS ion sources where the parent ion is generally the protonated or deprotonated molecular ion. In the case of HFIP derivatized transformation products of pyrethroids the molecular ion was used for CID and produced better sensitivity and selectivity that GC/EI-MS which observed significant chromatographic resolution problems and reduced MS sensitivity (Arrebola et al., 1999).

There are a number of approaches that can be used to extend the range of pesticides that can be analyzed by GC/MS or to further improve MDLs beyond the most frequently used splitless injections with a hot split/splitless injector. For pesticides such as the trihalomethylthio fungicides that are more thermally labile other injectors including programmable temperature vaporizer (PTV) or cold on-column (COC) injector can be used (Bailey, 2005). Another advantage of these injectors is that they can also be utilized for large volume injections increasing the sample injection size from 1-2 µL to 5-100 µL. With both approaches the sample is injected cold (below or near the boiling point of the solvent). Pre-columns have also been utilized with these approaches for focusing and to extend the analytical column lifetime by minimizing build-up of non-volatile matrix components. Both approaches have limitations that are discussed requiring careful consideration.

A standard PTV injector has been used for analysis of chemical classes of pesticides such as OCs and OPs often operated with a solvent vent step where the initial injector temperature is set below the boiling point of solvent (eg 40 °C for toluene) and held at this temperature while the solvent vapour is eliminated via the split exit (Godula et al., 2001). PTV can be used for small injection volumes (2 μL) such as those in neat solvents (eg toluene) (Huskova et al., 2009) or matrix matched standards (Kirchner et al., 2005), and can also be used for large injection volumes of 20 μL (Grob and Li, 1988). More advanced PTV injectors are also available with modifications for dirty matrix injections (DMI) where the GC liner can be replaced for each injection with a robotic autosampler system and contains a small 40 μL DMI microvial. The challenge with larger volume injections with PTV is that the temperature and time the split vent is open must be optimized to remove the solvent without loss of analytes of interest and pesticides with boiling points near the solvent will have a higher potential for loss. An example system that I have used for this approach is a GC Twin-PAL (Leap Technologies, Carrboro, NC) and an Optics 3 PTV inlet (ATAS/GL International BV) with direct thermal desorption (DTD) probe. The crimp top DTD liner is an open liner (80 mm X 5 mm O.D.) containing a needle guide and the 40 μL DMI microvial held in place at 20 mm from the bottom of the liner by three knobs. The Optics 3 PTV inlet is equipped with DTD probe that allows for interchange of the DTD liners containing the DMI microvial between injections. The inlet has separate gas controls from the GC and a solvent vapour thermal conductivity detector (TCD) sensor and in the example shown below is operated in fixed time mode. During the injection temperature is set below the boiling point of the solvent and there is a high split vent flow (100 mL min^{-1}), after the solvent is vented the injection time starts (Figure 1). For a solvent such as ethylacetate which is often used for extraction procedures in QuEChERS pesticide analysis (Pihlström et al., 2007) a temperature of 70°C can be used and requires a vent time of 330 sec for a 10 μL injection. Increasing the temperature in 10°C increments will reduce vent time required by ~60 sec, however more volatile pesticides such as captan and captafol showed significant loss of signal above 70°C and consequently this temperature and vent time were required for the analysis. When injection size was increased to 20 μL the required vent time increased to 540 seconds and for larger volume samples near the capacity of the microvial the vent time was in excess of 10 minutes which is not practical for analysis. Switching the solvent to a lower boiling solvent such as hexane reduced the temperature to 60°C. For both GC/MS and LC/MS/MS applications there has also been interest in coupling SPE cleanup methods directly with analysis. Table 2 provides the steps required for coupling the LVI-DMI (large volume injection-dirty matrix injection) with the at-line automated SPE approached. The at-line automated SPE LVI-DMI-GC/MS method sequence involving first direct clean-up of a sample with a 96-well plate C-18 SPE format using the Twin-PAL robotic autosampler system for SPE preparation; followed by injection of a portion of the SPE eluted extract directly into DMI liners; and then exchange of the liners in the PTV-DTV probe for sample injection. In this example a 10 μL fraction of each 100 μL fraction eluted from the SPE 96 well plate was analyzed for pesticides. Figure 2 shows that the trihalomethylthio fungicides are eluted with 200 μL of ethylacetate (fractions F2 and F3 of size 100 μL) and illustrates that the at-line SPE approach is capable of replacement of standard off-line SPE procedures. Good linearity from method detection limit (MDL)-500 μg/L (r^2>0.99) was observed with method detection limits of 2.5-5 μg/L similar to that observed for LVI-COC injections (Bailey and Belzer, 2007). The clear advantage of this injection approach over LVI-COC injections is that

non-volatile material remains in the injector liner (in the DMI microvial) which is replaced with each injection so there is no build-up of non-volatile material on column reducing maintenance requirements. It is limited in its applicability to pesticides with boiling points near the solvents boiling point as they will be lost during the solvent venting stage so solvent selection is also an important parameter for consideration. PTV inlets may also still cause degradation of pesticides in the injection port as after solvent venting, the injection port temperature is rapidly ramped.

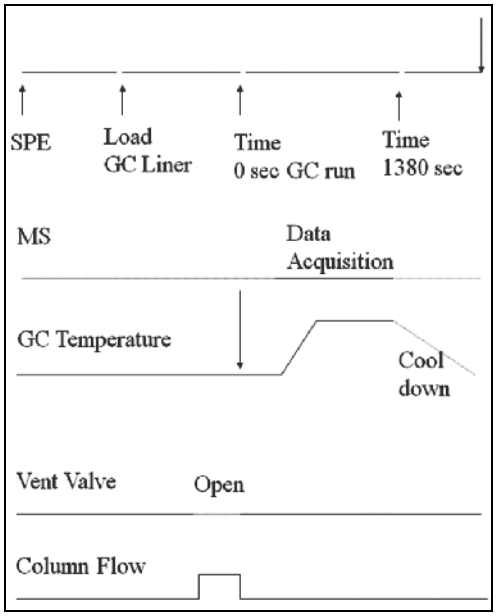

Fig. 1. SPE-LVI-DMI-GC/MS run set-up conditions.

Sequence Step	Conditions
SPE sorbent conditioning with Prep-PAL	1) 500 µL ethyl acetate, apply pressure 2) 500 µL of methanol, apply pressure
Sample Loading with Inject-PAL to SPE 96 well plate	10 µL sample added, rinse syringe
Washing with Prep-PAL	100 µL methanol added, apply pressure
Move 96-well plate with Prep-PAL	Ready for elution step –96 well plate moved forward from over waste to over 96-well collection plate
Elution with Prep-PAL	100 µL ethyl acetate, apply pressure
Addition of IS standard With Inject-PAL	Take 2 µL internal standard solution and mix with 100 µL SPE eluate in SPE collection plate (3-5 strokes)
DMI-Injection –load sample and transfer DTD liner with Inject-PAL	Take 10 µL of SPE eluate from 96-well collection plate and deliver to DTD/DMI liner, move liner into DTD probe, clean syringe

Table 2. At-line automated SPE LVI-DMI-GC-MS Method Sequence.

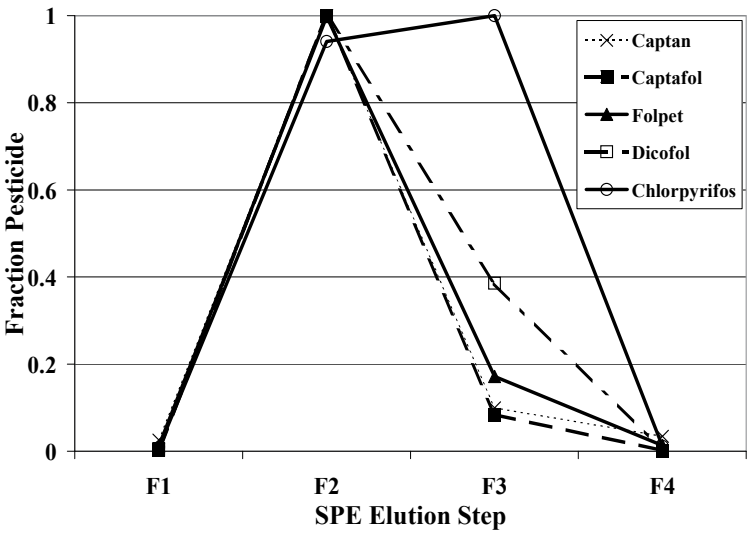

Fig. 2. Fraction of Pesticide in Washing and Elutions Steps of At-line SPE Procedure. F1 (washing):100 µL methanol; F2 (elution):100 µL ethylacetate; F3 (elution):100 µL ethylacetate; F4 (elution):100 µL ethylacetate; Sample 10 µL of 0.1 µg mL^{-1} pesticide mixture dissolved in hexane; SPE 96 well plate Bond Elute® C18 100 mg.

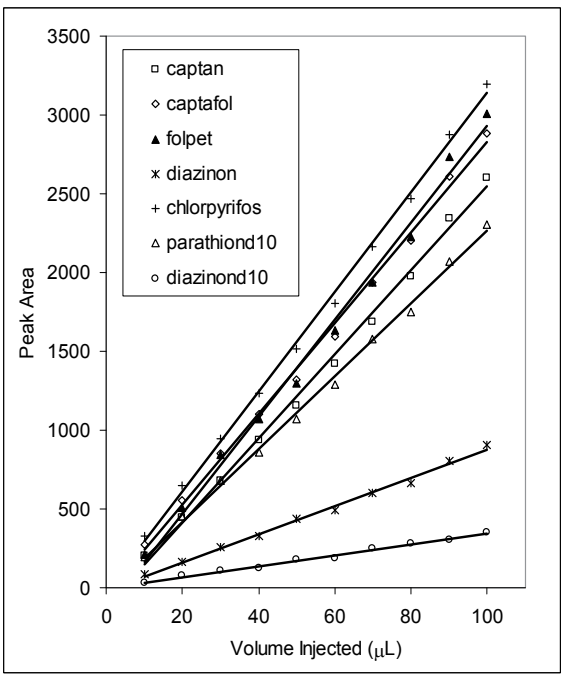

Fig. 3. Change in Peak Area with Injected Sample Size for LVI-COC GC/NCI MS. Peak area captan and parathion-d_{10} divided by 3; peak area chlorpyrifos divided by 8 for scaling. Taken from Bailey and Belzer, 2007.

The cold on-column injector is another option for thermally labile pesticides or large volume injections. Figure 3 shows that it can be used for injection sizes up to 100 μL which exceeds the capability of the LVI-DMI injections. The injection size is also more compatible with the needs for at-line SPE approaches. Cold on-column injection reduces the potential for breakdown of pesticides by directly injecting the sample onto typically a wider diameter 1-1.5 m retention gap (0.53 mm i.d.) which is connected to a short pre-column (~0.4 m X 0.25 mm) and then further connected with a T-connector to both the analytical column and a solvent vapour exit valve (50 μm bleed restrictor, Agilent) (Bailey and Belzer, 2007). The oven temperature at the start is set at 60-65°C (hexane as solvent) and the split vent is opened until the solvent is removed which for hexane was 60 seconds. The limitation of this system is that the retention gap and pre-column need periodic replacement due to build-up of non-volatile material from samples and thus there are higher maintenance requirements than standard PTV or LVI-DMI injections. Significant loss in sensitivity or poor chromatographic performance is observed when the retention gap requires replacement. Some of these problems may be alleviated with the availability of high temperature GC columns.

Another key recent advancement in GC/MS analysis that should be considered by users are the use of high temperature columns to extend column lifetime, reduce maintenance needs, to identify high boilers, and reduced column bleed. These columns are available in the full range of polarities from 100% polysiloxane to polyethylene glycol stationary phases and have low column bleed due to the proprietary ESC™ bonding technology. Low and mid-polarity columns can be used up to temperatures of 430°C, and higher polarity columns up to 400°C as compared to maximum temperatures of 300-360°C for most standard fused silica GC columns temperatures above which the standard polyimide resin coating pyrolyzes. Zebron™ Inferno™ columns (Phenomenex) utilize a high temperature polyimide coating with the flexibility and robustness of other non-metal columns making it highly compatible for GC/MS analysis. The use of higher temperatures has several advantages even if the pesticides elute prior to these temperatures as it reduces build-up of high boiling point matrix components which can be baked-off at the end of the run.

To extend GC/MS analysis to more polar pesticides often requires preceding or on-column derivatization. One chemical class of pesticides which has been successfully analyzed with derivatization prior to GC/MS analysis is the phenoxy acid herbicides. Derivatization agents have included pentafluorobenzyl (PFB) bromide, benzyl bromide, trimethylsilyl diazomethane, or alkylchloroformates to produce the corresponding PFB, benzyl, or methyl ester (Nilsson et al., 1998; Rimmer et al., 1996; Henriksen et al., 2001). Methylation with diazomethane or by reaction with 10% sulfuric acid in methanol has also been used (Shin, 2006). The chlorophenols which are transformation products of the phenoxy acid herbicides can also be converted to their carbonates for GC/MS analysis using alkylchloroformates (Henriksen et al., 2001). These approaches can suffer from deteriorating peak shapes over time and reduced column lifetime (Charlton et al., 2009). Carbamates are thermally labile and can breakdown in the injector port or on-column to their corresponding phenols and amines and consequently derivatization using acetylation, silylation, alkylation, or perfluorination is required. On-column derivatization with trimethylphenylammonium hydroxide and trimethylsulfonium hydroxide has been used to give thermally stable products for a variety of carbamates including carbaryl, methiocarb, chlorpropham, propham, and promecarb that can be analyzed by GC-EI/MS (Zhang and Lee, 2006). In more recent years there has been a shift to LC/MS/MS methods (see section 3.2) for both

phenoxyacid herbicides and carbamates as these methods do not require the derivatization step and can provide an ability to simultaneous analyze transformation products and often a wider range of pesticides within the same chemical class (Raina and Etter, 2010; Charlton et al., 2009; Chung and Chan, 2010).

To achieve the necessary MDLs required for environmental or food analysis the majority of GC/MS pesticide analysis methods are in SIM mode with either single quadrupole or ion-trap systems with ion-traps providing similar or slightly higher MDLs than the more popular quadrupole systems. In addition to the use of tandem mass spectrometry in GC/MS analysis, recent advances in pesticide analysis have included the use of GC/TOF-MS for pesticide analysis to achieve MS scan separation even at these low environmental levels enabling full confirmation ability and added selectivity. In these analysis TOF is generally operated with unit resolution and high scan rates (eg 200-500 scans/sec) to provide for automated mass spectral deconvolution of overlapping signals and library matching (de Koning et al., 2003; Zrostlikova et al., 2003b). GC/TOF-MS can also be operated with high mass resolution (0.02 -0.05 Da) with slower scan rates (2-10 scans/sec). It has had more limited applicability for pesticide analysis (Cajka et al., 2004), however with new designs that include a dynamic range enhancement (DRE) the limitations of saturation at high ion concentrations have been overcome (Leandro et al., 2007). GC/TOF-MS is most often used for fast-eluting peaks and for applications such as comprehensive two-dimensional gas chromatography (GC X GC) analysis of pesticides (Zrostlikova et al., 2003b) but has received much less attention than other GC or LC applications. In these multiresidue analysis applications unit resolution is used with fast scan rates to allow multiresidue screening by GC X GC/MS full scan (50-500 m/z) utilizing spectra library matching in EI mode (Dasgupta et al, 2010). A 5 μL DMI injection has also been used with GC/TOF-MS analysis of pesticides utilizing peak deconvolution and library searching software for isolation of the analyte peaks from matrix components (de Koning et al., 2003). With this smaller DMI injection size and for the list of pesticides under their study the temperature for solvent venting step was set to 50°C with a shorter solvent vent time of 120 sec. Utilizing DMI with GC/TOF-MS is a dual approach of reducing matrix interferences by firstly reducing the amount of matrix introduced into the GC/MS system and secondly utilizing MS spectral library matching ability of TOF-MS. Keeping the upper limit of injector temperature to that just necessary to volatilize analytes also keeps the non-volatile material in the DMI microvial and consequently reduced demands on mass spectral resolution.

3.2 LC/MS/MS methods

LC/MS/MS continues to gain popularity in use for pesticide analysis with most applications focused on non-GC amenable compounds, thermolabile, polar and non-volatile pesticides. Some chemical classes such as phenoxyacids herbicides, triazines, OPs, chloroacetanilides, and pyrethroids can be analyzed by both GC/MS and LC/MS/MS. For phenoxacid herbicides and carbamates LC/MS/MS is regarded as more favourable as it does not require a derivatization step prior to analysis. The use of LC/MS/MS over GC/MS for the chemical classes listed in Table 3 may also be done in order to achieve reduced analysis time by utilizing a multiresidue LC/MS/MS method covering a range of target pesticides from different chemical classes. However the key reason for choosing LC/MS/MS over GC/MS is the need to deal with more polar chemical classes of pesticides and increasingly for the simultaneous analysis of their transformation products.

Transformation products are often more polar and less volatile than their parent compounds and generally have poor chromatographic performance on nonpolar GC columns or are thermolabile. Transformation products many also require derivatization to make them GC amenable for some of these chemical classes as discussed previously. Even for LC/MS/MS methods the large difference in polarity between parent pesticide and transformation product may require different separation conditions or ion source (mode) for adequate sensitivity making development of simultaneous methods challenging.

The use of LC/MS/MS for pesticide residue analysis has focused on systems with atmospheric pressure ionization (API) either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) either in positive or negative mode. Many LC/MS/MS methods are multiresidue analysis methods and have been done for a target list of pesticides requiring analysis for regulatory purposes. Both APCI or ESI have been used for multiresidue methods with ESI+ the most popular as shown in Table 3. Direct comparisons of the sensitivity of APCI and ESI are often not available or not under the same chromatographic conditions. In addition, often regulatory requirements can be met with both approaches with similar MDLs for many pesticides observed under optimal conditions (Titato et al., 2007; Thurman et al., 2001). The design and operational parameters of individual API ion sources can also lead to varying results between the sensitivity of ESI versus APCI and consequently should be evaluated for the system under use and expected flow rate conditions. Table 3 shows that flow rate conditions for the separation are an important consideration as ESI is generally most sensitive at lower flow rates typically near 0.2 mL/min and consequently it may be desire to utilize smaller particle size (2-3µm) LC/MS columns however the reduction in sample loading capacity should also be consider (Asperger et al., 2001; Titato et al.,2007). If using higher flow rate conditions for the separation on columns (5µm, 150 to 250 mm X 4.6 mm) then the flow is generally split prior to MS (Banerjee et al., 2009; Crescenzi et al., 1995; Di Corcia et al., 2000). APCI is most often operated under high flow rates 1-2 mL/min (Table 3) and optimal flow varies with chemical class (Asperger et al., 2001; Titato et al., 2007). OPs are distinctly different and require lower flow rates for optimal sensitivity with APCI (Asperger et al., 2001; Jansson et al., 2004; Titato et al, 2004). Even if sensitivity is better with the more popular ESI methods there may be preference to use APCI for some chemical classes (OPs, chloracetanildes, pyrethroids, phenoxyacid herbicides, carbamates) to take advantage of other factors which include the following: (1) APCI is generally less prone to sodium adduct formation that ESI; (2) APCI can be less prone to matrix impacts as compared to ESI (Souverian et al., 2004); and (3) in some cases the SRM transition can differ from ESI so that co-eluting peaks can be isolated with MS/MS thereby reducing chromatographic resolution needs (see Table 3).

As a general rule the choice of positive or negative mode depends upon polarity and acidity of analytes and sample matrix impacts. In general, ESI- is more sensitive for phenoxyacid herbicides and their transformation products (Raina and Etter, 2010; Koppen et al., 1998; Dijkman et al., 2001) and chloroacetanilide transformation products; ESI+ for sulfonylureas, phenylureas, N-methylcarbamates, organophosphorus pesticides (Cessna et al., 2006; Degenhardt et al., 2010; Hernandez et al., 2006; Steen et al., 1999; Raina and Sun, 2008); APCI+ for triazines (Dagnac et al., 2005; Jeannot et al., 2000); and APCI+ or ESI+ for chloroacetanilides (Dagnac et al., 2005; Ferrer et al., 2007; Banerjee et al., 2009). It should be noted that phenoxyacid herbicides have been analyzed with APCI- (Puig et al., 1997); sulfonylureas, phenylureas, carbamates, OPs with APCI+ (see Table 3); triazines with ESI+ (Dagnac et al., 2005; Jeannot et al., 2005); and for methods where acidic pesticides are

Column, flow rate, ion source	Mobile phase organic modifier (MeCN – acetonitrile; MeOH-methanol) and additive	Reference
Chloroacetanilides and transformation products* – LC/ESI+MS/MS unless specified		
Zorbax Eclipse SB-C18 (1.8 µm, 150 X 4.6 mm) 0.6 mL/ min	0.1 % formic acid, MeCN 10-100%	Ferrer et al ., 2007
Omnisper C-18, (3 µm 150 X3 mm) 0.4 mL/min		
Purosphere STAR RP-18e (5 µm, 150 X 4.6 mm) 1.0 mL/ min	MeCN 85% to 40%	Dagnac et al., 2005
split to MS	5 mM ammonium formate, 34%-90% MeOH	Banerjee et al., 2009
Gromsil C18 (3 µm, 150 X 2.0 mm) 0.15 mL/min, ESI-		
	0.6%formic acid, MeOH 40-95%	Gomides Freitas, 2004*
Dithiocarbamates (anionic[1] and neutral[2]) and transformation products[2] –LC/MS		
ZIC-pHILIC column (5 µm 150 X4.6 mm) 0.7 mL/min with 50%	10 mM ammonia, 10 to 40% MeCN	Crnogorac et al ., 2007[1]
split to LC/MS, ESI-		
C8 (5 µm 150 X4.6 mm) 0.8 mL/min, ESI+ and APCI+	10-90% MeOH	Blasco and Pico, 2004[2]
OPs –LC/MS/MS ESI+ unless specified		
C6phenyl Gemini (3 µm, 150 X 2.0 mm) 0.2 mL/ min	0.1% formic acid, 2 mM ammonium acetate, MeOH 40-95%	Raina and Sun, 2008
C12 (4 µm, 150 X 2.0 mm) 0.25 mL/min	5 mM ammonium formate, MeOH 5%-90%	Chung and Chan, 2010
Chromolith SpeedROD RP-18e (50 X 4.6 mm) varied 0.2-1.2 mL/min ESI+; APCI+ 0.2-2.8 mL/min	MeOH 97%	Asperger et al., 2001
Zorbax Eclipse SB-C18 (1.8 µm, 150 X 4.6 mm) 0.6 mL/ min	0.1 % formic acid, MeCN 10-100%	Ferrer et al., 2007
Atlantis C18 (5 µm, 100 X 2.1 mm) 0.2 mL/ min		
Xterra MS C18 (3.5 µm, 150 X 2.1 mm) 0.2 mL/ min	0.01%formic acid gradient MeOH 5%-90%	Hernandez et al., 2006
Genesis C18 (4 µm, 100 X 3 mm) 0.3 mL/min	0.1% formic acid, MeCN 10-90%	Botitsi et al., 2007
Genesis C18 (4 µm, 100 X 3.0 mm) 0.2 mL/ min, APCI+ and ESI+	10 mM ammonium formate pH 4, MeOH	Pihlstrom et al., 2007
Supelcosil LC18 (5µm, 150 mm X 2.1 mm) 0.6 mL/min for	10 mM ammonium formate, pH 4, MeOH 0-90%, flush with MeCN 80% each run	Jansson et al. , 2004
APCI+; ODS (5µm, 150 mm X 2.1 mm) 0.1 mL/min	0.05% TFA, MeCN 70% isocratic	Titato et al ., 2007
Aqua C18 (3 µm, 150 mm X 2 mm), 0.3 mL/min		
Synergie RP (4 µm, 50 mm X 2.0 mm), 0.6 mL/min	5 mM ammonium formate, MeOH 0-90%	Paya et al. , 2007
Alltima C18 (5 µm, 150 X 3.2 mm) 0.3 mL/ min	5 mM ammonium acetate MeOH 20-80%	Muller et al ., 2007
XTerra MS C18 (3.5 µm, 150 X 2.1 mm) 0.2 mL/ min	5 mM ammonium formate, 25-95% MeOH 0.1%	Hiemstra et al. 2007
Purosphere STAR RP-18e (5 µm, 150 X 4.6 mm) 1.0 mL/ min	formic acid, MeCN 10%-90%	Economou et al., 2009
split to MS	5 mM ammonium formate 34%-90% MeOH	Banerjee et al 2009
OP transformation products (oxons[1], sulfoxides and sulfones[2]) LC-ESI+/MS/MS		
C6phenyl Gemini (3 µm, 150 X 2.0 mm) 0.2 mL/ min	0.1% formic acid, 2 mM ammonium acetate, MeOH 40-95%	Raina and Sun, 2008[1]

Column, flow rate, ion source	Mobile phase organic modifier (MeCN – acetonitrile; MeOH-methanol) and additive	Reference
C12 (4 µm, 150 X 2.0 mm) 0.25 mL/min	5 mM ammonium formate, MeOH 5%-90%	Chung and Chan, 2010[2]
Genesis C18 (4 µm, 100 X 3.0 mm) 0.2 mL/ min	10 mM ammonium formate, pH 4, MeOH 0-90%, flush with MeCN 80% each run	Jansson et al., 2004[2]
Alltima C18 (5 µm, 150 X 3.2 mm) 0.3 mL/ min	5 mM ammonium formate, MeOH 25-95%	Hiemstra et al., 2007[2]
XTerra MS C18 (3.5 µm, 150 X 2.1 mm) 0.2 mL/ min	0.1% formic acid, MeCN 10%-90%	Economou et al., 2009[2]
Pyrethroids LC-ESI+/MS/MS		
Genesis C18 (4 µm, 100 X 3 mm) 0.3 mL/min	MeOH, 10 mM ammonium formate pH 4, gradient not specified	Pihlstrom et al., 2007
Waters Symmetry (5 µm, 250 X 4.6 mm) 1.0 mL/min	50 mM ammonium formate, formic acid pH 3.5, 70-100% acetontrile	Martinez et al., 2006
Waters Symmetry(5 µm, 250 X 4.6 mm) 1.0 mL/min	50 mM ammonium formate, formic acid pH 3.5, 70-100% acetontrile	Gil-Garcia et al., 2006
Zorbax C18 (5 µm, 250 X 4.6 mm) 0.6 mL/min	MeOH 77%-100%	Chen et al., 2007
Pyrethroid Transformation Products –only GC/MS/MS – Arrebola et al., 1999		
Phenoxyacid herbicides LC/MS/MS ESI- unless specified		
Hypersil-BDS C18, (5µm, 250 X 2.0 mm) 0.2 mL/min	A Water:MeOH:acetic acid; B MeOH:water 90:810:1 for A; 900:1 B, 0-50% B	Koppen et al., 1998
Zorbax Eclipse XDB-C18 (1.8 µm, 50 X 4.6 mm) 0.15 mL/ min	2 mM ammonium acetate, MeOH 65-90%	Raina and Etter, 2010
C-18 (5 µm, 50 to 100 mm X 2.1 mm in general) 0.2 mL/min	0.1% formic acid, Gradient ranges on column,	Dijkman et al., 2001
LiChrocart C-18, (5µm, 250 X 4.6 mm) 0.9 mL/min, APCI-	MeCN 0-65%or higher starting	
Alltima C18, (5 µm 250X4.6 mm) 0.8 mL/min split 3:1 to MS	ammonium formate, 5 mM formic acid (pH 3), 40%	Santos et al., 2000
Alltima C18, (5 µm, 250 X 4.6 mm) 1.0 mL/min split 30/970 to MS/UV	MeCN	
Hypersil C18 (5µm, 250 X 4.6 mm) 1 mL/min 50% to MS	50-95% MeOH, 1% v/v acetic acid	Baglio et al., 1999
	0.1 mM K_2HPO_4 0.2 mM TBAF, MeOH 30-75%	Crescenzi et al. ,1995
	Formic acid and ammonia –pH varied 2.9-8.4, 20-100% MeOH	Di Corcia et al., 2000
Phenoxyacid herbicide transformation products (chlorophenols) and nitro substituted phenols, phenols LC/MS/MS		
Hypersil green ENV (C-18) (150 X 4.6 mm) 1mL/min, APCI-	1% acetic acid, 25-100% gradient with 1:1 MeOH/MeCN	Puig et al. , 1997
Zorbax Eclipse XDB-C18 (1.8 µm, 50 X 4.6 mm) 0.15 mL/ min, ESI-	MeOH 2 mM ammonium acetate	Raina and Etter, 2010

Column, flow rate, ion source	Mobile phase organic modifier (MeCN – acetonitrile; MeOH-methanol) and additive	Reference
	65-90% + postcolumn addition of ammonia in MeOH	
Sulfonyl ureas LC/MS/MS ESI+ unless specified		
C6-phenyl (3 µm, 150 mm X 2.0 mm), 0.2 mL/min	0.1% formic acid, 2 mM ammonium acetate, 35% MeCN	Degenhardt et al., 2010
Zorbax Eclipse Plus C-18 (3.5 µm 150 mm X 2.1 mm)	MeCN 0.1 % formic acid, MeCN 45-60%	Fang et al., 2010
Zorbax Eclipse SB-C18 (1.8 µm, 150 X 4.6 mm) 0.6 mL/ min	0.1 % formic acid, MeCN 10-100%	Ferrer et al ., 2007
Varied 5 µm C-18 from 50 to 100 mm X 2.1 mm in general, ESI+ and ESI-	0.1% formic acid, Gradient ranges on column, 0-65%MeCN or higher starting %	Dijkman et al., 2001,
Hypersil-BDS C18, (5µm, 250X2.0 mm i.d.) 0.2 mL/min, ESI+ and ESI-	MeOH:acetic acid:water ;MeOH /water	Koppen et al., 1998
Hypersil C18 (5µm, 250 X 4.6 mm) 1 mL/min 50% to MS, ESI+ and ESI-	90:810:1 for A; 900:1 B, 0-50% B gradient formic acid and ammonia –pH varied 2.9-8.4, MeOH 20-100%	Di Corcia et al ., 2000
Sulfonyl urea transformation products LC-ESI+ or APCI+/MS/MS		
Hypersil BDS C18 (5µm, 250X2.0 mm i.d) 0.2 mL/min, ESI+ and APCI+	confirmation only with LC/MS/MS MeOH 10-100%	Bossi et al., 1999
Triazines LC/ESI+ MS/MS unless specified		
Omnisper C-18, (3 µm 150 X3 mm) 0.4 mL/min, APCI+	MeCN (85-40%)	Dagnac et al ., 2005
Vydac C18 (5µm, 250 mm X 4.6 mm) 1.0 mL/min	10 mM ammonium acetate (pH 4.5) MeOH 45-90%, or MeCN 27-78%	Steen et al.,1999
Zorbax Eclipse SB-C18 (1.8 µm, 150 X 4.6 mm) 0.6 mL/ min	0.1 % formic acid, MeCN 10-100%	Ferrer et al .,2007
Chromolith SpeedROD RP-18e (50 X 4.6 mm) varied 0.2-1.2 mL/min ESI+, 0.2-2.8 mL APCI+	MeOH 97%	Asperger et al., 2001
Genesis C18 (4 µm, 100 X 3 mm) 0.3 mL/min	MeOH, 10 mM ammonium formate pH 4, gradient	Pihlstrom et al., 2007
Supelcosil LC18 (5µm, 150 mm X 2.1 id) 0.6 mL/min for APCI+;	not specified,	
ODS (5µm, 150 mm X 2.1 id) ESI+ 0.1 mL/min	0.05% TFA, MeCN 70%	Titato et al .,2007
Alltima C18, (5 µm, 250X4.6 mm 1.0 mL/min), APCI+		
Hypersil ODS (5 µm, 250X4.6 mm 1.0 mL/min, APCI+	50-95% MeOH	Baglio et al., 1999
Synergie RP (4 µm, 50 mm X 2.00 mm), 0.6 mL/min	MeCN 15-60%	Jeannot et al., 2000
Uptispher ODB, (3 µm, 50 mm X 2 mm), 0.2 mL/min	5 mM ammonium acetate MeOH 20-80%	Muller et al., 2007
Purosphere STAR RP-18e (5 µm, 150 X 4.6 mm) 1.0 mL/ min	0.5% acetic acid, MeCN 10%-100%	Bichon et al ., 2006

Column, flow rate, ion source	Mobile phase organic modifier (MeCN – acetonitrile; MeOH-methanol) and additive	Reference
split to MS	5 mM ammonium formate 34%-90% MeOH	Banerjee et al., 2009
Triazine Transformation Products LC/ESI+ MS/MS unless specified		
Omnisper C-18 (3 µm, 150 X3 mm) 0.4 mL/min, APCI+	MeCN 85% to 40%	Dagnac et al., 2005
Hypersil ODS (5 µm, 250X4.6 mm 1.0 mL/min, APCI+	MeCN 15-60%	Jeannot et al., 2000
Vydac C18 (5µm, 250 mm X 4.6 mm) 1.0 mL/min	10 mM ammonium acetate (pH 4.5) MeOH 45-90%, or MeCN 27-78%	Steen et al., 1999
Uptispher ODB, 3 µm, 50 mm X 2 mm, id, 0.2 mL/min	0.5% acetic acid, MeCN 10%-100%	Bichon et al., 2006
Carbamates and transformation products* LC/ESI+MS/MS unless specified		
Xterra MS C18 (5µm, 100 mm 2.1 mm) 0.2 mL/min	0-75% MeOH with 0.01 % formic acid	Goto et al., 2006*
Zorbax Eclipse SB-C18 (1.8 µm, 150 X 4.6 mm) 0.6 mL/ min	0.1 % formic acid, MeCN 10-100%	Ferrer et al., 2007*
Atlantis C18 (5 µm, 100 X 2.1 mm) 0.2 mL/ min		
Xterra MS C18 (3.5 µm, 150 X 2.1 mm) 0.2 mL/ min	0.01%formic acid MeOH 5%-90%	Hernandez et al, 2006
C12 (4 µm, 150 X 2.0 mm) 0.25 mL/min	0.1% formic acid, MeCN 10-90%	Botitsi et al., 2007*
Supelcosil LC18 (5µm, 150 mm X 2.1 id) 0.6 mL/min for APCI+;	5 mM ammonium formate, MeOH 5%-90%	Chung and Chan, 2010
ODS (5µm, 150 mm X 2.1 mm) 0.1 mL/min ESI+	0.05% TFA, MeCN 70%	Titato et al ., 2007
Genesis C18 (4 µm, 100 X 3 mm) 0.3 mL/min		
Genesis C18 (4 µm, 100 X 3.0 mm) 0.2 mL/ min, APCI+	10 mM ammonium formate pH 4, MeOH gradient not specified	Pihlstrom et al., 2007
Aqua C18 (3µm, 150 mm X 2. mm), 0.3 mL/min	10 mM ammonium formate, pH 4, MeOH 0-90%,	Jansson et al., 2004*
Alltima C18 (5 µm 250mm X 4.6 mm) 1.0 mL/min	flush with MeCN 80% each run	
Synergie RP (4 µm, 50 mm X 2.00 mm), 0.6 mL/min	5 mM ammonium formate, MeOH 0-90%	Paya et al., 2007
Polaris C18 3 µm, 150 X 2.0 mm id, 0.2 mL/min	50-95% MeOH	Baglio et al., 1999
	5 mM ammonium acetate MeOH 20-80%	Muller et al., 2007
Luna C18 (5 µm, 150 X 4.6 mm), 0.4 mL/min	2 mM ammonium formate, pH 2.8 MeOH 20-85%	Martinez Vidal et al., 2005
Alltima C18 (5 µm, 150 X 3.2 mm) 0.3 mL/ min	10 mM Ammonium formate MeOH 35-90%	
XTerra MS C18 (3.5 µm, 150 X 2.1 mm) 0.2 mL/ min	5 mM ammonium formate, MeOH 25-95% 0.1%	Pico and Kozmutza, 2007*
Purosphere STAR RP-18e (5 µm, 150 X 4.6 mm) 1.0 mL/ min	formic acid, MeCN 10%-90%	Hiemstra et al., 2007
split to MS, ESI+	5 mM ammonium formate 34%-90% MeOH	Economou et al., 2009
		Banerjee et al., 2009
Phenylureas LC/ESI+/MS/MS unless specified		
Microsphere 3 µm LC-LC (50 X4.6 mm – 100 X 4.6) 1.0 mL/min split to MS to 0.5 mL/min, APCI+	MeOH 10-60%	Van der Heeft et al., 2000

Column, flow rate, ion source	Mobile phase organic modifier (MeCN – acetonitrile; MeOH-methanol) and additive	Reference
Omnisper C-18, (3 µm 150 X3 mm) 0.4 mL/min	MeCN (85-40%)	Dagnac et al., 2005
Vydac C18 (5µm, 250 mm X 4.6 mm) 1.0 mL/min	10 mM ammonium acetate (pH 4.5)	Steen et al., 1999
Zorbax Eclipse SB-C18 (1.8 µm, 150 X 4.6 mm) 0.6 mL/ min	MeOH 45-90% or MeCN 27-78%	
Atlantis C18 (5 µm, 100 X 2.1 mm) 0.2 mL/ min	0.1 % formic acid, MeCN 10-100%	Ferrer et al., 2007
Supelcosil LC18 (5µm, 150 mm X 2.1 mm) 0.6 mL/min for APCI+;		
ODS (5µm, 150 mm X 2.1 mm) ESI+ 0.1 mL/min	0.01%formic acid gradient MeOH 5%-90%	Hernandez et al., 2006
Genesis C18 4 µm, 100 X 3 mm) 0.3 mL/min, ESI+	0.05% TFA MeCN 70%	Titato et al ., 2007
Aqua C18 (3µm, 150 mm X 2. mm), 0.3 mL/min, ESI+	MeOH, 10 mM ammonium formate pH 4, gradient	Pihlstrom et al., 2007
Alltima C18 (5 µm 250 mm X 4.6 mm) 1.0 mL/min, APCI+	not specified	
Hypersil ODS (5 µm 250 mm X 4.6 mm) 1.0 mL/min, APCI+	5 mM ammonium formate, MeOH 0-90%	Paya et al., 2007
Polaris C18 (3 µm, 150 mm X 2.0 mm), 0.2 mL/min, ESI+	50-95% MeOH	Baglio et al., 1999
	MeCN 15-60%	Jeannot et al., 2000
Uptispher ODB (3 µm, 50 mm X 2 mm), 0.2 mL/min, ESI+	2 mM ammonium formate, pH 2.8 MeOH 20-85%	Vidal et al., 2005
Genesis C18 (4 µm, 100 X 3.0 mm) 0.2 mL/ min, ESI+	0.5% acetic acid, MeCN 10%-100%	
		Bichon et al., 2006
Alltima C18 (5 µm, 150 X 3.2 mm) 0.3 mL/ min, ESI+	10 mM ammonium formate, pH 4, MeOH 0-90%,	
Discovery C18 ((5.0 µm, 150 X 3 mm) 0.5 mL/ min, ESI+ and ESI-	flush with MeCN 80% each run	Jansson et al., 2004
	5 mM ammonium formate, 25-95% MeOH	
	MeOH 20-100%	Hiemstra et al., 2007
		Zrostlikova et al. 2003
Phenylurea Transformation Products		
Omnisper C-18, (3 µm 150 X3 mm) 0.4 mL/min, ESI+	MeCN (reverse 85% decreased to 40%, water 15% to 100%)	Dagnac et al ., 2005
Vydac C18 (5µm, 250 mm X 4.6 id) 1.0 mL/min, ESI+ and ESI-	10 mM ammonium acetate (pH 4.5)	Steen et al ., 1999
Chromolith SpeedROD RP-18e (50 X 4.6 mm) varied 0.2-1.2	MeOH 45-90% or MeCN 27-78%	
mL/min ESI+, 0.2-2.8 mL APCI+	97% MeOH, (note lower flows when % MeOH	Asperger et al., 2001
Uptispher ODB, 3 µm, 50 mm X 2 mm, id, 0.2 mL/min, ESI+	decreased	
Genesis C18 (4 µm, 100 X 3.0 mm) 0.2 mL/ min, ESI+	0.5% acetic acid, MeCN 10%-100%	Bichon et al., 2006
	10 mM ammonium formate, pH 4, MeOH 0-90%, flush with MeCN 80% each run	Jansson et al., 2004

Column, flow rate, ion source	Mobile phase organic modifier (MeCN – acetonitrile; MeOH-methanol) and additive	Reference
Azoles (Triazoles and benzimidazoles) and triazole transformation products* LC/MS/MS ESI+ unless specified		
Synergi Hydro HP (4 µm, 150 X 2 mm) 0.25 mL/ min	MeCN 0.1 % formic acid 50%-100%	Trosken et al., 2005
Symmetry C18 (5.0 µm, 250 X 4.6 mm) 0.3 mL/ min	0.2% formic acid, MeOH 50% to 82%	Schermerhorn et al., 2005*
Discovery C18 (5.0 µm, 150 X 3 mm) 0.5 mL/ min	MeOH 20-100%	Zrostlikova et al., 2003
Zorbax Eclipse SB-C18 (1.8 µm, 150 X 4.6 mm) 0.6 mL/ min	0.1 % formic acid, MeOH 10-100%	Ferrer et al., 2007
Supelcosil LC18 (5µm, 150 mm X 2.1 mm) 0.6 mL/min for APCI+;	0.05% TFA, MeCN 70%	Titato et al., 2007
ODS (5µm, 150 mm X 2.1 mm) 0.1 mL/min, ESI+		
Hypersil ODS (5 µm, 250mm X 4.6 mm) 1.0 mL/min		
Genesis C18 (4 µm, 100 X 3 mm) 0.3 mL/min	MeCN 15-60%	Jeannot et al ., 2000
Aqua C18 (3µm, 150 mm X 2.0 mm), 0.3 mL/min	10 mM ammonium formate pH 4, MeOH	Pihlstrom et al., 2007,
Synergie RP (4 µm, 50 mm X 2.00 mm), 0.6 mL/min	5 mM ammonium formate, MeOH 0-90%	Paya et al. , 2007
Alltima C18 (5 µm, 150 X 3.2 mm) 0.3 mL/ min	5 mM ammonium acetate MeOH 20-80%	Muller et al., 2007
Atlantis C18 (5 µm, 100 X 2.1 mm) 0.2 mL/ min	5 mM ammonium formate, 25-95% MeOH	Hiemstra et al., 2007
XTerra MS C18 (3.5 µm, 150 X 2.1 mm) 0.2 mL/ min	0.01%formic acid, MeOH 5%-90%	Hernandez et al., 2006
Purosphere STAR RP-18e (5 µm, 150 X 4.6 mm) 1.0 mL/ min split to MS	0.1% formic acid, MeCN 10%-90%	Economou et al., 2009
	5 mM ammonium formate 34%-90% MeOH	Banerjee et al, 2009

Table 3. LC/MS/MS Separation Conditions by Chemical Class

analyzed separately from neutrals the sulfonylureas along with phenoxyacid herbicides are analyzed together with ESI- (Dijkman et al., 2001; Koppen et al., 1998; Di Corcia et al., 2000). Similarly triazines and atrazine metabolites may be done with ESI+ rather than APCI+ due to the diversity and sensitivity of ESI+ for other chemical classes that are analyzed simultaneously with only a small loss in sensitivity.

Common co-elution problems that must be resolved prior to detection exist for a number pesticides either within the same chemical class or for multi-class residue methods. Table 3 shows the vast majority of LC/MS/MS methods utilize C18 columns in order to achieve the desired selectivity for the separation. A few separations have taken advantage of different selectivity from ZIC-pHILIC, C6-phenyl, C8, or C12 (Raina and Sun, 2008; Degenhardt et al., 2010; Crnogorac et al., 2007; Blasco and Pico, 2004). In general methods provide the necessary resolution for compounds with the same SRM transitions. For chloracetanilides care must be taken with ESI+ as acetochlor, metalochlor, and alachlor can co-elute and acetochlor and alachlor which both have molecular mass of 269.5 g/mol have the same precursor ions with ESI+ (m/z 270 or 292) from [M+H]+ and [M+Na]+ (Dagnac et al., 2005; Ferrer et al., 2007). This can be resolved by adequate chromatographic resolution prior to detection or switching to APCI + where in addition to monitoring [M+H]+ for both the 224 and 256 precusor ion can be monitored for acetochlor, while for alachlor 162 and 238 can be monitored (Dagnac et al., 2005). Phenoxyacid herbicides and sulfonylureas also require adequate chromatographic resolution. Niocsulfuron, ethametsulfuron-methyl, bensulfuron-methyl have a similar parent ion (411.2, 411.8, 411.5) but can be separated using C6-phenyl column (Degenhardt et al., 2010). With ESI- the phenoxy acid herbicides MCPA, mecoprop, MCPB have a common confirmation SRM transition of 140.9 > 105.2 which is also the quantitative SRM for degradation product, chloromethylphenol. In addition, 2,4-D, dichlorprop, and 2,4-DB also have the sample confirmation SRM of 160.9 > 124.7 which is also the quantitative SRM for dichlorophenol (Raina and Etter, 2010). Separated of these phenyoxyacid herbicides can be achieved using a short C18 column with methanol gradient and mobile phase containing 2 mM ammonium acetate (Raina and Etter, 2010). The list of pesticides that are required for screening or quantitative analysis and those potentially present in samples should determine your requirements for detection and separation.

Degradation products by LC/MS/MS such as more chlorophenols (degradation products of phenoxyacid herbicides) and nitrophenols are move sensitive with ESI^-, while other phenols are more sensitive with APCI (Reesmtsma et al., 2003; Raina and Etter, 2010). However these chloro and nitrophenols have also been analyzed successfully by APCI (Silgoner et al., 1997). OP degradation products including 3,5,6-trichloro-2-pyridinol, diethyl phosphate, 2-isopropyl-6-methyl-4-pyrimidinol, malathion monocarboxylic acid, and OPoxons (Raina and Sun, 2008) as well as OP sulfones and sulfoxides (Jansson et al, 2004; Chung and Chang, 2010, Hiemstra et al., 2007; Economou et al, 2009) are more sensitive with LC-ESI^+/MS/MS and some OPs observe a drastic loss in sensitivity with an APCI source at high flow rates (Asperger et al., 2001). Degradation products of triazines and phenylureas have been done by LC-$APCI^+$/MS/MS but for triazines lower MDLs can be achieved with GC-EI/MS/MS (Dagnac et al., 2005; Goncalves et al., 2006). Diuron and its degradates 3,4-dichlorophenyurea and 2,4-dichlorophenylurea also observed better sensitivity in methanol compared to acetonitrile mobile phases and switching the organic modifier has greater impact for these degradation products when using ESI+ than ESI- (Steen et al., 1999). Chloroacetanilide metabolites have been analyzed by LC-ESI^-/MS/MS (Gomides Freitas et al., 2004). LC-ESI^+/MS/MS has also been used for analysis of a number of carbamate and

azole degradation products the most common of which for carbamates are aldicarb sulfone and sulfoxide, and 3-hydroxycarbofuran (Goto et al., 2006; Ferrer et al., 2007; Botitsi et al., 2007; Jansson et al., 2004; Pico and Kuzmutza, 2007; Schermerhorn et al., 2005). A more extensitive list of dithiocarbamates and their transformation products have only been analyzed using LC/MS methods (Crnogorac et al., 2007; Blasco and Pico, 2004) with triallate routinely analyzed by GC/MS methods. Transformation products of pyrethroids currently have no LC/MS or LC/MS/MS.

Selection of the organic modifier (generally methanol or acetonitrile), and the presence or absence of formic or acetic acid, and salts can greatly impact the ionization of a pesticide and its sensitivity. Some pesticides have the potential to form sodium or ammonium adducts in positive ion mode, or acetate or formate adducts in negative ion mode with an API source. The formation of adducts decreases the abundance of the protonated or deprotonated molecular ion and there is greater potential for adduct formation with ESI than APCI. In general positive ion mode is more prone to adduct formation than negative ion mode. Methanol mobile phases have a higher degree of adduct formation particularly for sodium adducts relative to acetonitrile although many pesticides see better ionization in methonal than acetonitrile. The formation of sodium adducts in mobile phases with methanol can be reduced or suppressed by the addition of ammonium or hydrogen ions. The most common additives for this purpose are ammonium acetate (2-10 mM), ammonia, acetic acid (1%), formic acid (0.05-.2 v/v%), or trifluoroacetic acid (TFA, 0.05 v/v%) (see Table 3). The impact of adjustment of the pH of the mobile phase on chromatographic resolution for closely eluting pesticides with the same SRM transitions or parent ions should be considered along with the impact of changing pH on sensitivity particularly for acidic pesticides (Raina and Etter, 2010). In practice a balance must be met between separation needs and MS sensitivity for the range of pesticides and transformation products under study and can vary significantly even for those of the same chemical class. Table 3 shows that there is a large diversity in additives and organic solvent used even within the same chemical class.

For OPs ESI+ is superior and generally [M+H]+ is observed as the parent ion even in mobile phases only containing methanol. The presence of both ammonium and formic acid was shown to give optimal sensitivity or OPs, OP oxons, and other OP transformation products (Raina and Sun, 2008). For OP sulfone and sulfoxide transformation products sodium adducts can form in mobile phases containing only methanol or methanol with formic acid. Switching either to acetonitrile with formic acid or addition of a salt such as ammonium formate (or ammonium acetate) suppresses the formation of adducts (Hiemstra et al., 2007; Economou et al., 2009; Jansson et al., 2004; Raina and Sun, 2008; Muller et al., 2007).

Individual phenylureas and carbamates are also more likely to form sodium adducts as compared to triazines. Aldicarb, 3-hydroxycarbofuran, aldicarb sulfone, and aldicarb sulfoxide form sodium adducts in a methanol mobile phase with 0.01% formic acid (Hernandez et al., 2006). Switching to acetonitrile reduces adduct formation for the sulfone and sulfoxide of aldicarb but aldicarb is still present as sodium adduct (Botitsi et al., 2007). Addition of ammonium formate (Pihlström et al., 2007; Pico and Kozmutza, 2007) or ammonium acetate leads to suppression or reduction in the formation of the sodium adduct. Depending upon the ammonium ion concentration aldicarb may form the ammonium adduct (Pico and Kozmutza, 2007) as well as oxamyl (Pihlström et al., 2007). In a mobile phase of methanol with 5 mM ammonium formate, methiocarb sulfone and ethiocarb sulfone both form the ammonium adduct as well as the protonated molecular ion with SRM transitions of 275→122 and 258→122 for methiocarb sulfone and 275 →107 and 258→107 for

ethiocarb sulfone (Hiemstra and de Kok, 2007). Aldicarb does not form [M+H]+ under most mobile phase conditions so either [M+Na]+ (213→116 or 213→89) or [M+NH4]+ (208 →116 or 208→89) are used for SRM transition under the proper mobile phase conditions (Table 3). Other carbamates generally form [M+H]+ regardless of mobile phase composition.

With ESI+ the most commonly monitored phenylureas produce [M+H]+ under varying mobile phase conditions and the mass selected may be utilizing 35 or 37 Cl isotopes. A few phenylureas such as isoproturon and chlortoluron can form sodium adducts and consequently ammonium formate or ammonium acetate may be added to the mobile phase (Table 3). Often these additives are required more for other chemical classes that are analyzed along with the phenylureas such as carbamates. The more commonly analyzed phenylureas such as diuron and linuron do not require addition of additives. Phenylureas sensitivity is impacted by the organic solvent selected with methanol having significant improved response in ESI+ for phenylureas and their degradation products (Steen et al., 1999). In addition, the use of a higher percentage of methanol to achieve the desired separation conditions also improves sensitivity for both for ESI+ and ESI- as sensitivity improves with the percentage of organic modifier. The reduction in signal intensity for the degradation products when switching organic modifier to acetonitrile was not as great in ESI- (Steen et al., 1999). For chloracetanilides and phenylureas using APCI also reduces potential for sodium adduct formation (Dagnac et al., 2005).

Sulfonylureas also observe predominately [M+H]+ but can form sodium adducts as has been observed for sulfometuron-methyl with ESI+ with acetonitrile-aqueous 0.1%formic acid mobile phase (Dijkman et al., 2001). Consequently separation conditions generally contain both 0.1% formic acid and 2 mM ammonium acetate (Degenhardt et al., 2010) and if an organic modifier is used it is generally acetonitrile as shown in Table 3. In negative ion mode adduct formation is not observed. For phenoxy acid herbicides the presence of formic acid will result in decreased abundance of [M-H]- (Raina and Etter, 2010) while for many neutral pesticide chemical classes it will improve the ionization so typically is added at ~0.1-0.2 v/v%. For phenoxyacid herbicides methanol is generally choosen as the organic modifier as it improves the efficiency of ionization and the sensitivity improvement relative to acetonitrile mobile phases ranges from 3-5 orders of magnitude (Raina and Etter, 2010). OPs and their degradation product signal intensity is also much better with methanol (Raina and Sun, 2008) and it has been shown as the % of methanol increases the signal intensity improves as was also observed for phenylureas (Steen et al., 1999). This suggests an advantage in using gradient elutions with methanol rather than acetonitrile for these pesticides as a higher percentage of organic solvent will be required to achieve the same chromatographic resolution during the separation.

Users must also be aware of particularly for gradient elution whether the pesticides of interest are soluble over the range of mobile phase conditions for the separation. For some chemical classes or multi-residue analysis gradient elution programs will start at a very low percentage of methanol or acetonitrile and peak broadening or distortion and even carry-over and increasing MS background signal may be observed. Reduced sensitivity and reproducibility over time may become apparent due to low solubility of some of the analytes in mobile phases of high aqueous content. For these challenging chemical classes which are more prone to build-up a flushing step with high concentration of acetonitrile is used prior to re-equilibration of the column to reduce carry-over issues.

A number of LC/ESI+MS methods for pyrethroids have been developed (Chen and Chen, 2007; Gil-Garcia et al., 2006; Martinez et al., 2006) which have comparable MDLs to GC/EI-

MS methods (Yoshida, 2009). For halogenated pyrethroids GC/NCI-MS provides the best sensitivity (Feo et al., 2010). These methods have largely focused on LC/MS where either the protonated molecular ion or ammonia adduct are predominately observed in mobile phases containing ammonium acetate or formate (Table 3). There is little structural information available and only a few multi-residue LC/MS/MS methods contain selected pyrethroids (Pihlstrom et al., 2007). In addition, the only available methods for analysis of pyrethroid metabolites currently require derivatization with GC/MS analysis.

There are a number of approaches that have been used to further improve sensitivity of LC/MS/MS methods. When separation needs do not permit changes in mobile phase composition to improve MS sensitivity then alternatively post-column reagents may be added using an additional pump (Raina and Etter, 2010; Carabias-Martinez et al., 2004) at lower flow rates (eg 50 µL/min) such that the total flow is still optimal for the ESI or APCI used for the analysis. Bases have been used as post-column reagents to enhance ionization including ammonia, trimethylamine, tris(hydroxymethyl) aminomethane, and 1,8-diazabicyclo-(5,4,0) undec-7-en (Raina and Etter, 2010; Carabais-Martinez et al., 2004; Marchese et al., 2002; Gomides Freitas et al., 2004). This approach has been used to improve the sensitivity of transformation products of phenoxyacid herbicides with ammonia in methanol (Raina and Etter, 2010). Reagent addition should consider the change in solvent composition as this may also alter sensitivity with most pesticides observing enhanced sensitivity with higher percentages of organic modifiers such as methanol.

Similar to GC/MS methods large volume injections have also been used for LC/MS/MS applications although not specifically for pesticides. Direct on-column loop injection of 2 mL of water samples to a standard C18 column with LC/APCI+MS/MS achieved sub-µg/L range detection (Speksnijder et al., 2010). For urine samples an on-line LC-MS approach was used where the sample is pumped into the LC system and diluted through a mixing Tee with ammonium acetate after which it is loaded onto a restricted access material (RAM) pre-column while the analytical column equilibrates. The analytes of interest are then back-flushed to transfer them to the analytical column followed by a typical gradient elution (Liu et al., 2008). The use of the RAM pre-column enables matrix removal of proteins as it retains only low molecular weight analytes. Matrix effects with API sources can lead to suppression or enhancement of analyte response due to co-eluting matrix constituents (Niessen et al., 2006). The choice of solvent used in extraction procedures can reduce matrix impacts with ethylacetate or acetonitrile often preferred for QuEChERS methods. If matrix suppression/enhancement can not be eliminated by sample preparation procedures prior to LC/MS/MS then deuterium or carbon-C13 labeled internal calibrations should be used for stable isotope dilution. If sufficient levels are available sample dilution or infinite dilution (matrix-free solution) can be utilized with typical dilution factors of 0.05 or 0.025 (Kruve et al., 2009). UV detection can also be utilized to identify co-eluting matrix issues requiring improvements in chromatography which can be achieved either with other column choices or comprehesive LCXLC (Hajšlová and Zrostíková, 2003). LC/TOF-MS has also gained considerable interest for confirmation of pesticides or transformation products with exact mass measurements for those applications where LC/MS/MS may not have the required sensitivity from the confirmation SRM transition (Portolés et al., 2009; Kuster et al., 2009). Pesticides such as aldicarb, diuron, linuron, aldicarb sulfone and sulfoxide can be distinguished and quantified by exact mass measurements with mean error of 2.3 ppm (Maizels and Budde, 2001).

4. Conclusion

GC/MS, GC/MS/MS, LC/MS/MS, and in some cases LC/MS methods are required to cover the full range of pesticide chemical classes and their transformation products. No one method can meet the needs of all the current pesticide chemical classes. There are also a number of chemical classes including phenoxyacid herbicides, pyrethroids, triazines, acetanilides, and azoles with both GC and LC methods coupled to mass spectrometry which may meet the needs of users. GC/MS or GC/MS/MS multiresidue methods with NCI are recommended for use with OCs, most OC transformation products, trihalomethylthio fungicides, and if a wide range of OPs require analysis for the best sensitivity and selectivity. Pyrethroids are recommended to be done with GC/EI-MS methods for those which are not chlorinated and for their derivatized degradation products. NCI may be also used for added sensitivity and selectivity for those pyrethroids that are halogenated. When developing GC/MS multiresidue methods it is also essential to have a GC/EI-MS method which is more suitable for acetanilides, triazines, atrazine transformation products, some OCs and a few OP pesticides. For some pesticides or transformation products the second confirmation ion or SRM transition may not be sensitive enough and both NCI SIM and NCI SRM methods or NCI and EI SIM methods may be required. If sufficient sample concentration is available then EI SRM methods may also be useful for a wide range of these pesticides. If sample concentrations are lower but added confirmation beyond these methods is required then GC/TOF-MS or GCXGC/TOF-MS is an alternative. In general it is not recommended to analyze azoles with GC/MS methods due to often higher detection limits as compared to LC/MS/MS methods and thermal instability. As a strategy LC/MS/MS methods should be utilized for carbamates, phenylureas, sulfonyl ureas, azoles, and transformation products from these chemical classes. If a laboratory desires to minimize the need for derivatization then phenoxyacid herbicides and their degradation products can also be accomplished by LC/MS/MS and postcolumn reagent addition can be used if added sensitivity is required for the chlorophenol transformation products. If a multiresdiue LC/MS/MS method is developed for these pesticide classes then the inclusion of chloroacetanilides, triazines, and transformation products from these chemical classes should also be considered. As with GC/MS methods, one ionization method can not be utilized for all chemical classes for LC/MS/MS methods and matrix impacts should be assessed to determine if there is an advantage in utilizing an alternative ionization mode to minimize impacts from interferences. For example one may consider analyzing sulfonylureas and acetanilide transformation products with phenoxyacid herbicides with LC/ESI-MS/MS, while analyzing phenylureas, carbamates, azoles, chloroacetanilides, triazines, OPs and transformation products of OPs, carbamates, and some sulfonylureas by LC/ESI+MS/MS, or LC/APCI+MS/MS method for triazines, phenylurea and their transformation products for best sensitivity. LC/APCI+/MS/MS can also be used to resolve matrix issues or co-elution problems for OPs, chloroacetanilides, phenylureas, carbamates, triazines, sulfonyl ureas (if phenoxyacid analysis is not required). Inclusion of transformation products will likely be the largest factor in selection of multiple methods with ESI and APCI in positive and negative mode as some transformation products require alternative ionization methods from that used for parent pesticides for adequate sensitivity. In addition the dithiocarbamates with the exception of triallate which can be included with standard GC/MS methods should be done with separate LC/MS methods either for anionic or neutral dithiocarbamates and their transformation products. Although their analysis

requires a separate LC/MS method this is a significant improvement over prior GC methods that were not specific to individual dithiocarbamates and were laborious. If no GC/MS methods are necessary then pyrethroids may be included in LC methods. Currently the advantages of new column choices, GCXGC or LCXLC, large volume injections, on-column clean-up, post-column reagent addition, and TOF-MS are underutilized to resolve matrix and confirmation needs and should be considered in future method development.

5. References

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6

Fast Gas Chromatography and Its Use in Pesticide Residues Analysis

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1. Introduction

Pesticides have been worldwide used for the protection of food crops against pests and diseases. It is common that residues of these pesticides occur in food products, especially agricultural commodities. Adverse effects on human health of pesticides residues remaining in food after they are applied to food crops are generally known. Possible health risk due to pesticide residues in the diet has deeply modified the strategy for the crop protection, with emphasis on food quality and safety. The widespread concern for the health of society led to the strict regulation of maximum residue limits (MRLs) of pesticide residues in food commodities. There are various organizations that set maximum residue limits (MRLs), such as European Commission (EC), Codex Alimentarius or national governments in Australia, Canada, Japan, USA, etc. Individual limits for different active substance per food commodity combinations are being set by EC within the range of 0.0008-50 $mg.kg^{-1}$ (Directive 91/414/EEC). Newly discovered ecotoxicological problems, particularly the knowledge on endocrine disrupting effects (Colborn et al., 1993; Lintelmann et al., 2003) related also to pesticide residues, emphasise the acute requirement of analytical methods development with increased sensitivity and reliability for monitoring, confirmation and quantification of lower residue levels. Analysis close to these levels corresponds to the ultra-trace analysis. This calls for urgent attention in two areas: (a) legislative requirements continuously decreasing the maximum acceptable concentration levels in food, and (b) the apparent importance of methods development in the area of pesticide residues analysis. The urgent requirement for low-level analyses promotes also contribution to the science – in the field of separation methods for ultra-trace analysis of organic pollutants in complex mixtures. The method development heads to speeding up the analysis (what leads to reduction of financial demands) while preserving the efficiency of conventional approaches or getting even better efficiency. In pesticide residues analysis additionally there is ever increasing interest to analyse as many analytes as possible in a single analysis. In the case of semivolatile pesticide residues analysis gas chromatography (GC) still plays an important role. Scientifically valid methods for the analysis at low concentration levels are currently still often very close to limits of detections (LODs). The most efficient approach to pesticide analysis involves the use of multiclass, multiresidue methods (MRMs). The sample preparation procedure should be taken into consideration together with the chromatographic analysis and detection in many aspects, mainly in limit of quantifications (LOQs) and selectivity. In multiresidue pesticides analysis used for an inspection of the

presence and/or violation of MRLs in a great number of pesticide residues, usually several chromatographic runs are necessary for qualitative and quantitative analyses. Positive samples exceeding the MRLs value require a subsequent confirmation. Nowadays, the use of mass spectrometry as universal detection method that has identification capability with mass spectral information and high selectivity with extracted ion trace or selected ion monitoring seems to become indispensable for identification purposes.
Gas chromatography – mass spectrometry (GC-MS) with electron ionization (EI) and the combination of liquid chromatography (LC) with tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) are identified as techniques most often applied in multi-residue methods for pesticides at present (Alder et al., 2006). For GC-amenable semivolatile pesticides GC methods are still preferred over LC (liquid chromatography) methods due to higher resolution. After a major advance of recent years in ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), which have been demonstrated to reliably quantify and identify hundreds of pesticides in less than 10 min (Romero-Gonzalez et al., 2008), the establishment of faster GC methods instead of conventional GC methods reaching separation in 25-45 min is a necessary continuation of the development. Especially fast GC techniques satisfy the present-day demands on faster and cost-effective analysis (Korytár et al., 2002; Dömötörová & Matisová, 2008). Analysis time and the cost are the most important aspects that should be considered in the choice of analytical method in routine application.
This contribution is devoted to the fast gas chromatography in pesticide residues analysis. Classification according to the GC speeding-up strategies is mentioned and the main part of the chapter is devoted to the fast GC in the analysis of pesticide residues with the use of narrow-bore columns (internal diameter I.D. <0.2 mm). Specificity of pesticide residues analysis as well as problems associated with analysis of pesticides in general are discussed. Sample preparation mainly from the point of view of time requirements and feasibility for fast GC is briefly outlined. Special attention to the selectivity enhancement by the negative chemical ionization approach is devoted. Applicability of fast GC for pesticide residues in real-life samples is demonstrated.

2. Classification of faster GC

During the last decade fast GC has acquired a real importance in the pesticide residues analysis. Classification of faster GC based on speed enhancement factor was suggested by Dagan & Amirav, 1996 and the terms fast GC, very fast GC and ultrafast GC are commonly used at present days. The speed enhancement factor shows the gain in speed compared to conventional capillary GC. Van Deursen et al., 2000 suggested a classification based on the peak half width and the total analysis time. Every reduction of analysis time results in an identical reduction of the chromatographic zone width due to the shorter residence time of the components in the column. It is reasonable to use a definition that takes account of the degree of separation per time. In classification, valuable information based on a peak width is very useful also from the point of view of the major requirements for instrumentation. The summarisation of both approaches to the classification of faster GC is in Table 1.
Nowadays fast GC can be performed on commercial gas chromatographs, which are standard equipped with high-speed injection systems, electronic gas pressure control, rapid oven heating/cooling and fast detection (Korytár et al., 2002, Matisová & Dömötörová, 2003). Fast GC technique has been established to real sample analysis very slowly. In the last

few years the number of publications offering application of fast GC in real analysis has increased (Dömötörová & Matisová, 2008, Donatao et al., 2007).

Type of analysis	Analysis time range	Peak width at half height	SEF	Efficiency (N)
fast	minutes	1-3 s	5-30	≥comparable to conventional GC
very fast	seconds	30-200 ms	30-400	25 000
ultra fast	sub-seconds	5-30 ms	400-4000	7 000

SEF – speed enhancement factor, N – plate number

Table 1. Classification of faster capillary GC.

3. Strategies of fast GC

Numerous options exist for pushing the speed of capillary gas chromatography as it was summarized in a few reviews (Matisová & Dömötörová, 2003; Dömötörová & Matisová, 2008; Maštovská & Lehotay, 2003). The most often approaches use i) narrow-bore columns, ii) fast temperature programming, iii) low-pressure gas chromatography (LP-GC), or iv) comprehensive GCxGC.

3.1 Narrow-bore columns

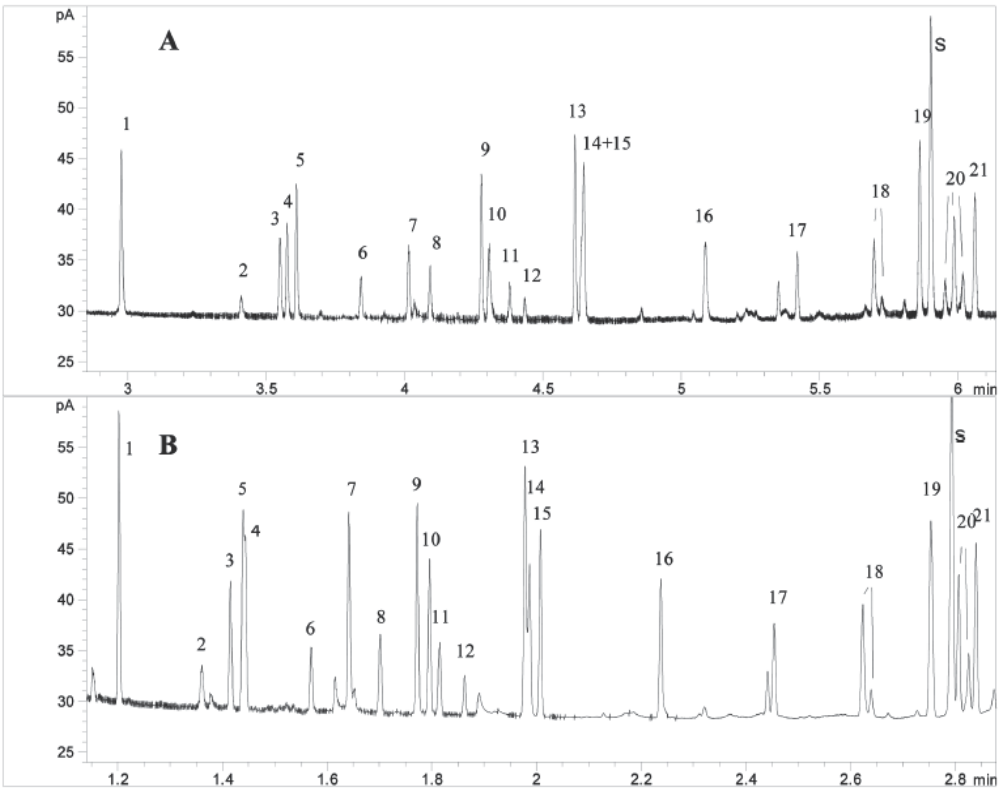
According to recent review by Donato et al., 2007, the wide majority of high-speed GC applications described in literature have been carried out by means of reduced columns I.D. (internal diameter). The reduction of column I.D. is usually combined with strategies as: changing column geometry (column shortening approach, thinner stationary phase), or its operating parameters (higher heating rates, above optimum carrier gas flow rate and in some cases usage of hydrogen as a carrier gas) what corresponds to the theoretical concept for the practical optimization of analysis speed of routine fast GC proposed by Klee & Blumberg, 2002. Theory of capillary gas chromatography has already demonstrated that the application of narrow-bore capillary columns has a number of advantages. Reduction of the column diameter can increase the efficiency (and consequently, the resolution) and drastically reduces analysis times. When the I.D. is reduced, optimal average linear velocity is also faster, what additionally contributes to the higher speed of analysis. The penalty to be paid is a much lower sample capacity which may result in higher LODs and LOQs and related higher maintenance frequency is needed.

The list of latest applications of narrow-bore fast GC for analysis of pesticide residues in food samples is given in Table 2. Various groups of pesticides were investigated by fast GC, for instance carbamate, organochlorine, organophosphorous, organothiophosphate, organotin, triazine and others. Prior to GC analysis, pesticide samples (standard solutions or extracts) were injected to the system using split, splitless, on-column or PTV (programmed temperature vaporization) injector mainly in cold splitless or in solvent vent mode. Helium and exceptionally hydrogen were the most frequently used carrier gases. MS detector in SIM mode is used preferably, specific and selective detectors as ECD (electron capture detector) and universal as FID (flame ionization detector) are also used.

Inlet systems and their operation have a significant effect on the performance of GC systems in pesticide residues analysis. The most frequently used technique of injection in trace analysis is a classic hot splitless injection. This injection technique has been employed in GC analysis due to its robustness. It has some restrictions such as small sample capacity and it may have a negative affect on results of quantitative analysis of pesticide residues, including discrimination, adsorption and degradation of analytes, which can subsequently influence the sensitivity. It was shown by Kirchner et al., 2004 that for the compounds with a broad range of volatilities and polarities good solute focusing and repeatability of the peak area measurements was obtained. Additionally, the pre-column to protect the analytical column from excessive contamination was suggested. However, PTV injector provides the best protection against effects of co-extracted compounds and operating in solvent vent mode allows even larger sample volume introduction resulting in excellent LOQs. It significantly eliminates matrix effects by releasing high-boiling co-extracted compounds through the split vent and/or trapping in a liner. Hada et al., 2000 showed that PTV with solvent vent mode was useful for large-volume injection (40 µl) into a narrow-bore capillary column because the injected solvent volume could be reduced to less than 2 µl. The introduction of a large sample volume is a simple and efficient way to increase sensitivity and useful way to analyze low-level concentrations. This approach is utilisable mainly for relatively "clean" matrices.

For a number of reasons (as sample capacity, inlet pressure values required, temperature-programmable rates), 0.1 mm I.D. columns seem to represent the current limit for the routine use (Matisová & Dömötörová, 2003). Properties of two narrow columns with I.D.s of 0.15 and 0.1 mm (15 m length and 0.15 µm film thickness, resp. 10 m length and 0.10 µm film thickness) compared Dömötörová et al., 2006 with regards to their advantages, practical limitations and applicability for fast GC on commercially available instrumentation. The two columns have the same phase ratio and the same separation power (length to I.D. ratio) to allow the method translation with preserved resolution (Klee & Blumberg, 2002). 0.1 mm I.D. column provided speed gain of 1.74 and significantly narrower peaks, but all other parameters investigated were better for 0.15 mm I.D. column concerning more efficient sample transfer from inlet to the column using splitless injection. Comparison of pesticides separation on columns with different I.D.s is shown in Fig. 1. Better sample capacity (3 times higher for 0.15 mm than for 0.10 mm I.D. column) resulted in improved ruggedness (up to 450 matrix sample injections with acceptable performance of analytical column (Kirchner et al., 2005 *a*) and simpler fast GC- MS method development. The use of 0.1 mm I.D. column in comparison to conventional one increased the detection limit as the peaks become sharper (Kempe & Baier, 2002).

Trends in GC are ever-increasing need for positive identification and for more flexible methods that enable analysis of a wide variety of samples in one system. These trends clearly result in the need for MS detection (van Deursen et al., 2000), because it enables structural elucidation for analyte identification. To obtain the low LODs and LOQs required for regulation purposes, selected ion monitoring (SIM) must be used. Unfortunately, when this sensitive mode of detection is used a part of the spectral information is lost. TOF is generally considered to be the detector of choice for applications with columns of I.D. ≥ 0.1 to < 0.2 mm (Maštovská & Lehotay, 2003) due to their fast data acquisition rates reaching up to 500 Hz and the subsequent possibilities of chromatographic and spectral deconvolution.



Elution order (1) C_{16}, (2) dimethoate, (3) terbutylazine, (4) diazinon, (5) pyrimethanil, (6) chlorpyrifos-methyl, (7) fenitrothion, (8) chlorpyrifos, (9) cyprodinyl, (10) penconazole, (11) captan, (12) methidathion, (13) C_{22}, (14) kresoxim-methyl, (15) myclobutanil, (16) tebuconazole, (17) phosalone, (18) bitertanol, (19) C_{28}, (20) cypermethrin, (21) ethofenprox; S, impurity from solvent.

Fig. 1. Chromatogram of GC-FID analysis of n-alkanes and pesticides in toluene on the narrow-bore column CP-Sil 8 CB (A) 15 m x 0.15 mm I.D. x 15 µm. (B) 10 m x 0.1 mm I.D. x 10 µm. Injected volume 2 µl of solution 0.25 ng.µl^{-1} of each analyt in toluene. (Dömötörová et al., 2006).

Quadrupole instruments have been most widely used with conventional capillary GC. Dalüge et al., 2002 utilized quadrupole MS as a detector in the resistively heated GC, the scan speed of the quadrupole mass spectrometer (16 spectra per second in the range m/z 50-310) was found to be sufficient for a proper reconstruction of the chromatographic peaks, and good-quality mass spectra were obtained. Six scans across a peak were sufficient for peak integration. In pesticide residue analysis with narrow-bore fast GC, Kirchner et al., 2005 b found that the spectra acquisition rate has a great impact on sensitivity (peak areas, peak shapes and S/N (signal-to-noise) ratios). The quality of the obtained spectra was not significantly influenced in the full scan monitoring mode for the fastest scan rates. For quantitative analysis a SIM mode was able to acquire the sufficient number of data-points for the proper peak shape reconstruction and good repeatability of peak areas measurements expressed by RSD (<5%) for all tested dwell times shorter than 75 ms. However, for shorter dwell times up to 10 ms, the S/N ratio is lower, while peak areas are not influenced. Proving the quadrupole MS ability for adequate detection of narrow peaks

without the loss of sensitivity gives the possibility to extend the use of the fast GC to routine laboratories.
Fast GC-MS methods using narrow-bore capillary columns have been developed and validated as effective substitutes for conventional capillary GC-MS for limited number of pesticides (Kirchner et al., 2005 *a*) and for multiresidue analysis of wide range of pesticides including carbamates, organochlorines, orhanophosphorous, triazoles and others (Húšková et al., 2008). The LOQs and ruggedness of fast GC-MS are sufficient for the analysis of pesticide residues even in baby food (Hercegová et al., 2006; Kirchner et al., 2008). The method for the determination of 29 pesticides proved or suspected to be endocrine disrupting chemicals was developed and validated by Húšková et al., 2010 *a*. LOQs in the range of 0.04 to 10 $\mu g.kg^{-1}$ for majority of pesticides were obtained, dicofol, linuron and prochloraz gave LOQs ≤ 21 $\mu g.kg^{-1}$ using matrix-matched standards for calibration.

3.2 Fast temperature programming

Combined approaches to realize the analysis faster are usually applied. Fast temperature programming and narrow-bore column utilization was shown by Ochiai et al., 2006 and subsequently modified by Samsamoto et al., 2007. 82 pesticides in natural water by fast screening method employing dual SBSE (stir bar sorptive extraction) – termal desorption (TD)-GC-MS were analyzed. Fast temperature programming (75 $°C.min^{-1}$) using a 0.18 mm I.D. narrow-bore capillary column and fast scanning (10.83 $scan.s^{-1}$) quadrupole MS were employed. The method showed high sensitivity with LODs < 10 $ng.l^{-1}$ and remarkable precision for most of the target pesticides.
Very fast temperature programming is realized by inserting capillary column into a resistively heated metal tube, or column enclosed in a resistively heated toroid-formed assembly, allowing heating rate of 1800 $°C.min^{-1}$ and a cool-down time of less than 1 min (Dalüge et al., 2002). Maštovská et al., 2001 used the flash GC technique (resistive heating of a short capillary column 5 m x 0.25 mm I.D.) for the analysis of 15 organophosphorus pesticides, the GC analysis time was reduced by a factor of more than 10 compared to the conventional GC technique (moderate oven temperature programming of a six times longer high resolution capillary column). Due to much narrower peak widths, improved detectability of analytes (higher S/N) was achieved. In comparison with the alternative fast temperature programming technique realized by a conventional GC oven, significantly better retention time repeatability was observed.

3.3 Low-pressure gas chromatography

Typically, LP-GC-MS involves the use of a short narrow uncoated restriction capillary (3 m x 0.15 mm I.D. or 0.1 m x 0.1 mm I.D.) connected between the inlet and a relatively short wide-bore analytical column (5-10 m x 0.53 mm I.D. x 1 µm film thickness). This column is maintained under vacuum conditions due to pumping from the MS system, which causes the helium carrier gas to have shifted the optimal flow velocity from the van Deemter equation to greater flow rate. Meanwhile, the restriction capillary allows normal operating pressure at the inlet (de Zeeuw et al., 2000). Detailed review on LP-GC was recently published by Ravindra et al., 2008. The main drawback of this technique is the loss of separation efficiency. Wider peaks compared to other approaches of fast GC do not require high acquisition rate of MS detectors, therefore, common detection techniques are adequate.

Another advantage is the lower elution temperature that is beneficial for thermally unstable analytes and stationary phases, enhancement of S/N ratio leads to the improved detection limits and it offers a 3-5-fold reduction in analysis time in comparison to conventional GC. LP-GC–MS using a quadrupole MS instrument was developed and evaluated for the fast analysis of 57 pesticides in food crops by Maštovská et al., 2004. The further study for fast LP-GC-MS employing TOF for determination of 100 analytes was developed by Čajka et al., 2008. The sample throughput of combination of QuEChERS sample preparation technique followed by LP-GC/TOF-MS (time-of-flight mass spectrometry) was checked by Koesukwiwat et al., 2010.

3.4 Comprehensive GCxGC

Comprehensive two-dimensional gas chromatography (GC × GC) is a powerful separation technique in which two gas capillary columns with different separation mechanism are coupled via an interface called modulator. This modulator is used to focus and efficiently transfer (i.e. re-inject) the entire effluent from the first column into the second one as consecutive narrow chromatographic bands. The fast GC separation that takes place in the second column depends on the nature of stationary phase, its length and the offset of temperature between this column and the main oven. Advanced detection methods, as a consequence, necessitate rapid acquisition capacities. Flame ionization detector (FID) systems are characterized by rapid data acquisition rates (250 Hz max) and are the most commonly used. Time-of-flight mass spectrometers (TOF MS) have a demonstrated effectiveness for the positive identification of comprehensive GC analytes (Tranchida et al., 2004). This type of MS possesses a higher scan speed in respect to traditional quadrupole systems and is capable of supplying sufficient spectra per peak (at least 10) for reliable component assignment.

The main features of GC × GC, the influence of the experimental parameters in the final peak capacity and separation power as well as the main advantages of GC × GC as compared to other multidimensional chromatographic separation techniques for different application fields have been discussed in the recent reviews (Adahchour et al., 2006; Adahchour et al., 2008).

Ramos et al., 2009 evaluated the feasibility of using GC×GC-μECD (micro electron capture detection) in combination with a miniaturised generic matrix solid-phase dispersion-based sample preparation method for the fast monitoring of pesticides in real samples. The comparison of LODs with conventional GC-MS (quadrupole mass analyzer) screening triazines, organophosphorus pesticides (OPPs) and pyrethroids, in different types of fruits was evaluated. GCxGC provided lower LODs values.

4. Specificity of pesticide residues analysis

Despite of great efforts in the research of GC amenable pesticide residues analysis the analysis is complicated by the co-injected matrix constituents responsible for the matrix-induced chromatographic response enhancement or the subsequent decrease of the response. When a real sample is injected, the matrix components tend to block active sites in the GC injector and column, thus reducing losses of susceptible analytes caused by adsorption or degradation on active sites. This phenomenon results in ordinarily higher analyte signals in matrix-containing, versus matrix-free solutions (Hajšlová & Zrostlíková, 2003).

analytes /sample	column	carrier gas	temperature conditions	injection technique	detection technique	references
17 pesticides / water	HP-1, 10 m x 0.1 mm I.D. x 0.1 µm	He, const. press. 70.12 psi	80°C, 3.7 min, 100°C.min^{-1} to 150°C, 30°C.min^{-1} to 250°C, 3 min	LVI, PTV, solvent vent	MS (full scan, SIM)	Hada, 2000
15 organophosphor. pesticides / wheat	DB-5MS, TDX-RTX5, 5 m x 0.25 mm I.D. x 0.25 µm	He, const. flow 1 ml.min^{-1}	resist. heating	splitless	NPD, FID, FPD	Maštovská, 2001
pesticides / apples	DB-5, 5m x 0.1 mm I.D. x 0.1 µm	He, const. press. 300 kPa	resist. heating 100, 200 or 400°C.min^{-1}	split, splitless	MS (full scan, SIM)	Daltige, 2002
organophosphor. and sulphur pesticides	SGE DB5 , 10 m x 0.1 mm I.D. x 0.4 µm	H_2, linear velocity 120 cm.s^{-1}		splitless	FPD, FTD	Kempe & Baier, 2002
6 multiclass pesticides / lettuce	J&W DB5-MS, 20 m x 0.18 mm I.D. x 0.18 µm	He, 1 ml.min^{-1}	60°C, 7.5 min, 30°C.min^{-1}to 280°C	LVI-DMI-PTV, solvent vent	TOF MS (20 Hz)	Patel, 2003
15 organochlorine pesticides / tap and ground water	HP1-MS, 15 m x 0.1 mm I.D. x 0.4 µm	H_2, 1.1 ml.min^{-1}	45°C, 3.4 min, 120°C.min^{-1}to 280°C, 6 min	LVI- PTV, solvent vent	MS (SIM)	Korenková, 2003
20 pesticides / peach	DB5-MS, 20 m x 0.18 mm I.D. x 0.18 µm	He, 1 ml.min^{-1}	70°C, 1 min, 2.5°C.min^{-1} to 200°C, 10°C.min^{-1} to 280°C, 9.8 min	splitless	TOF MS	Čajka, 2004
20 organophosphor. pesticides / peach, sweet pepper	RTX 1701, 5 m x 0.25 mm I.D. x 0.25 µm	He, const. press. 3.8 psi	EZ flash, 60°C, 158°C.min^{-1} to 200°C, 24°C.min^{-1} to 240°C, 141°C.min^{-1} to 280°C, 88 s	splitless	FPD	Patel, 2004
18 pesticides / baby food	a) CP-Sil 13 CB, 25 m x 0.15 mm I.D. x 0.4 µm b) CP-Sil 8 Low-BleedMS, 15 m x 0.15 mm I.D. x 0.15 µm	a) H_2, progr. flow 2.3 ml.min^{-1} (5.5 min), 2 ml.min^{-2}, 3.4 ml.min^{-1}; b) He, const. flow, 0.5 ml.min^{-1}	a) 100°C, 1 min, 65°C.min^{-1} to 290°C, 8 min; b) 120°C, 1min, 30°C.min^{-1} to 290°C, 5 min	a) splitless b) cold splitless	a) ECD b) MS (SIM)	Hercegová, 2005
18 pesticides / apples	CP-Sil 8 Low-BleedMS, 15 m x 0.15 mm I.D. x 0.15 µm	He, const. flow 0.5 ml.min^{-1}	100°C, 1.5 min, 30°C.min^{-1} to 290°C, 6 min	PTV, cold splitless	MS (SIM)	Kirchner, 2005 a

analytes /sample	column	carrier gas	temperature conditions	injection technique	detection technique	references
pesticides	HP-1 MS, 5 m x 0.1 mm I.D. x 0.4 μm	H_2, const. press., 413 kPa	80°C, 0.65 min, 65°C.min^{-1} to 300°C	on-column	FID	Dömötörová, 2005
pesticides	CP-Sil 8 CB: a) 15 m x 0.15 mm I.D. x 0.15μm; b) 10 m x 0.10 mm I.D. x 0.10μm	I. H_2, const. press., a) 260 kPa b) 437 kPa II. He a) 363.5 kPa b) 0.5 ml.min^{-1}, 9 min, 5 ml.min^{-2}, 0.8 ml.min^{-1}	I. a) 100°C, 1 min, 65°C.min^{-1} to 290°C, 8 min; b) 120°C, 1 min, 30°C.min^{-1} to 290°C, 5 min II. a) 130°C, 1.13 min, 27.25°C.min^{-1} to 290°C, 6 min; b) 115°C, 1.88 min, 27.6°C.min^{-1} to 290°C, 5.78 min	I. split; splitless, II. PTV in cold splitless	I. FID, ECD II. MS (SIM)	Dömötörová, 2006
82 multiclass pesticides / brewed green tea	DB-5, 10 m x 0.18 mm I.D. x 0.18 μm	He, const. flow 1.1 ml.min^{-1}	40°C, 2 min, 75°C.min^{-1} to 300°C, 2 min	PTV in splitless, TDU	MS Full scan	Ochiai, 2006 Sasamoto, 2007
20 pesticides / apples, baby food, processed samples	CP-Sil 8 Low-BleedMS, 15 m x 0.15 mm I.D. x 0.15 μm	He, const. flow 1.2 ml.min^{-1}	130°C, 1.13 min, 27.25°C.min^{-1} to 290°C, 6 min	PTV, cold splitless	MS (SIM)	Hercegová, 2007
pesticides / fruit and vegetables	CP-Sil 8 Low-BleedMS 15 m x 0.15 mm I.D. x 0.15 μm	He, const. flow 1.2 ml.min^{-1}	60°C, 1.75 min, 60°C.min^{-1} to 150°C, 23.8°C.min^{-1} to 300°C, 1.9 min	PTV, solvent vent	MS (SIM)	Kirchner, 2008 Húšková, 2008 Húšková, 2009 Húšková, 2010 a Hercegová, 2010

DMI – difficult matrix introduction, ECD – electron capture detector, FID-flame ionization detector, FPD-flame photometric detector, FTD- flame thermo-ionic detector, LVI-large volume injection, MS – mass spectrometry, NPD-nitrogen-phosphorus detector, PFPD-pulsed flame photometric detector, PTV – programmed-temperature vaporization (injector), SIM-selected ion monitoring, TDU – thermal desorption unit, TOF-time-of-flight. Highlighted items use the fast temperature programming.

Table 2. A narrow-bore column and resistive heating fast GC approaches to the pesticide residues analysis

Ways to compensate the matrix effects include: (i) use of isotopically labelled internal standards, (ii) method of standard addition, (iii) use of matrix-matched standards, and (iv) use of analyte protectants (APs). The most widely used method in laboratories nowadays is the use of matrix-matched standards. This approach is, however, complicated by the fact, that the composition of matrix-matched standard should be as close as possible to the composition of real sample matrix in order to provide good compensation of matrix effects.
The principle of "analyte protectants" use is to find masking agents that would mask active sites in the GC system and thus would provide strong response enhancement of pesticides. More than 90 compounds belonging to different chemical classes were evaluated in order to protect coinjected analytes against degradation and/or adsorption in GC system (Anastassiades et al., 2003 *b*). Ethylglycerol, gulonolactone and sorbitol have been chosen as the most promising substitute of fruit and vegetable matrix.
The influence of chromatographic matrix induced response enhancement in fast GC with narrow-bore column was studied by Kirchner et al., 2005 *a* and it is illustrated in Fig. 2,

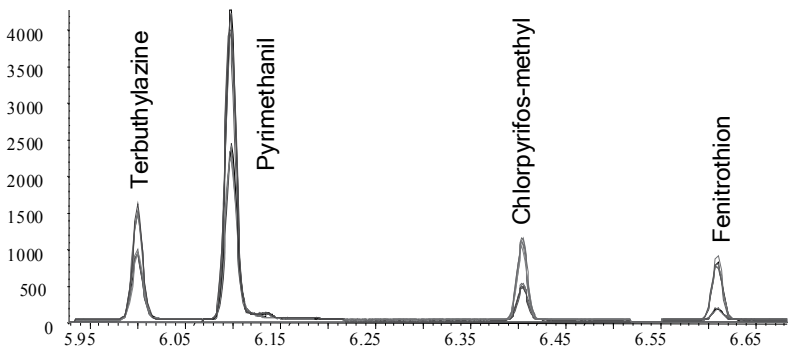


Fig. 2. Overlaid chromatograms (n=5) of selected pesticide standards prepared in neat toluene (lower responses) and standards prepared in blank apple matrix-matched standards, both at concentration level 0.0125 ng.µl^{-1}, corresponding to 5 µg.kg^{-1} in apple matrix, Kirchner et al., 2005 *a*.

where extracted ion chromatograms (n=5) of several pesticides obtained from the standard solution in a neat solvent (lower responses) is overlaid with matrix-matched calibration standard chromatograms (higher responses) of the same concentration injected under identical conditions (n=5). The chromatographic matrix induced response enhancement was found to be strongly dependent on the concentration of residues mainly in the lowest concentration region and is reaching up to 700 % compared to the pesticides solutions in a neat solvent. Selected results are shown in Table 3. Response enhancement is caused primary by the deactivation of active sites in the inlet but some improvements of the peak shapes were observed also under the protective effect of co-eluting matrix components in the analytical column and retention gap. However, it was shown that fast GC-MS utilizing narrow-bore columns with 0.15 mm I.D. shows acceptable stability of the separation system and the responses of pesticides in matrix-matched standards at different concentration levels do not significantly change during 130 injections with the proper maintenance of inlet liner and retention gap. Illustrative chromatograms of fast GC separation with narrow-bore column showing the influence of matrix-matched standards use and the use of analyte protectants in comparison to the chromatogram without matrix compensation is given in

Fig. 3. In a neat solvent (acetonitrile MeCN) the shapes of pesticide peaks are very poor, whereas the chromatogram of matrix-matched standards and also the chromatogram with APs, which have been utilised for the elimination of matrix effects in real sample analysis, are suitable for evaluation. It should be pointed out, that measurements in a neat solvent were performed in a "dirty" chromatographic system. The instrument noise seems to be comparable to matrix-matched standards and standards in a neat solvent.

However, responses in matrix-matched standards improve significantly. In order to compare the performance of APs with matrix-matched standards (Kirchner et al., 2008) calibration curves of selected pesticides were searched in terms of linearity of responses, repeatability of measurements and reached LOQs utilizing the following calibration standards in the concentration range 0.001 – 0.5 $\mu g.kg^{-1}$: in a neat solvent (MeCN) with/without addition of APs, matrix-matched standards with/without addition of APs. For APs results are in a good agreement with matrix-matched standards. To evaluate errors of determination of pesticide concentration in samples at the concentration level of pesticides 0.05 $\mu g.kg^{-1}$ synthetic sample were analyzed and quantified. For less troublesome pesticides very good estimation of concentration was obtained utilizing APs, while for more troublesome pesticides such as methidathion, malathion, phosalone and deltamethrin significant overestimation reaching up to 80 % was occurred (Fig. 4).

Pesticide	Relative average peak area in %					
	Concentration ($ng.\mu l^{-1}$)					
	0.0125	*0.025*	*0.125*	*0.25*	*1.25*	*2.5*
Dimethoate	419.7	295.5	209.6	152.9	101.0	89.2
Terbuthylazine	150.2	144.0	129.6	112.3	91.8	76.5
Diazinone	178.7	177.5	148.9	125.3	95.9	79.6
Pyrimethanil	155.8	153.8	134.5	115.2	91.5	74.2
Chlorpyrifos-methyl	227.8	227.6	188.2	152.0	102.9	85.8
Fenitrothion	489.3	487.8	414.1	288.8	130.1	101.0
Chlorpyrifos	228.6	229.3	188.9	148.3	106.0	89.6
Cyprodinyl	163.2	168.1	150.2	118.9	98.3	84.2
Penconazole	198.4	203.7	167.4	130.4	103.8	89.9
Captan	-	-	23.8	18.05	18.4	22.3
Methidathion	332.3	307.5	192.4	135.8	99.9	90.0
Kresoxim-methyl	218.6	220.4	161.1	129.4	107.2	94.8
Myclobutanil	438.7	350.8	190.4	141.8	107.8	95.2
Tebuconazole	464.5	433.5	279.0	194.5	127.5	113.2
Phosalone	367.5	377.1	237.8	165.3	112.6	99.3
Bitertanol 1	758.2	700.8	531.1	293.2	160.5	150.9
Bitertanol 2	772.3	709.7	393.6	219.9	111.0	116.8
Cypermethrin 1	378.7	380.0	317.6	193.3	140.1	126.9
Cypermethrin 2	395.7	346.6	278.3	161.6	119.7	104.4
Cypermethrin 3	571.1	419.1	253.9	153.3	113.9	96.0
Etofenprox	222.9	202.7	153.1	131.5	113.7	99.7

Table 3. Dependence of chromatographic matrix induced response enhancement on concentration of pesticides measured, expressed as relative peak area of matrix matched standard to standard prepared in neat solvent, in % (n=5), Kirchner et al., 2005 *a*.

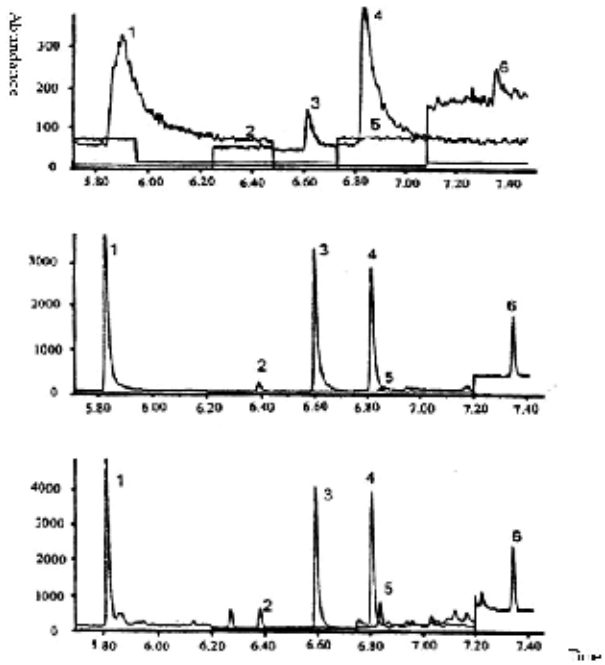


Fig. 3. Fast GC-MS chromatograms of pesticides obtained in SIM mode. A – standards in neat solvent (MeCN); B- standards in solvent with addition of analyte protectants (2 μg of 3-ethoxy-1,2-propanediol, 200 ng of D-sorbitol and L-gulonic acid γ-lactone); C – matrix-matched standards (matrix-apple prepared by QuEChERS method). Concentration level 10 $\mu g.kg^{-1}$, injected volume 2 μl, PTV injection. 1 - pyrimethanil, 2 - fenitrothion, 3 - tetraconazole, 4 – cyprodinil, 5 - tolylfluanid, 6 – kresoxim-methyl (Húšková et al., 2007)

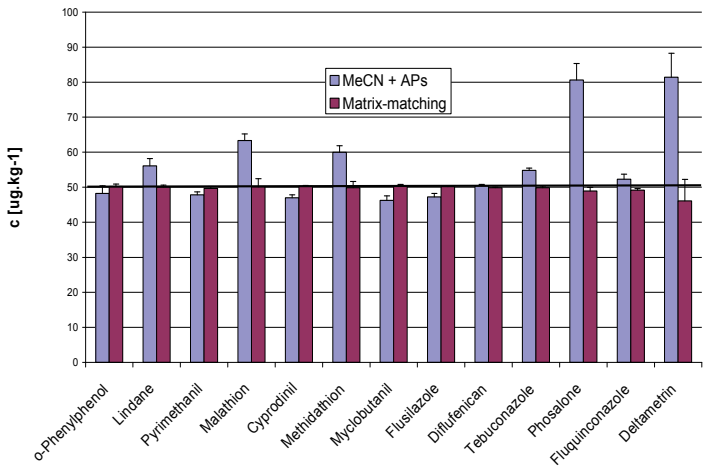


Fig. 4. Graf of calculated concentrations of pesticides in synthetic sample using MeCN with addition of APs and matrix-matched standards (both normalized to triphenyl phosphate) vs. expected concentration of 50 $\mu g.kg^{-1}$. Error bars on each column represent repeatability of measurements, (Kirchner et al., 2008).

5. Sample preparation in food samples analysis by fast GC

When considering the merits of developing and validating a routine GC method, the total run time involved in analysing the sample must be considered (Klee & Blumberg, 2002). The total run time is the sum of the time for sample preparation, sample introduction, separation and detection, cool down and reequilibrating and reporting.

Sample preparation is usually the limiting step of analytical method determining the sample throughput in pesticide residues analysis of food and environmental samples. Therefore, it is necessary to combine a fast chromatography technique with fast sample preparation technique; the total analysis time can be reduced significantly.

The sample preparation approach known as QuEChERS, which stands for "quick, easy, cheap, effective, rugged and safe", firstly introduced by Anastassiades et al., 2003 *a* represents a breakthrough in the field of sample preparation. QuEChERS approach use acetonitrile for extraction of a 10-15 g homogenized sample followed by salt-out partitioning of the water from the sample using anhydrous $MgSO_4$, NaCl, and/or buffering agents, and further clean-up using dispersive solid-phase extraction (d-SPE) or disposable pipette extraction (DPX) with anhydrous $MgSO_4$, primary secondary amine (PSA) and/or in combination with C_{18}, graphitized carbon black (GCB) sorbents. Ethyl acetate (EtOAc) is sometimes used as a substitute solvent in QuEChERS procedure, but generally it leads to less clean extracts and lower recoveries of numerous pesticides. Acetate-buffered MeCN version (relatively strong buffering at pH 5) exhibits advantages in comparison to non-buffered or weaker citrate buffered version, but both were accepted by scientific standards organizations – acetate version AOAC Official Method 2007.01 and citrate version European Committee for Standardization (CEN) Standard Method EN 15662. Intercomparison of unbuffered and both buffered methods (Lehotay et al., 2010) was evaluated in terms of pH-dependence, co-extracted matrix components, matrix effects and substitution of MeCN with EtOAc. It was shown, that the use of EtOAc leads to less clean extracts and lower recoveries of more pesticides, but for GC-amenable pesticides EtOAc gave equivalent results as MeCN.

The QuEChERS approach is very flexible and it serves as a template for modifications on the analyte properties, matrix composition, equipment and subsequent analytical technique. QuEChERS reached the worldwide acceptance, it serves as a base to create different permutations for the analyte(s)/matrix(es) applications.

Concerning throughput, according to Koesukwiwat et al., 2010, sample preparation of fruit and vegetables using QuEChERS technique takes <10 min per individual sample, or <1 h for the two chemists to prepare 32 pre-homogenized samples and <10 min LP-GC run time and <15 min cycle time allowed > 32 injections in 8 hrs for identification and quantification of 150 pesticides. Time requirements of QuEChERS and gel permeation chromatography (GPC) in combination with 7 min LP-GC-TOF-MS run time for separation of multiple pesticide residues were compared by Čajka et al., 2008, and the batch of 12 fruit samples with 6 matrix-matched standards were analyzed within 4 h with the use of QuEChERS in contrary to 20.5 h with the use of EtOAc extraction with GPC, thus time reduction by a factor of 5. However, GPC is considered as universal method for cleaning-up purposes of a wide range of matrices and multi-class pesticides, the time analysis and the cost is too high. According to Húšková et al., 2009, the batch of 6 samples in parallel is prepared within 46 min with the fast GC with narrow-bore column single run time 11.45 min.

QuEChERS technique is the most effective technique employing the clean-up step. If the extract does not contain co-extractants and also a lot of matrix components, avoiding clean-up step lead to decreasing the analysis time.

Other extraction techniques and particularly novel mikroextraction techniques belong to effective and fast techniques. They overcome the shortcomings of classical liquid-liquid extraction or solid-phase extraction. To have simple, fast and green procedures microextraction techniques are developed. Solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE) and microextraction with packed sorbents (MEPS) are examples that are used for sample preparation of pesticide residues containing food matrices (Chen et al., 2010). It was shown by several authors (Ochiai et al., 2005, Barriada-Pereira et al., 2010), that using SBSE technique allows batch sample preparation, for example 60 samples on one stir-plate, (typically in minutes (up to 60 min)), high recovery and extremely low LODs at sub $ng.l^{-1}$. Whereas SPE and MSPD need a concentration step, SPME and SBSE allow carrying out the extraction and concentration step in a single step. SPME and SBSE are equilibrium processes, but SBSE enables a much higher capacity because of the large amount of polymeric phase (24-126 μl) compared to SPME (0.5 μl).

6. Selectivity enhancement by GC-NCI-MS approach

Over the past 10 years, there has been an increased interest in negative ion/molecule reactions as an ionization technique in MS detection (Liapis et al., 2003; Schulz, 2004; Húšková et al., *b*, 2010). In negative chemical ionization (NCI), a reagent gas at higher pressure is introduced into the ion source. The electron beam in the mass spectrometer collides with the reagent gas and the essence of the technique is based on the ionization through the capturing of a low energy electron by the analyte molecule. Compounds with sufficient electron affinity, such as chlorinated molecules, are very sensitive towards this mode of detection. NCI is a low energy process with limited fragmentation (easily identifiable molecular ion) and provides simple mass spectra in comparison to the EI technique. With this technique, usually a few ions of high abundance are observed in the relevant mass spectrum and this enhances analyte detectability. Summarily, advantages of the NCI ionization technique are chromatograms with less chance for the interferences from ions derived from the sample matrix; better S/N ratio; higher sensitivity and selectivity; analysis of organic compounds at the ultratrace concentration levels (ppt concentration) with low LODs and LOQs. Detection limits are usually two orders of magnitude lower than the corresponding EI-MS or positive chemical ionization methods.

Application of fast GC set-up using narrow-bore column (0.15 mm I.D.) in combination with MS detector in NCI mode was introduced and compared to fast GC-MS with electron ionization by Húšková et al., 2009. Multi-residue method of 25 pesticides belonging to different groups (organochlorines, organophosphates, pyrethroids, dicarboximides, 2,6-dinitroaniline, triazinone, substituted urea, phthalamide, cyclodiene, triazole, imidazole), varying in polarity, volatility and other physicochemical properties from non-fatty fruit and vegetable matrices based on fast GC with quadrupole NCI-MS was developed and verification of the method was realized. Blank apple sample extracts were used for the preparation of matrix-matched standards. The illustrative chromatograms of target ions of endocrine disrupting pesticides in matrix-matched standard solution using both ionization techniques are presented in Fig. 6. The concentration level corresponding to 10 $\mu g.kg^{-1}$ in fruit matrix was below or far below the MRL values of all pesticide/commodity combinations and it was selected with intention to show the potential of the method for utilizing in ultratrace analysis that is essential in the analysis of endocrine disruptors. EDCs are compounds that are expected even in minute amounts to be able to disrupt the

endocrine system and cause cancer, harm to male and female reproductive systems, and other adverse effects. Chromatogram obtained in the NCI mode (Fig. 6 A) with the very high response of detector, lower background disturbances and better S/N ratio in comparison to the EI mode (Fig. 6 B) at the same concentration level can be seen. Matrix-matched standard solutions were analyzed to determine the linear response range of the MS detection in the NCI and EI mode, repeatability of the peak area measurement, and to compare the overall performance of NCI vs. EI. The selected EDCs pesticides were analyzed in 11.45 min. Linearity of calibration curves constructed from absolute peak areas (A_i), expressed as coefficient of determination (R^2), was in the range of 0.9936 – 1.000 in the NCI mode and 0.9820 – 0.9999 in the EI mode. Repeatability of calculated R^2, expressed as relative standard deviations (RSDs), for both absolute and normalized peak areas was ≤ 1.1 %. NCI is more sensitive, resulting in up to 100-fold decrease in the lowest calibration levels (LCLs, Table 4). For the majority of EDCs pesticides the LCLs were 0.01 and 0.05 ng.ml^{-1} (0.01 and 0.05 µg.kg^{-1}) for fast GC-NCI-MS and 1 ng.ml^{-1} (1 µg.kg^{-1}) for fast GC-EI-MS. Instrument LODs, LOQs and further validation parameters are listed in Table 4.

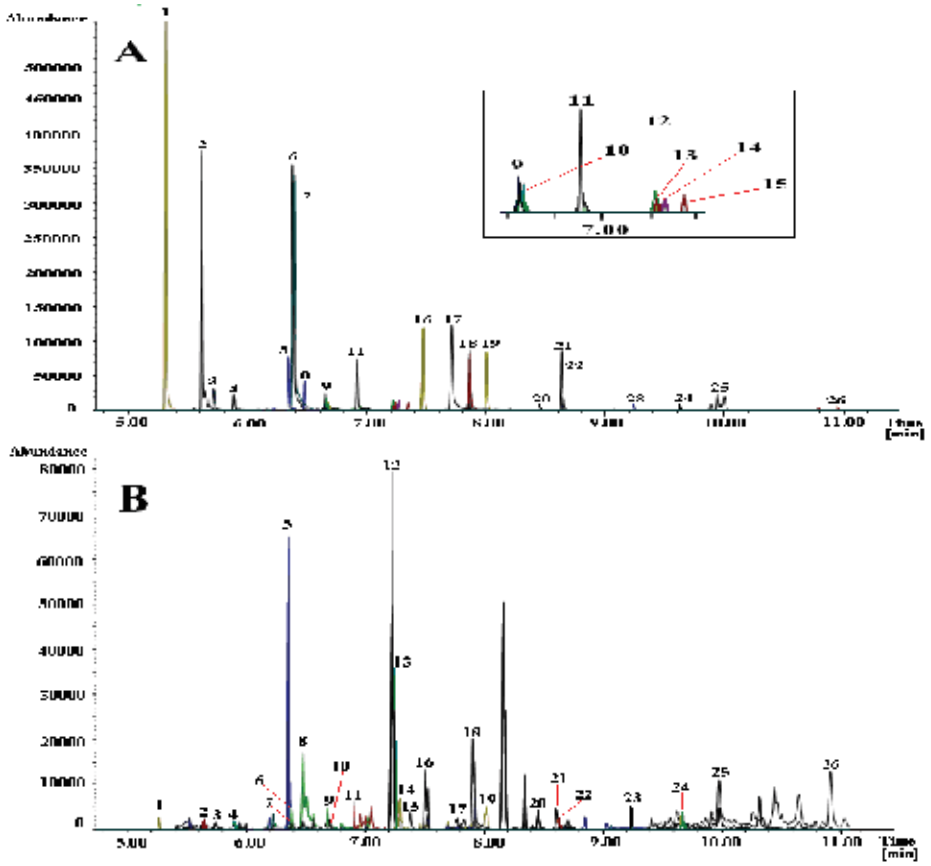


Fig. 6. Chromatograms of target ions of pesticides in matrix–matched standard solutions analyzed by fast GC–MS in SIM mode at the concentration level of 10 ng.ml^{-1} (corresponding to 10 µg.kg^{-1}): A – **NCI** mode; B - **EI** mode. Numbering of peaks is identical with the number of compounds given in the Table 4. (Húšková et al., 2009).

No	Pesticide	R^2		LCL (ng.ml^{-1})		RSD_{Ai} (%)		LOD		LOQ	
								(pg.ml^{-1})	(ng.ml^{-1})	(pg.ml^{-1})	(ng.ml^{-1})
		NCI	EI	NCI	EI	NCI	EI	NCI	EI	NCI	EI
1	trifluralin	0.9998	0.9980	0.01	1.00	4.3	6.9	0.15	0.07	0.52	0.24
2	hexachlorobenzen	0.9996	0.9995	0.01	1.00	5.1	7.9	0.85	0.02	3.0	0.08
3	dimethoate	0.9997	0.9984	0.05	1.00	5.3	8.2	33.1	0.16	110	0.53
4	lindane	0.9996	0.9992	0.05	1.00	3.7	7.3	3.7	0.52	12.5	1.73
5	chlorpyrifos-methyl	0.9999	0.9990	0.05	1.00	4.9	8.6	33.8	0.08	113	0.27
6	metribuzin	0.9998	0.9986	0.05	1.00	4.8	8.8	8.8	0.10	29.5	0.33
7	vinclozolin	0.9990	0.9991	0.01	1.00	4.9	9.9	1.1	0.29	3.8	0.97
8	heptachlor	internal standard									
9	malathion	0.9998	0.9995	0.05	1.00	3.4	11	38.8	0.34	129	1.13
10	linuron	0.9936	0.9868	1.00	10.00	3.9	7.6	281	6.32	936	21.07
11	dicofol	0.9964	0.9820	0.10	10.00	4.1	6.9	30.4	3.56	147	11.87
12	procymidone	0.9999	0.9998	0.10	1.00	4.5	9.5	30.9	0.27	103	0.90
13	diazinon	0.9989	0.9988	0.10	1.00	4.0	8.1	55.1	0.09	184	0.28
14	folpet	0.9988	0.9984	0.50	10.00	15	9.0	430	1.05	1430	3.62
15	chlordane	0.9995	0.9995	0.01	1.00	5.5	9.6	1.2	0.10	4.6	0.32
16	endosulfan-alfa	0.9995	0.9993	0.01	1.00	5.7	11	1.1	0.46	3.7	1.53
17	myclobutanil	0.9998	0.9990	0.01	1.00	2.9	9.9	3.0	0.07	10.1	0.22
18	nitrofen	0.9992	0.9939	0.01	5.00	4.9	8.2	2.7	1.09	9.2	3.63
19	endosulfan-beta	0.9995	0.9998	0.01	1.00	4.8	7.7	2.2	0.01	7.3	0.04
20	chlordecone	0.9998	0.9895	5.00	10.00	3.5	7.2	113	0.91	378	3.03
21	iprodione	1.0000	0.9999	0.01	1.00	4.2	11	2.3	0.10	7.7	0.33
22	bifenthrin	0.9998	0.9997	0.01	1.00	5.1	10	3.3	0.02	11.9	0.08
23	mirex	1.0000	0.9999	0.10	1.00	2.5	9.9	89	0.11	291	0.32
24	prochloraz	0.9992	0.9987	1.00	10.00	2.8	5.3	619	5.37	2360	17.90
25	cypermethrin	0.9994	0.9993	0.50	5.00	5.6	11	127	2.05	424	6.83
26	deltamethrin	0.9991	0.9982	0.10	1.00	4.1	11	331	0.07	1112	0.24

R^2 – coefficients of determination, LCL- the lowest calibration level, RSD_{Ai} – relative standard deviation (6 replicates), LOD – limit of detection calculated as 3:1 S/N ratio from calibration measurements, LOQ – limit of determination calculated as 10:1 S/N ratio from calibration measurements.

Table 4. Verification parameters of the GC-NCI-MS and GC-EI-MS analytical methods.

In the NCI mode, LODs were in the range of 0.15 – 88.82 pg.ml^{-1} (0.00015 – 0.089 μg.kg^{-1} in real sample) and LOQs were in the range of 0.52 – 291.35 pg.ml^{-1} (0.00052 – 0.291 μg.kg^{-1} in real sample) for the majority of analytes under the study. Linuron, folpet, chlordecone, prochloraz, cypermethrin and deltamethrin have the LOD values > 100 pg.ml^{-1} and LOQ values > 300 pg.ml^{-1}. LODs and LOQs obtained in EI mode are at the level of ng.ml^{-1}. For all analytes except linuron, dicofol and prochloraz, the LOQs were below 10 μg.kg^{-1}, which is the MRL required for the pesticide residues in baby-food. The NCI mode provided significantly higher selectivity and sensitivity. Better results for the NCI mode were obtained as a consequence of minimizing the background interferences and a better S/N ratio. The method LOQs, determined from recovery studies, was 5 μg.kg^{-1} in EI mode (except for folpet, chlordecone, endosulfan-alfa and endosulfan-beta). NCI-MS detection allowed 5 times to even 50 times for some pesticides lower method LOQs. The developed GC-NCI-MS method fulfilled the EU criterion concerning recovery rates and RSDs at the concentration of 1, 5 and 150 μg.kg^{-1} for all compounds under study.
The main advantage of the GC-NCI-MS measurement procedure was the increase of the selectivity, e.g., the differentiation between the target component and the accompanying sample matrix coextractants. As the universal detection by MS in EI mode is changed to selective detection by the process of chemical ionization (CI), the selectivity is increased, and the measured sensitivity of the selected analytes is enhanced for a variety of active endocrine disrupting pesticides with adverse effect on human endocrine system. For the proper balance it is necessary to mention the disadvantages of NCI, unavailability of library spectra for analytes and the requirement for an analyte to be active in NCI mode, e.g. to contain halogen elements are the most weighty.

7. Real-life samples analysis

The most important application of fast GC is in situations, where the results of analysis are needed close to where the answer is needed (e.g. process control, on-site environmental and industrial hygiene applications) to obtain increased laboratory throughput. Practicality of fast GC is a function of a sample preparation step and the matrix interferences, so, for those applications, where the GC separation is the bottleneck using fast GC is indeed a significant contribution. Several real-life analyses usually follow the method validation or the developed method is applied to a screening or monitoring. Examples of real-life samples analysis employing the fast GC with the narrow-bore columns and resistive heating approach as an analytical technique for pesticide residues determination were summarised in Table 2.
Cunha et al., 2009 applied the fast LP-GC-MS method for the determination of multiple pesticides in grapes, musts and wines, 8 min time analysis for fast GC versus 24 min using conventional approach was obtained. Total analysis time including QuEChERs sample preparation technique was less than 20 min. LP-GC-MS-MS was applied to the analysis of 65 pesticide residues in fat fruit matrices such as avocado (Moreno et al., 2006), pesticides in tomato samples (Walorczyk & Gnusowski, 2006), determination of pesticides residues in tropical fruit (Vidal et al., 2007), analysis time reduction in half was obtained by Arrebola et al., 2003 for 71 pesticides in fresh vegetables matrices. Koesukwiwat et al., 2010 evaluated method ruggedness and matrix effects in LP-GC-TOF-MS analysis of 150 pesticides in fruit and vegetables.

In this sub-chapter the example of real sample analysis by narrow-bore fast GC-NCI-MS method combined with QuEChERS sample preparation developed by our group is presented. The selected positive findings of pesticide residues in different real fruit and vegetable samples are shown in Table 5.

Matrix	Pesticide	NCI		EI	
		c_i [µg.kg^{-1}]	RSD [%]	c_i [µg.kg^{-1}]	RSD [%]
orange	malathion	50.1	0.52	52.5	3.5
lettuce	iprodione	40.1	1.2	42.0	3.0
pear$_A$	iprodione	40.1	1.2	41.3	3.4
pear$_B$	bifenthrin	69.0	5.2	64.8	4.1
	myclobutanil	0.07	6.8	n.d.	-
kohlrabi	metribuzin	0.06	3.0	n.d.	-
	vinclozolin	0.15	2.1	n.d	-
	myclobutanil	0.25	3.6	n.d.	-
plum	iprodione	234.3	0.31	241.1	2.8
strawberry	iprodione	40.9	1.2	41.3	3.4
pepper	myclobutanil	24.3	6.3	30.4	4.2
	cypermethrin	47.2	2.2	54.9	6.0

Table 5. Concentration c_i (µg.kg^{-1}) of pesticide residues in real samples and repeatability of measurements expressed as relative standard deviation RSD (%) of parallel extractions; n. d. – not detected.

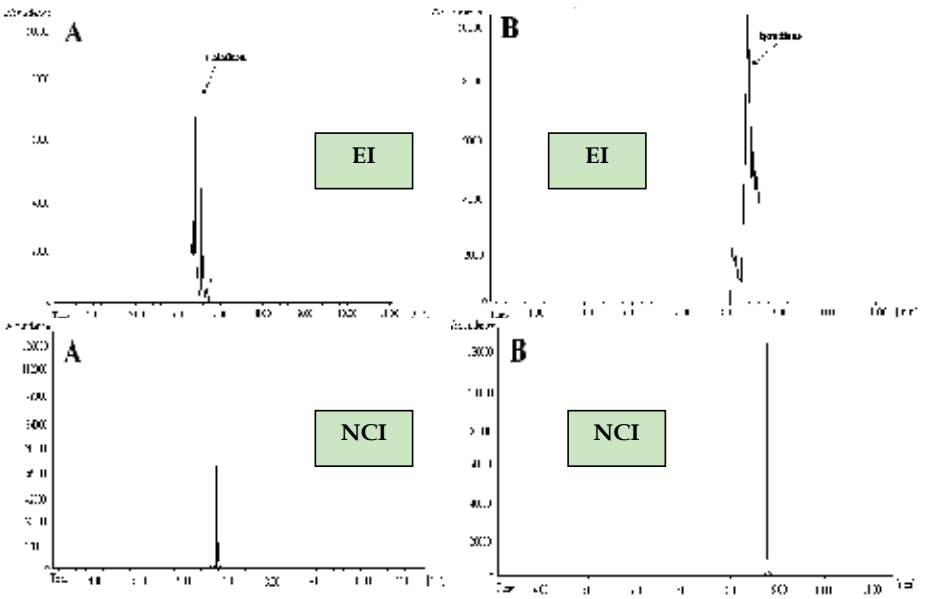


Fig. 7. Chromatogram of target ions of EDCs pesticides analyzed by fast GC-MS in SIM in EI (upper figures) and NCI mode (bottom figures) in real samples A - orange (malathion), B - lettuce (iprodione).

Average pesticide concentration (calculated from triplicate analysis of two parallel samples) obtained from absolute peak areas and relative standard deviations (RSDs < 6.8 %) for parallel samples are presented in Table 5. Determined pesticide residues concentrations were in the range of 0.06 – 241.1 $\mu g.kg^{-1}$. The concentrations of pesticide residues determined by fast GC-MS in the NCI and EI mode were in a good agreement. The example of $pear_B$ and kohlrabi shows the ability of the NCI-MS method for the pesticide residue analysis at low concentration level. For illustration the chromatograms of the target ions of the pesticides analyzed by fast GC-MS in the SIM mode in the real sample extracts, in NCI and EI ionization modes, are given in Fig. 7 (Húšková et al., 2009). It is evident from the chromatograms that there are interferences in EI mode which are dependent on the matrix (orange, lettuce), whereas in NCI mode very "clean" chromatograms with high responses of analytes without influence from the matrices were obtained.

8. Conclusions

For pesticide residues analysis ultrasensitive analytical methods are required and there is still the need to improve the performance and ruggedness of analyses. Despite the tremendous developments and improvements in the analytical instrumentation, for most of substances there is continuous need to employ the extraction and preconcentration steps. Speeding-up analysis in gas chromatography is the unavoidable way in routine analytical laboratories requiring higher throughput and reduced costs of performed analyses. The use of narrow-bore capillary columns with the enhanced separation efficiency and the use of short wide-bore column for low-pressure GC are the most promising ways and an additional research in these areas is expected. The advances obtained in the study of fast GC are a base of knowledge for comprehensive gas chromatography where the fast GC takes a place.

Nowadays fast GC can be performed on commercial gas chromatographs with standard equipment for high-speed injection, electronic pressure control, rapid oven heating/cooling and fast detection. The main stress of this contribution was given to the advances and achievements in the area of narrow-bore approach of speeding the GC analysis up, as there is the significant contribution of our research group to the study of possibilities and limitations and to the search on ruggedness of fast GC with narrow-bore columns for pesticide residues analysis at ultratrace concentration level. It was shown, that not only time-of-flight, but also in laboratories widely used quadrupole MS detector was shown to broaden its ability to detect the narrow peaks without the loss of sensitivity. Multiresidual fast GC methods for analysis of pesticide residues in different non-fatty fruit and vegetable samples employing MS in electron ionization and negative chemical ionization modes were developed. The use of negative chemical ionization was demonstrated as a tool for sensitivity enhancement for selected analytes and matrices. Applicability of the methods was demonstrated on real samples. The special emphasise was given to the analysis of samples contaminated by endocrine disrupting pesticides with the aim to obtain LOQs significantly lower in comparison to MRL values.

9. Acknowledgement

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7

Multidimensional Chromatography in Pesticides Analysis

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1. Introduction

The agricultural production of food and feed on an economically competitive basis needs an ever-increasing application of pesticides. Pesticide is a general term that includes variety of chemical and biological products to kill or control pests such as fungi, insects, rodents and weeds. In the European Union (EU) approximately 320,000 tonnes of active substances are sold every year, which accounts for one quarter of the world market (The Pesticides Safety Directorate, York, United Kingdom). Residues in fruit and vegetables, cereals, processed baby food and foodstuffs of animal origin are controlled through a system of statutory maximum residue limits (MRLs). The maximum residue limits (MRLs) are defined as: 'The maximum concentration of pesticide residue (expressed as milligrams of residue per kilogram of commodity (mg/kg)) likely to occur in or on food commodities and animal feeds after the use of pesticides according to good agricultural practice (GAP)' (Proposed PAHO/WHO Plan of Action for Technical Cooperation in Food Safety, 2006–2007). MRLs vary ordinarily within the interval 0.0008–50 mg/kg (The Applicant Guide: Maximum Residue Levels, The Pesticides Safety Directorate, York, United Kingdom), typically between 0.01 and 10 mg/kg for adult population. The lower values of MRLs are set for baby food—EC specified the MRL of 0.010 mg/kg (Pesticides and the Environment, A Strategy for the Sustainable Use of Plant Protection Products and Strategy Action Plans, London, United Kingdom), the lowest levels are set for particular special residues (Status of active substances under EU review (doc. 3010); Commission Directive 2003/13 and 14/; Council Directive 98/83/).

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 is a very rapid analysis. In this study are 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.

2. Mutidimensional chromatographic techniques applied to analysis of pesticides

2.1 Mutidimensional Gas Chromatography (MDGC)

The application of multidimensional gas chromatography has been focused in essentially two areas: (i) increasing peak capacity of the separation system, and (ii) increasing the speed of analysis of the separation system. In common with all multidimensional separations, two-dimensional GC requires that the target analytes are subjected to two or more mutually independent separation steps and that the compounds remain separated until completion of the overall procedure. **Figure 1** shows several potential on-line modes of two-dimensional GC operation. These couplings demonstrate GC-GC performed by using a single heart-cut from the primary to the secondary column, multiple heart-cuts, transferred to multiple intermediate traps, and heart-cuts to a multiple parallel secondary column configuration (Lewis, 2002). For all three of the examples of two-dimensional gas chromatography configurations shown in **Figure 1**, an interferacing unit is required between the primary and secondary column.

2.1.1 Introduction to heart-cutting Multidimensional Gas Chromatography (MDGC)

The non-intrusive manipulation of carrier gas effluent between two columns clearly has significant advantages in 2D GC. In addition, a pressure-driven switch between the columns introduces no extra band broadening to an eluting peak. A basic principle of pressure switching which has subsequently been used extensively for heart-cutting, venting and backflushing within two-dimensional systems was described by Deans (Deans, 1968). In this process are highlighted three distinct phases, i.e., that of survey of prefractionation, sample transfer and backflush of the primary column (Bertsch, 1990). The principle of operation is shown in **Figure 2**. The basis of the method is in the diversion of flows by using pressure balancing at junctions. The flow of carrier gas to each junction is controlled by solenoid valves, with the magnitude of pressure introduced being determined by the inlet and outlet pressures of the interacting devices and their individual flow resistances (Lewis, 2002).

There are three phases in the operation of DEANS switch (Lewis, 2002):

a. Prefractionation-operation in the SURVEY position illustrated in **Figure 2** results in a balance of pressure such that flow from the primary column is diverted at the junction between the columns (marked 'A') towards Detector 1. This set of pressures prevents the sample entering the second column, but does provide the carrier gas for the second column. This is supplied as excess pressure at the junction A.
b. Sample transfer – The second pressure configuration results in both columns being coupled in a sequential manner. A minor portion of the primary eluent is split at junction A to go to Detector 1, with the majority passing directly on to the secondary column.
c. Analysis of fraction-once the sample transfer is complete, the third pressure configuration is adopted. The carrier gas flow through the secondary column is maintained by excess pressure at junction A, supplied from regulator B. Concurrently, the primary column is backflushed by also using the pressure supplied from regulator B.

Following the backflush of the primary column and separation of the analytes on the second column, the system can then be returned to its original prefractionation position, ready for the next sample injection (Lewis, 2002). The most intriguing developments in GC include comprehensive new designs for two-dimensional gas chromatography (GC x GC) and

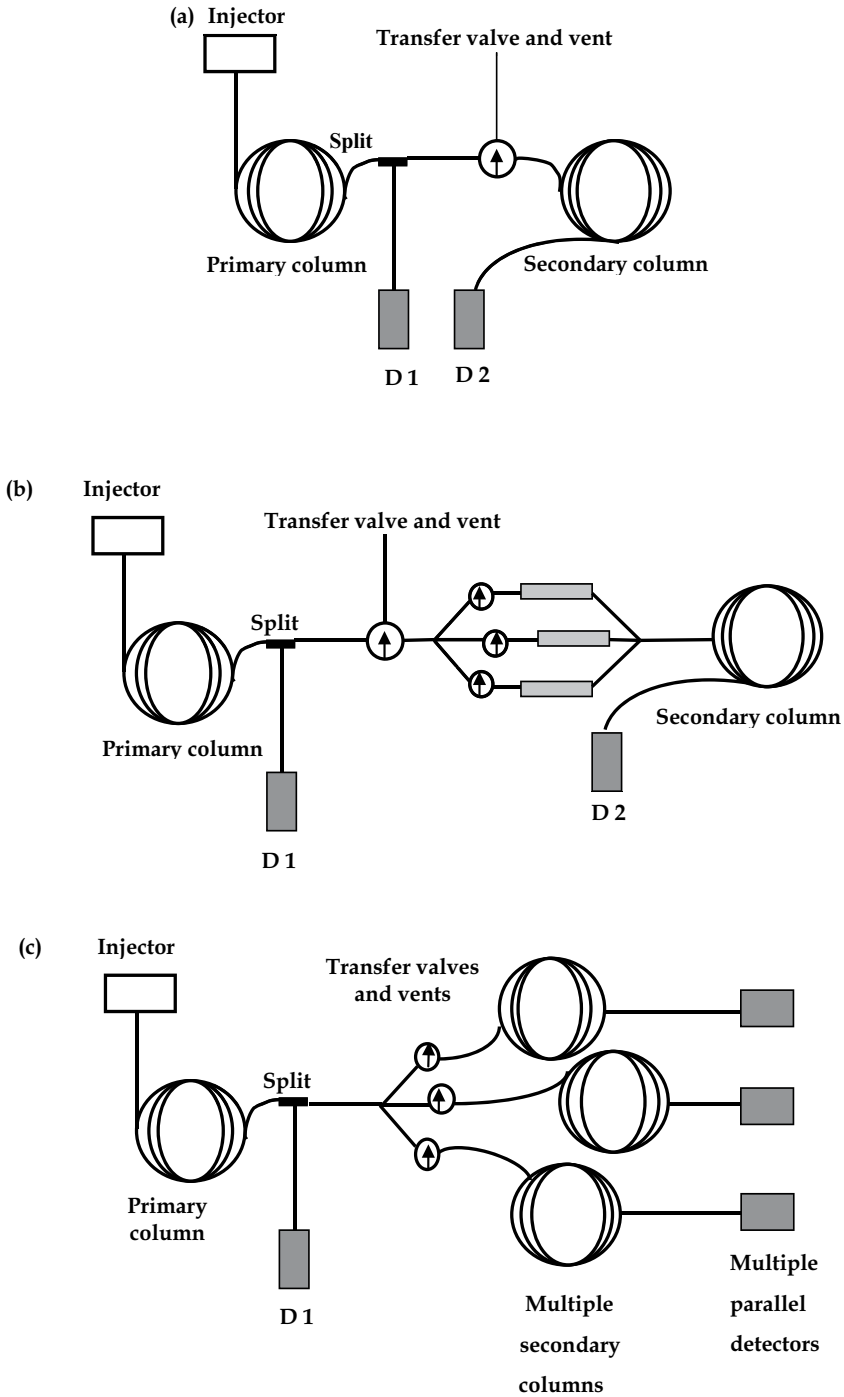


Fig. 1. Two-dimensional gas chromatography instrumental configurations: (a) direct transfer heart-cut configuration; (b) multiple parallel trap configuration; (c) multiple parallel column configuration; D 1 – first detector; D 2 – second detector (adapted from Lewis, 2002).

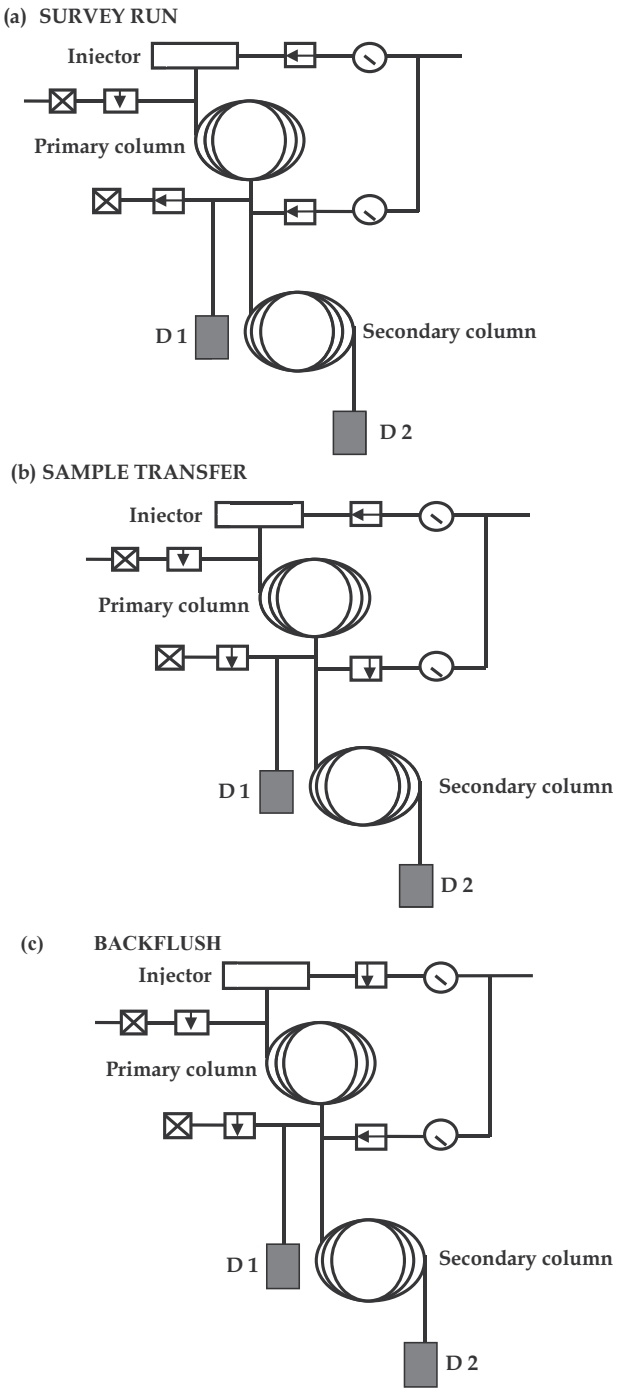


Fig. 2. Survey, sample transfer and backflush positions used during the non-intrusive DEANS heart-cut switching process; ⊖ - Carrier pressure regulation; ⊏ - On/off valve; ⊠ - Needle valve (adapted from Bertsch, 1990).

fluidic switching (DEANS switching) for multidimensional separations. Improvements in electronic pressure and flow control in modern GC instruments and the development of miniature and inert devices for column connections have greatly improved the performance and reliability of these techniques which has created new applications for these powerful techniques.

2.1.2 Heart-cutting Multidimensional Gas Chromatography (MDGC)

The heart-cutting multidimensional gas chromatography (MDGC) to determination of the enantiomeric fractions of *o,p′*-DDT was described (Muñoz-Arnanz et al., 2009). The MDGC system employed throughout consisted of two independent GC chromatographs, both equipped with a ^{63}Ni-ECD system. The two chromatographs were designated as the first and the second dimension chromatographs, referred to as the 1D and 2D chromatographs, respectively. The heart-cutting MDPC configuration used in the paper cited is shown in **Figure 3**.

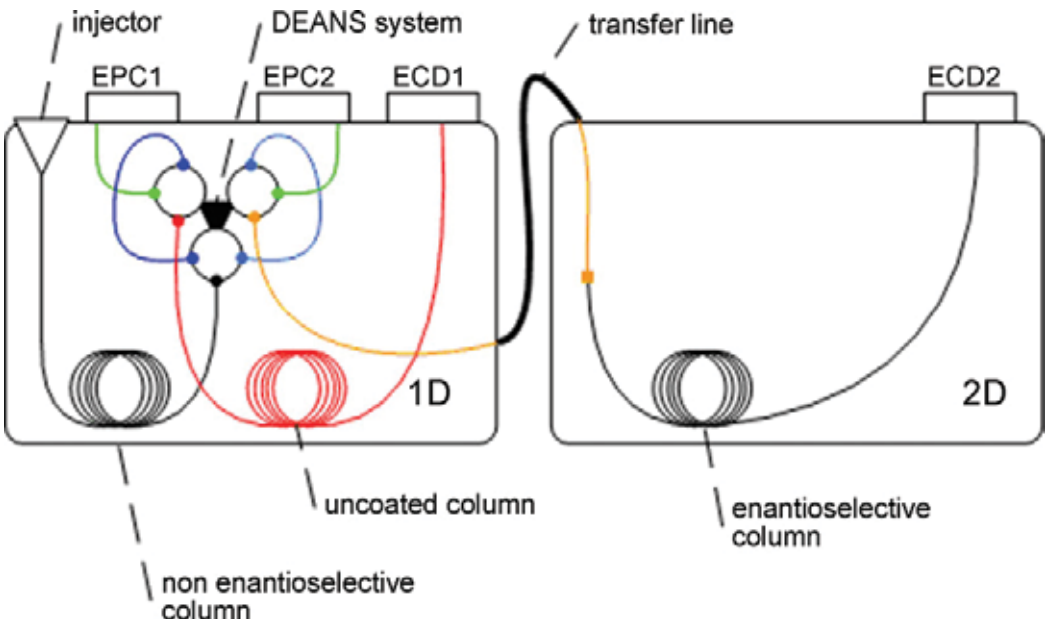


Fig. 3. Schematic diagram of the MDGC system used. Note the presence of the deactivated and uncoated fused silica capillary columns, which permits a total length from the injector to the detector in the 1D chromatograph equal to the distance that the analytes have to cover between the injector and the 2D ECD system (From Muñoz-Arnanz et al., 2009. With permission.).

Cuts or selected fractions were transferred from the first dimension column to the second using a system (DEANS), located in the 1D chromatographic oven, that was pneumatically controlled by means of two independent electronic pressure control (EPC) units. A temperature-controlled transfer line was directly connected to the DEANS switching system using press-fit connection. The instrumental LOD and LOQ were 2.1 pg μL^{-1} and 7.1 pg μL^{-1}, respectively for *o,p′*-DDT.

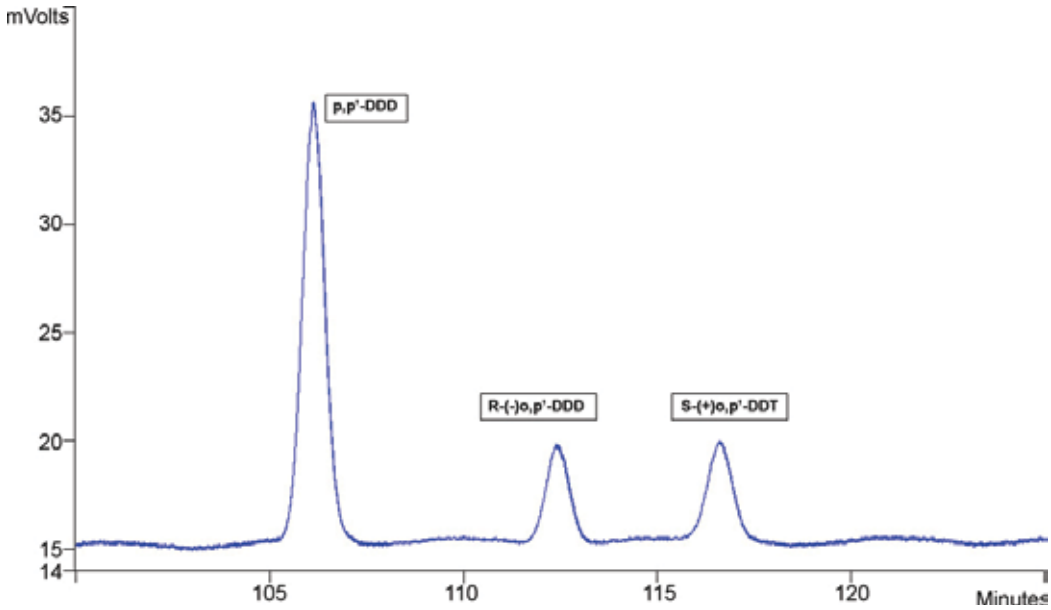


Fig. 4. Second dimension chromatogram showing the separation between *p,p′*-DDD and the two *o,p′*-DDT enantiomers (From Muñoz-Arnanz et al., 2009. With permission.).

As shown in **Figure 4**, a good separation between *p,p′*-DDT and both *o,p′*-DDT enantiomers was achieved. The 1D and 2D chromatograms obtained in the analysis of one soil sample are shown in **Figure 5**. The described MDGC method is reliable for determination of organochlorine pesticides in soil samples.

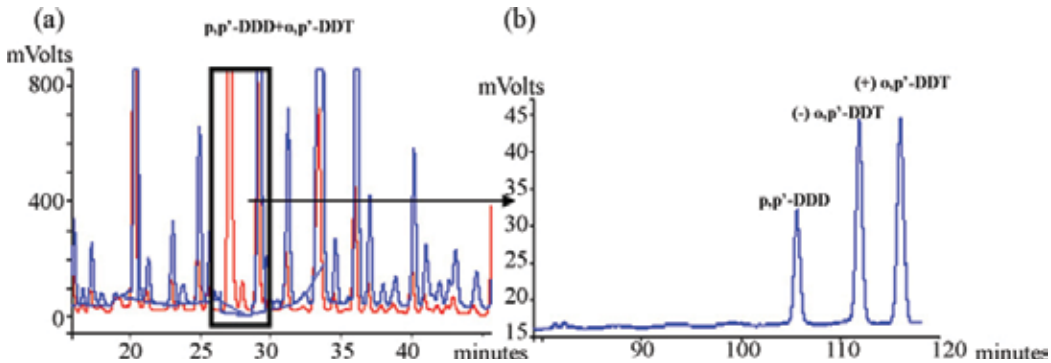


Fig. 5. (a) First dimension chromatogram showing the trace of the organohalogen compounds in a soil sample. Note the cut carried out between minutes 27 and 29. (b) Second dimension chromatogram showing the separation of the *o,p′*-DDT enantiomers from the rest of the species transferred in the same cut to the 2D system. (From Muñoz-Arnanz et al., 2009. With permission.).

2.1.3 Introduction to GC x GC separation

Single-column gas chromatography analysis has become the standard approach for measurement of volatile and semi-volatile constituents in numerous applications. However

the separation provided by conventional gas chromatography can be significantly enhanced by using comprehensive two-dimensional gas chromatography (GC x GC) instead. The comprehensive GC x GC technique was introduced by Phillips and co-workers (Liu and Phillips, 1991; Phillips and Xu, 1995; Phillips and Ledford, 1996). GC x GC technique have been reviewed (Geus et al., 1996; Bertsch, 1999, 2000; Phillips and Beens, 1999; Dallüge et al., 2003). Some fundamental and detailed information are included in book entitled 'Multidimensional Chromatography' edited by Mondello, Lewis and Bartle (John Wiley & Sons, 2002). A GC x GC system consists of two columns with different retention mechanisms, which are connected in series. In the truly multidimensional system, the separation mechanisms in the first and the second columns are different and independent of each other and the separation obtained in the first column is maintained during the modulation and separation in the second column. Generation and visualization of GC x GC chromatogram is shown in **Figure 6**.

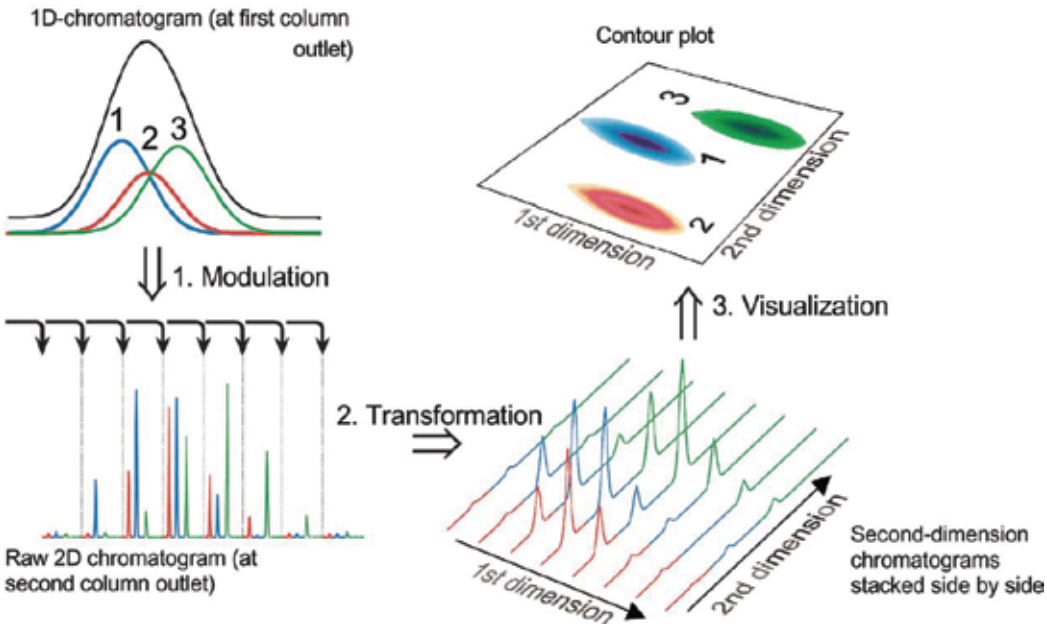


Fig. 6. Generation and visualization of a GC x GC chromatogram (From Dallüge et al., 2002. With permission.).

Peaks, or spots in the contour plot are identified by their retention times in the 1D and 2D, i.e., by their coordinates in the contour plot. Mass spectra for target analyte confirmation and/or the identification of unknowns can at present be obtained only from the chromatogram of the raw data (**Figure 6**, "raw 2D-chromatogram"). The total retention time of each peak of interest in the contour plot was calculated by adding the retention time from the first and second dimension. This total retention time is equivalent to the retention times in the raw 2D-chromatogram, where the peaks can be identified on the basis of their mass spectra, as is routinely done 1D-GC-MS. From the resulting peak table, both retention times (1D and 2D coordinates) were calculated to locate these peaks in the contour plot (Dallüge et al., 2002).

The two columns used may be operated at the same or independent temperatures. The sample is separated on the first column with conventional temperature program (most often 1-5°C per minute). During separation, small successive adjacent fractions of the eluate of the

first column are retained and focused at the beginning of the second column by means of a cryogenic modulator, which essentially works like a cold trap. After the trapping of each fraction, the modulator is switched off or moved away to effect the release of the retained analytes and the rapid injection on the second column (Dallüge et al., 2002). However, in practice, the sampling rate in the first dimension is limited by the duration of a single separation cycle in the second dimension. Thus, it would be advantageous to use as short a time for second-dimension to be efficient, it is advantageous to use longer time. Consequently, a compromise usually has to be struck between the first dimension sampling frequency and the second dimension separation time (Harynuk et al., 2005). Theoretical studies indicated that the optimum primary dimension sampling frequency is achieved when each primary dimension peak is sampled three to four times (Murphy et al., 2005).
The transfer of analyte from first dimension (1D) to second dimension (2D) by modulator and intervenes between the two dimensions gives important results in both dimensions, which arise from the modulation process. These are as follows (Marriott, 2002): (i) the zone to be passed (or more correctly, pulsed) from 1D to 2D must be compressed in space; (ii) the compressed zone must be delivered to 2D very rapidly, and as a sharp pulse; (iii) 2D must be capable of giving fast GC results, achieved by a combination or all of the following, i.e., a short column, thin film thickness, narrow i.d. column (giving high carrier linear velocity) and higher temperature (if a two-oven system is used); (iv) the peaks produced at the detector will be increased in peak height response due to the above process; (v) all of first column solute is transferred to the second column.
In practice, a number of mechanisms are used to enable refocusing and zone compression at the point of transfer, ranging from cryogenic and chemical micro-traps, to phase ratio refocusing at cooler over temperatures, when the secondary column is held at temperatures independent of the first column.
Overloading of the second dimension column in comprehensive two-dimensional gas chromatography separations can have a very significant effect on the available separation space in this dimension. The pesticides concentration in the samples are usually small and then narrow diameter columns should be used in second dimension of GC x GC experiment. Narrow columns have significant advantages, most important of which is the separation speed. It is much easier to obtain a very fast separation speed with narrower peaks under conditions of no overload in the second dimension. When analyzing samples in which the concentrations of the analytes or matrix components are unknown and may be high, it may be better to use larger diameter columns in the second dimension. In addition, this may lead to better overall resolution in the second dimension, unless the carrier gas flow rate in the system with the narrow second dimension column is reduced significantly (which lengthens the overall analysis time and might result in less efficient separation in the first dimension). It has been reported that it may actually be better to use columns with the same diameter in both dimensions in preliminary GC x GC experiments (Harynuk et al., 2005).

2.1.4 Comprehensive two-dimensional Gas Chromatography (GC x GC) applied to analysis of pesticides

As described earlier, substantial improvements in two-dimensional GC were not forthcoming until Phillips and his research group introduced and implemented an entirely new form of Two-Dimensional Gas Chromatography, called comprehensive two-dimensional GC, or GC×GC. The full sample is separated on a first, usually long conventional column, and then the entire sample is subjected to the second short column (in repeated cycles, hence

comprehensive), the eluting compounds are focused (usually cryo focused) in space and pulse-injected into a second column for their second dimension separation. A common phase selection strategy uses in first dimension separation of analytes according to their boiling points, whilst the second dimension separates analytes according to polarity.
In the literature, not too much attention has been devoted to the trace-level determination of pesticides, because there are many detailed protocols for their precise and accurate analysis by mean of 1D-GC-MS. The GC x GC technique has few main limitations (Poliak et al., 2008): (i) high price; (ii) difficult and expensive maintenance; GC x GC technique with thermal modulation requires inconvenient and costly maintenance in the form of daily consumption of carbon dioxide or liquid nitrogen; (iii) problematic compatibility with mass spectrometry; GC x GC technique with thermal modulation generates narrow GC peaks with less than 100 ms width, that are hard to combine with standard QMS, thus seems to require fast and expensive time-of-flight mass spectrometry (TOFMS). Nevertheless, now the GC x GC technique is more often applied as screening method for various groups of volatile and semivolatile analytes, because GC x GC (especially TOFMS) is a very promising powerful tool for identification and determination of pesticides. **Figure 7** shows diagram of modern GC x GC-TOFMS instrument. GC x GC provides unsurpassed resolution capability, the most pressing need is to prove that the implied complexity of a mixture is represented by the multitude of peaks spread about 2D space.

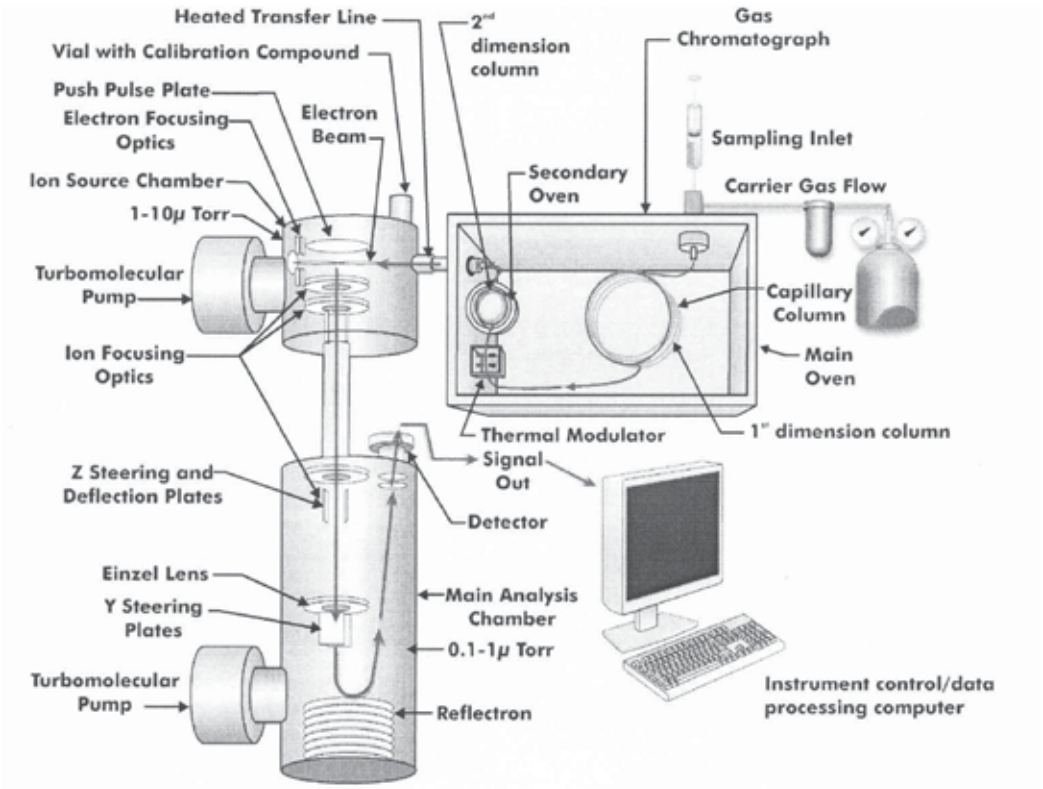


Fig. 7. Diagram of modern GC x GC – TOF MS instrument (From LECO Corporation, 2008. With permission.).

Determination of 287 pesticides at trace levels by GC x GC – TOFMS was described (Koning and Gumpendobler, 2007). GC x GC – TOFMS was able to analyze hundreds of pesticides in a single run. The use of GC x GC – TOFMS provides an enhanced separation mechanism that helps eliminate coelutions and provides users with a very structured and visually stimulating chromatogram (Koning and Gumpendobler, 2007).
As seen by viewing the second dimensions of the GC x GC contour plot in **Figure 8**, many coelutions would occur when analyzing in a single dimension mode. **Figure 9** displays a zoomed section of the contour plot indicating the coelution of four pesticides along the second dimension. However, these four pesticides are separated due to the selectivity of the second orthogonal separation (Koning and Gumpendobler, 2007). The separation of toxic analytes including 11 persistent halogenated pesticides by GC x GC – TOFMS was described (Focant et al., 2003). The feasibility of the coupling between GC x GC – TOFMS and the thermal desorption-programmable temperature vaporization injector was demonstrated from quantitative point of view. **Figure 10** illustrates the process responsible for the sharp re-injections of trapped analytes into the second column.
Especially, the determination of pesticides in complex matrices, such as food, often requires modern, rapid and universal methods. Comprehensive two-dimensional gas chromatography and isotope dilution time-of-flight mass spectrometry (GC x GC-IDTOFMS) for the simultaneous measurement of selected organochlorine pesticides (OCPs), polychlorinated biphenyls and bromide flame retardants in human serum and milk was described (Focant et al., 2003). The GC x GC-IDTOFMS results demonstrated the efficiency of this multianalyte measurement approach for such important matrices as serum and milk for human biomonitoring. **Figure 11** illustrates GC x GC-TOFMS chromatogram of the 11 persistent organochlorine pesticides in a human sample.

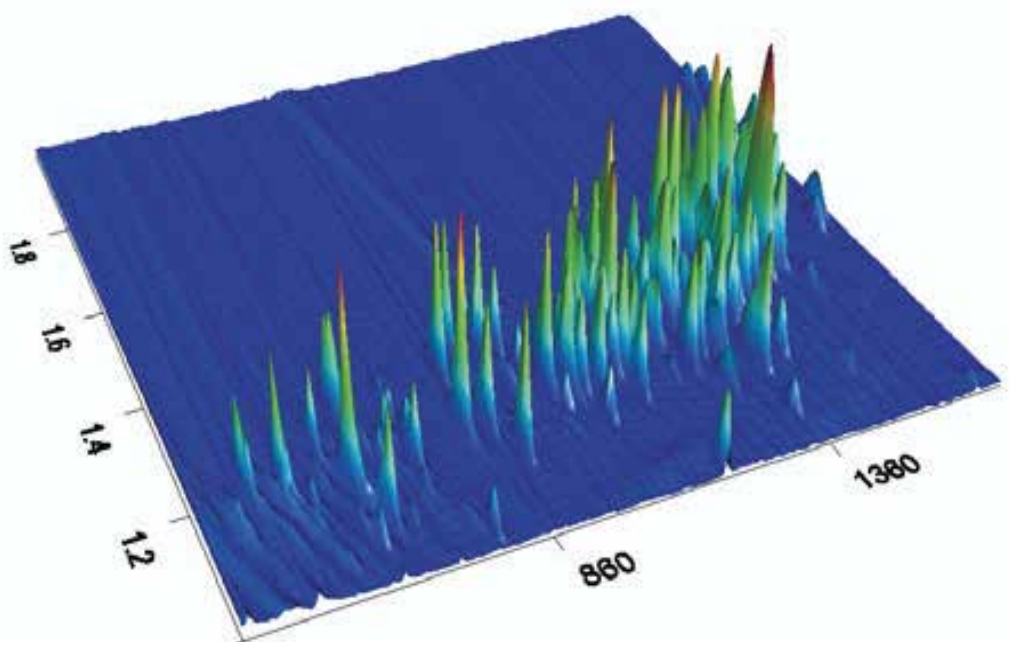


Fig. 8. GC x GC chromatogram for 287 investigated pesticides (From Koning and Gumpendobler, 2007. With permission.).

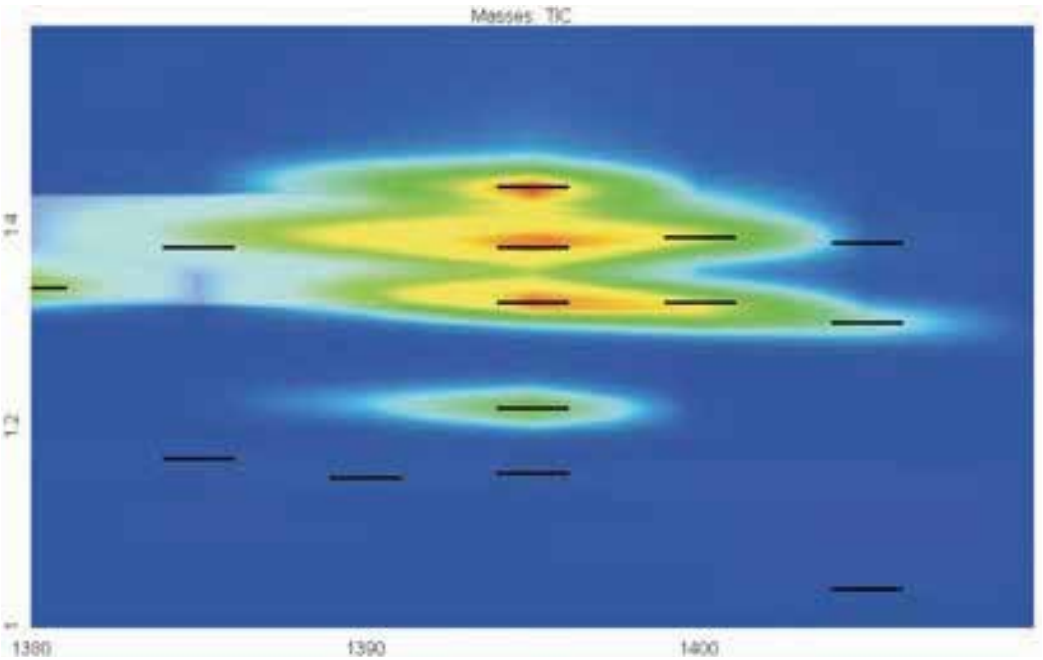


Fig. 9. Zoomed contour plot indicating coelution in the first dimension, but four analytes separation in the second dimension (From Koning and Gumpendobler, 2007. With permission.).

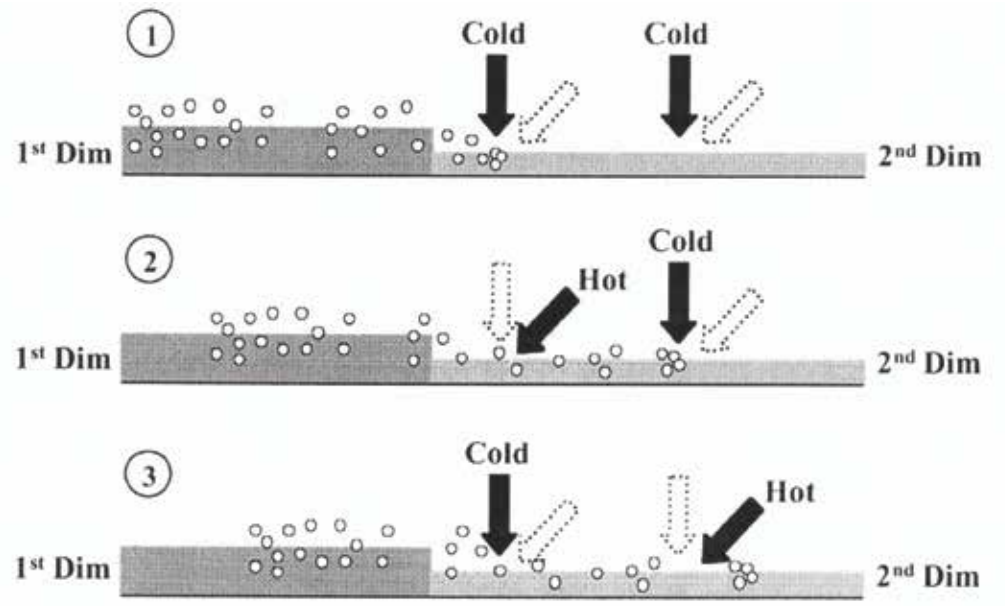


Fig. 10. Sequence of events responsible for (1) trapping, (2) releasing and re-focusing, and (3) re-injecting into the second column using a quad-jet cryo-modulator (From Focant et al., 2003. With permission.).

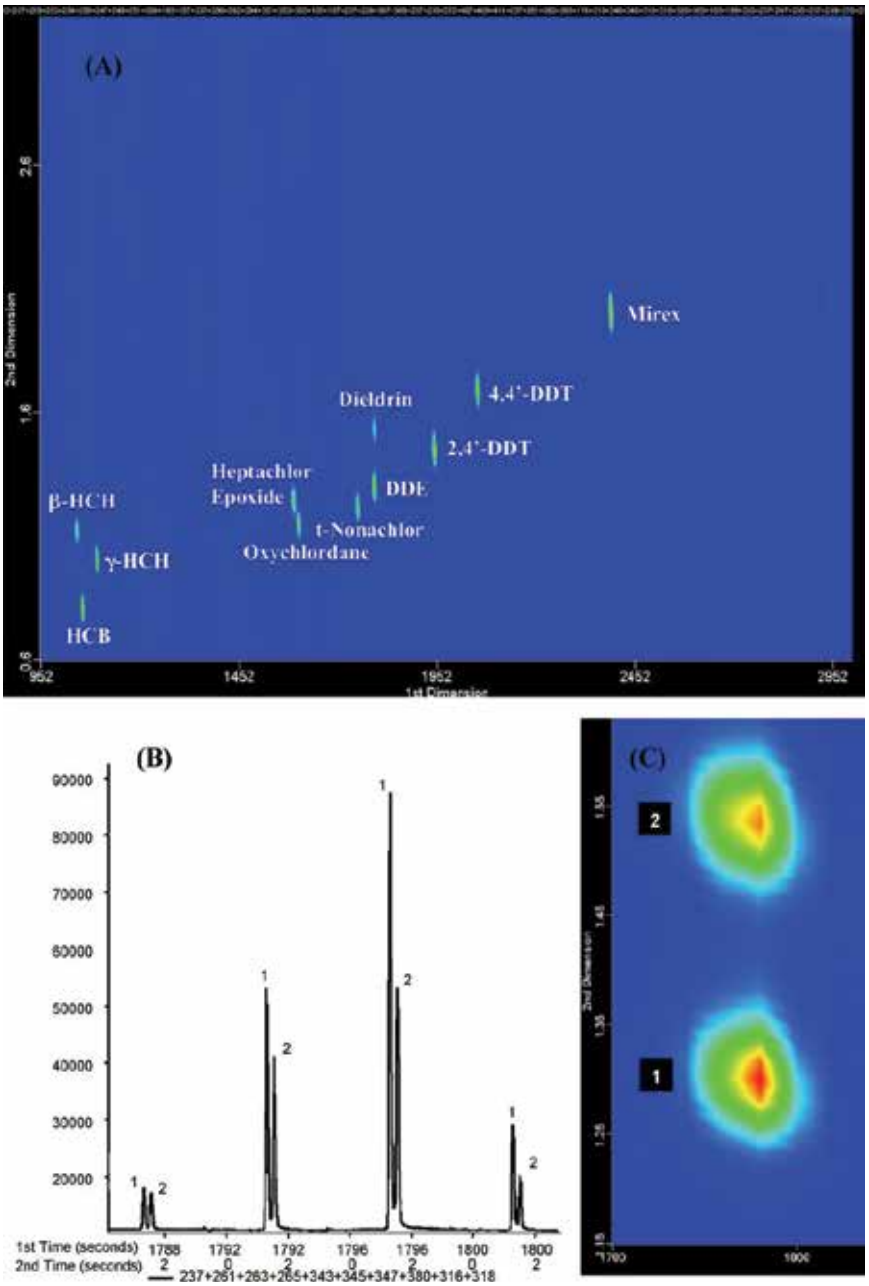


Fig. 11. (A) GC×GC–TOFMS chromatogram of the 11 persistent pesticides analyzed in human sample. The signal was reconstructed using six to eight characteristic ions for each compound (deconvoluted ion current traces). (B) A closer look at the region of the chromatogram where DDE (1) and dieldrin (2) eluate. The chromatogram has been reconstructed using the sum of the characteristic ions of the two species. (C) Shows the cluster corresponding to the 'slices' that can be recombined to produce the GC×GC contour plot shown in (B) (From Focant et al., 2003. With permission.).

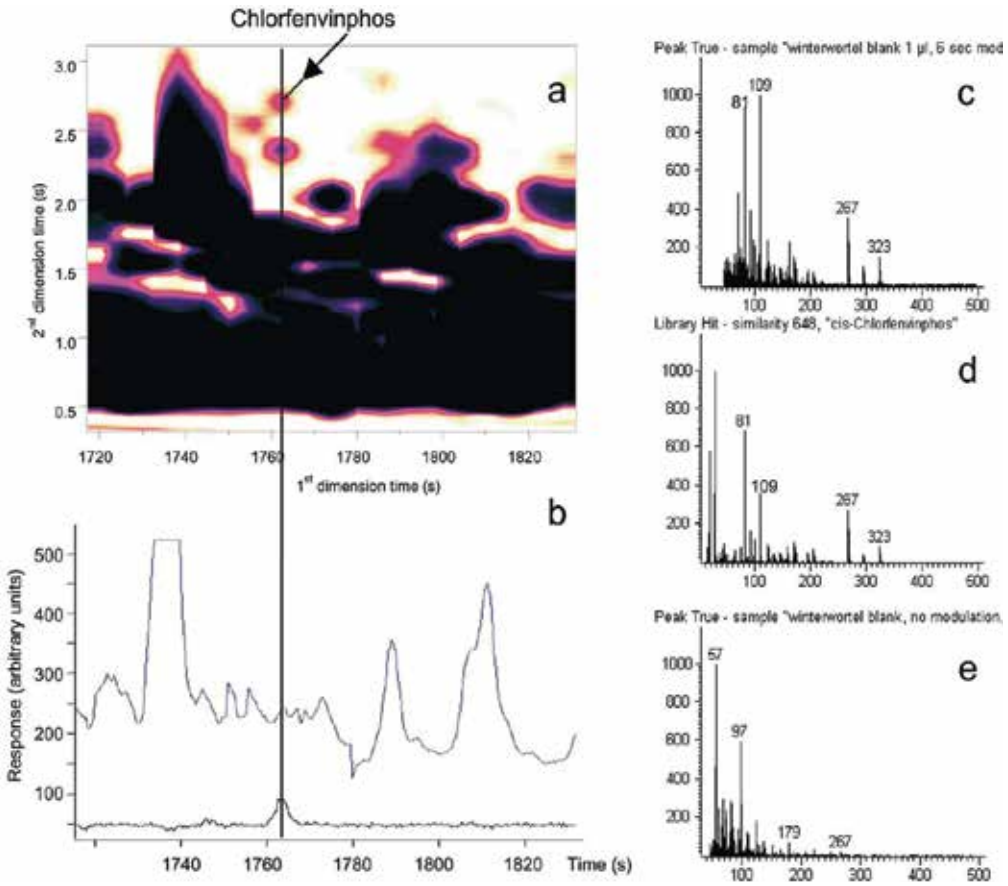


Fig. 12. GCxGC–TOF MS versus 1D-GC–TOF MS for the analysis of a carrot extract. (a) GCxGC–TOF MS contour plot. (b) 1D-GC–TOF MS chromatogram of the same region; upper trace, TIC scaled to 1%; lower trace, m/z 323 ion trace. (c) Mass spectrum obtained after GCxGC separation showing the characteristic m/z values of chlorfenvinphos (m/z 81, 109, 267, 295, 323). (d) Library spectrum of chlorfenvinphos and (e) spectrum obtained at the retention time of chlorfenvinphos after 1D-GC separation (From Dallüge et al., 2002. With permission.).

In another study (Zrostlíková et al., 2003), authors demonstrated GC x GC – TOFMS as a powerful tool for solving the problems with reliable confirmation of pesticide residues at very low concentration levels as required for the analysis of some types of samples of baby food. The most troublesome matrix interferences observed in apple and peach were eluted as very broad asymmetric peaks completely overlapping the peaks of pesticides in the first dimension "boiling-point" separation. However, thanks to differing retention of these co-extracts they were in most cases efficiently separated in the second dimension of GC x GC experiments. The limits of detection for most pesticides were well below 10 ng/mL and the reliable confirmation of analyte identity was possible at 10 ng/mL level for typically troublesome pesticides such as polar organophosphorus pesticides, methamidophos or acephate (Zrostlíková et al., 2003). In another paper, authors (Dallüge et al., 2002)

demonstrated the dramatically improvement of separation in comparison with conventional GC by employing GC x GC – TOFMS with cryogenic modulator. All analyzed 58 pesticides in food extracts could be identified using their full-scan mass spectra, which was not possible when using 1D-GC-TOFMS. GC x GC-TOFMS versus 1D-GC-TOFMS for the analysis of a carrot extract is shown in **Figure 12**.

Comprehensive two-dimensional gas chromatography (GC x GC) coupled with nitrogen-phosphorus detector (NPD) and with micro electron-capture detector (μECD) was applied for the separation and quantitation of fungicides in vegetable samples (Khummueng et al., 2006). The comparison of different column sets and selection of the temperature program were carried out with a mixture of nine N-containing fungicides, eight of which were chlorinated. Both techniques: GC x GC-NPD and GC x GC- μECD were compared. The non-polar/polar column set used in GC x GC, with thicker film phase in the 2D column gives symmetric, non-tailing peaks in GC x GC analysis with NPD detector. The limit of detection (LOD) and limit of quantitation (LOQ) were less than about 74 and 246 ng L^{-1}. The described study shows that GC x GC-NPD has a potential for the routine analysis of fungicides in food and vegetables samples, providing a low LOD and LOQ and a good repeatability and reproducibility of peak response (Khummueng et al., 2006). Determination of pesticide residues in grapes samples by GC x GC- μECD and GC x GC coupled with flame ionization detector (FID) was also described (Pizzuti et al., 2009). In this study, a new compressed air modulator was used in GC x GC- μECD experiments. **Figure 13** shows dual-stage

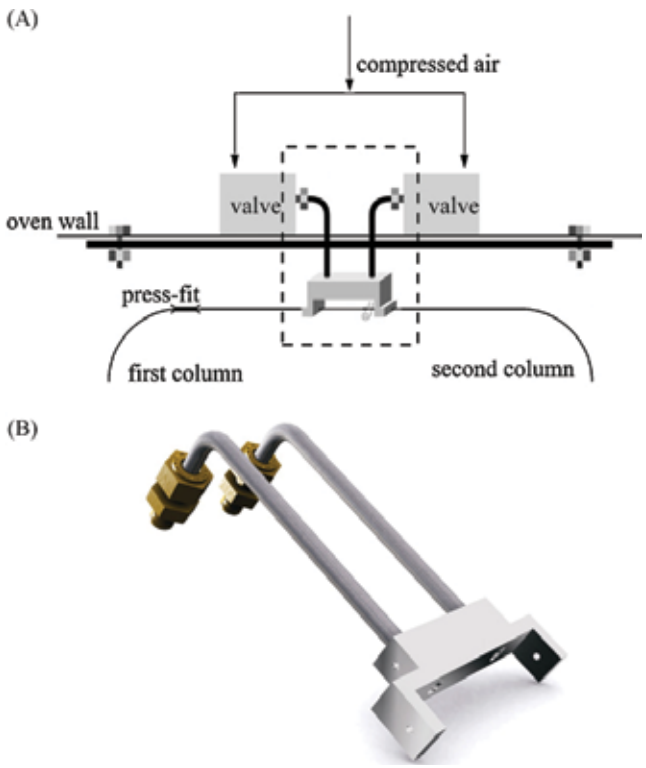


Fig. 13. (A) Schematic representation and (B) a photograph of the dual-stage compressed air modulator for GC×GC (From Pizzuti et al., 2009. With permission.).

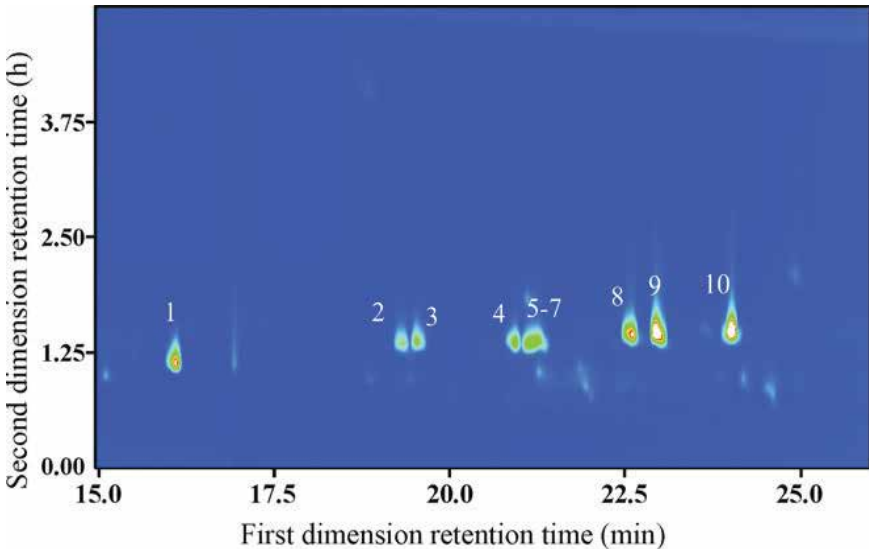


Fig. 14. GC×GC-μECD contour plots for a matrix extract spiked at 0.05mgkg^{-1} with pyrethroid pesticides. Target compounds are numbered as follows: (1) byfenthrin; (2) *cis*-permethrin; (3) *trans*-permethrin; (4–7) cypermethrin (I, II, III, IV); (8) fenvalerate; (9) esfenvalerate; (10) deltamethrin. (From Pizzuti et al., 2009. With permission.).

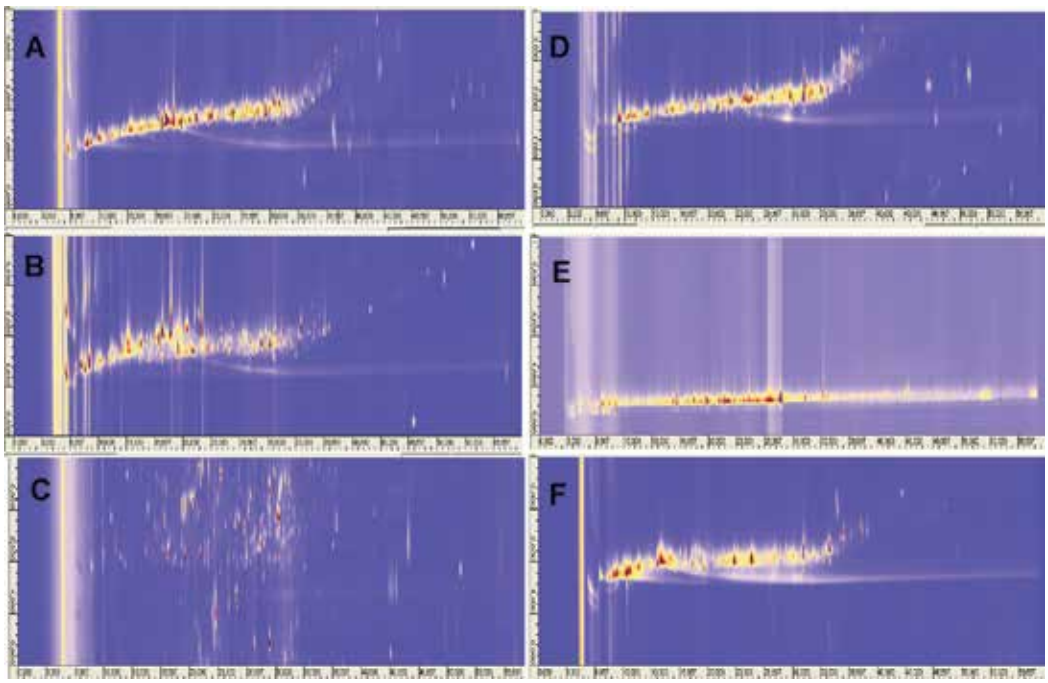


Fig. 15. Analysis of a non-spiked orange extract by GC×GC–μECD using (A) ZB-5×HT-8, (B) ZB-5×BPX-50, (C) ZB-5×SW10, (D) DB-17×HT-8, (E) DB-17×BPX-50 and (F) HT-8×BPX-50 as column combinations. (From Ramos et al., 2009. With permission.).

compressed air modulator for GC×GC instrument. This modulator uses compressed air to cool two small portions in the first centimeters of the second chromatographic column of a comprehensive multidimensional gas chromatography system. The GC x GC system proposed was applied in the determination of pyrethroid pesticides (bifentrin, cypermethrin, deltamethrin, fenvalerate, esfenvalerate, *cis*- and *trans*-permethrin) in grape samples.
Figure 14 shows GC x GC- μECD contour plots for a matrix extract spiked at 0.05 mg kg^{-1} with pyretroid pesticides. The values of method (GC x GC- μECD) limit of quantification (LOQ) were 0.01-0.02 mg kg^{-1} for all pyrethroids and the values of recovery were between 94.3 and 115.2% with good precision, RSD < 18.4% (Pizzuti et al., 2009).
Determination of three classes of pesticides (organophosphorus pesticides, triazines and pyrethroids) in selected fruits, i.e., orange, apple, pear and grape by GC x GC- μECD also was described (Ramos et al., 2009). Six column combination with different polarities were tested (**Figure 15)**. Firstly, an apolar phase, ZB-5, was combined with phases of increasing polarity, i.e. BPX-50 and SW, and of different selectivity, i.e., HT-8. Secondly, a more polar phase, DB-17, was tested as first dimension in combination with HT-8 and BPX-50 as second dimension columns (Ramos et al., 2009). The proposed GC x GC- μECD allowed accurate determination of the analytes at levels far below the MRLs set in current EU legislations even if no further concentration of the collected extract was carried out.

2.2 Mutidimensional Liquid Chromatography (MDLC) applied to analysis of pesticides

2.2.1 Introduction to Mutidimensional Liquid Chromatography (MDLC)

Multidimensional chromatography (also known as coupled-column chromatography (LC-LC coupling) or column switching) represents a powerful tool and an alternative procedure to classical one-dimensional high performance liquid chromatography. Multidimensional liquid chromatography (MDLC) separation has been defined as technique which is mainly characterized by two distinct criteria: (i) the first criterion for a multidimensional system is that sample components must be displaced by two or more separation techniques involving orthogonal separation mechanisms; (ii) the second criterion is that components that are separated by any single separation dimension must not be recombined in any further separation dimension (Giddings, 1984; 1987).
Multidimensional liquid chromatography (MDLC) can be performed as in MDGC either in on-line or off-line mode. With off-line operation, the fractions eluted from the primary column are collected manually or by a fraction collector and then reinjected, either with or without concentration, into a second column. This approach has the advantage of being simple, does not need any switching valve, and the mobile phases in each column need not be mutually compatible. These mode has also disadvantages: (i) more time-consuming; (ii) procedure are labour-intensive; (iii) sample loss or contamination during handling; (iv) recovery of sample is low. On-line mode of MDLC have the advantage of automation by using pneumatic or electronically controlled valving, which switches the column effluent directly from the primary column into the secondary column. The on-line technique is more reproducible, no loss off sample and contamination occurs. Automation improves reliability and sample throughput, and shortens the analysis time, as well minimizes sample loss or change since the analysis is performed in a closed-loop system (Corradini, 2002).
What characterizes coupled-column chromatography (LC-LC coupling) when compared to conventional multistep chromatography is the requirement that the whole chromatographic process be carried out on-line. The transferred volume of the mobile phase from the first

column to the second column (from 1D to 2D) 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 (Corradini, 2002).

LC-LC coupling can be subdivided into both systems (Regnier & Huang, 1996):

- homomodal LC-LC is a type of development, in which the chromatographic improvement occurs by switching columns of analogous selectivity. The goal is mainly to optimize an already satisfactory separation, that is, to concentrate a dilute sample (sample enrichment) or to shorten the analysis time;
- heterogenical LC-LC is a type of development achieved by varying the separation mechanism during the separation process; selectivity changes may be made by varying the nature of the stationary phase, which can posses complementary separation characteristics.

The term '*LC-LC*', and more generally 'multidimensional', is usually restricted to heterogenical LC-LC system, which involve separation modes which are as different as possible (orthogonal), and in which there is a distinct difference in retention mechanisms (Giddings, 1984).

The LC-LC separation system may be used in both modes (Regnier & Huang, 1996):

- profiling mode is to separate all single components from multicomponent, complex sample. In this mode every component from the first column (primary column, 1D) is fractionated and transferred to the second column (secondary column, 2D);
- targeted mode is to isolate either a single or a few components of similar retention in a complex sample containing components having a wide range of capacity factor values. Targeted LC-LC analysis is carried out by transferring a wide or narrow cut of the chromatographic effluent from the 1D to 2D by flow switching and the mobile phase is thereby diverted or reserved. The fraction of interest to be transferred to a secondary column may consist of early-eluting analytes (first eluted zone, the 'front-cut'), or components eluted in the middle of the chromatographic effluent ('heart-cut') or at the end of the chromatogram ('end-cut') (Ramsteiner, 1988).

Multidimensional liquid chromatography (MDLC) has been applied to analysis of non-volatile pesticides, but presently the MDLC is used generally rather less often than MDGC.

2.2.2 Coupled-column chromatography (LC-LC Coupling) applied to analysis of pesticides

Screening and analysis of polar pesticides based on coupled-column reversed-phase (RP) liquid chromatography (LC-LC) and GC-MS has been used as a powerful tool in the execution of environmental monitoring programmes (Hogendoorn et al., 1996). Reversed-phase liquid chromatography in combination with UV detection is an attractive technique for the analysis of polar pesticides in aqueous samples. It is robust, rugged and allows the direct injection of aqueous samples, without the need for extraction, derivation or other sample manipulations. A relative disadvantage is that the sensitivity attainable with UV detection is usually insufficient for trace analysis. Consequently, a sample preconcentration step is needed for sensitive analysis. The compatibility of the mobile phase system with aqueous samples allows on-line sample enrichment by large volume injections in combination with LC column switching techniques. Authors shows LC – LC (RP in both dimensions) examples of chromatograms of an extract of a raw drinking water sample indicating a monolinuron residue of 0.1 μg L^{-1} and ground water sample containing 0.08 μg L^{-1} ETU (Hogendoorn et al., 1996). The coupled-column (LC-LC) configuration consisting of

a 3 µm C_{18} column (50 x 4.6 mm I.D.) as the first column and a 5 µm C_{18} semi-permeable-surface (SPS) column (150 x 4.6 mm I.D.) as the second column appeared to be suitable for screening of acidic pesticides in surface water samples (Hogendoorn et al., 1999). In comparison to LC-LC employing two C_{18} stationary phases in both dimensions, the combination of C_{18} and SPS-C_{18} columns significantly decreased the baseline deviation caused by the hump of the humic substances when using UV detection. The developed LC-LC procedure allowed the simultaneous determination of the target analytes bentazone and bromoxynil in uncleaned extracts of surface water samples to a level 0.05 µg L^{-1} in less than 15 min. **Figure 16** shows example of LC-LC chromatogram with two acidic herbicides separated on the C_{18} (1D) and SPS-C_{18} (2D) columns combination (Hogendoorn et al., 1999).

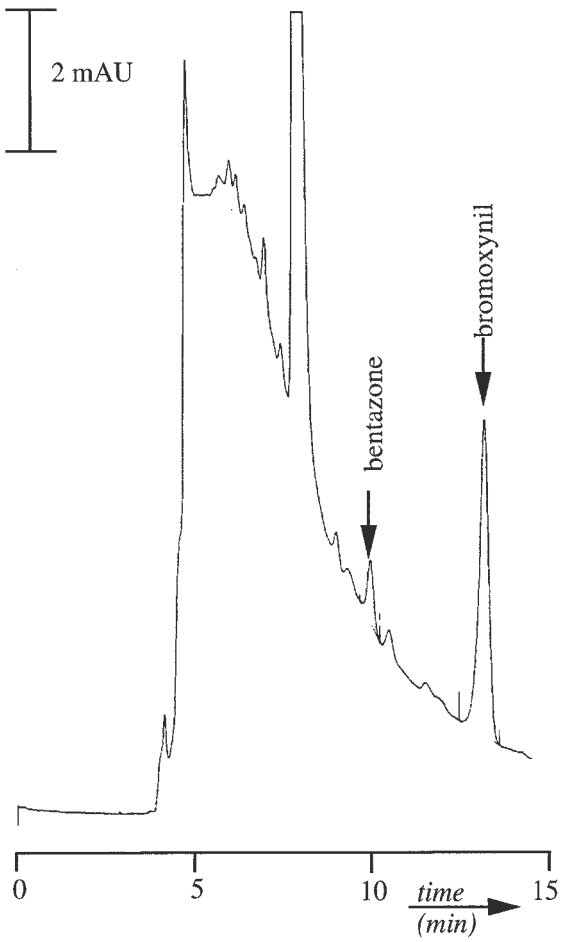


Fig. 16. LC-LC-UV (217 nm) chromatogram with the C_{18} (1D) and SPS-C_{18} (2D) columns combination of an extract of ditch surface water sample containing 0.37 µg L^{-1} of bentazone and 2.3 µg L^{-1} of bromoxynil (From Hogendoorn et al., 1999. With permission.).

Concerning the determination of analytes involved in the studies mentioned above coupled-column LC-LC methods were used for analysis of ETU (Hogendoorn et al., 1991) and chlorophenoxy acid herbicides (Sancho-Llopis et al., 1993).

2.3 Mutidimensional Planar Chromatography (MDPC) applied to analysis of pesticides

2.3.1 Introduction to Mutidimensional Planar Chromatography (MDPC)

The application of multidimensional planar chromatography combined with different separation systems and modes of chromatogram development is often necessary for performing the separation of more complicated multicomponent mixtures. High separation efficiency can be obtained using modern planar chromatographic techniques which comprise two-dimensional development, chromatographic plates with different properties, a variety of solvent combinations for mobile phase preparation, various forced-flow techniques and multiple development modes. By combination of these possibilities, multidimensional planar chromatography (MDPC) can be performed in various ways. Giddings defined multidimensional chromatography as a technique which includes two criteria (Giddings, 1990):

- the components of the mixture are subjected to two or more separation steps in which their migration depends on different factors,
- when two components are separated in any single step, they always remain separated until completion of the separation.

Nyiredy divided multidimensional planar chromatography techniques as follows (Nyiredy, 2001, 2002, 2003):

- comprehensive two-dimensional planar chromatography (PC x PC) – multidimensional development on the same monolayer stationary phase and two developments with different mobile phases or using a bilayer stationary phase and two developments with the same or different mobile phases;
- targeted or selective two-dimensional planar chromatography (PC + PC) – technique, in which following the first development from the stationary phase a heart-cut spot is applied to a second stationary phase for subsequent analysis to separate the compounds of interest;
- targeted or selective two-dimensional planar chromatography (PC + PC) – second mode – technique, in which following the first development, which is finished and the plate dried, two lines must be scraped into the layer perpendicular to the first development and the plate developed with another mobile phase, to separate the compounds that are between the two lines. For the analysis of multicomponent mixtures containing more than one fraction, separation of components of the next fractions should be performed with suitable mobile phases;
- modulated two-dimensional planar chromatography (^{n}PC) – technique, in which on the same stationary phase the mobile phases of decreasing solvent strengths and different selectivities are used;
- coupled-layer planar chromatography (PC-PC) – technique, in which two plates with different stationary phases are turned face to face (one stationary phase to second stationary phase) and pressed together so that a narrow zone of the layers overlaps, the compounds from the first stationary phase are transferred to the second plate and separated with a different mobile phase;
- combination of multidimensional planar chromatography methods – technique, in which the best separation of multicomponent mixture is realized by parallel combination of stationary and mobile phases, which are changed simultaneously. By use of this technique, e.g., after separation of compounds in the first dimension with changed mobile phases, the plate is dried and separation process is continued in perpendicular direction by use of the grafted technique with changed mobile phase (based on the idea of coupled TLC plates, denoted as graft TLC in 1979 (Pandey et al., 1979).

2.3.2 Multidimensional Planar Chromatography (MDPC) applied in pesticide analysis

2.3.2.1 Comprehensive Two-dimensional (2D) Chromatography on One Adsorbent

One of the most attractive features of planar chromatography is the ability to operate in the two-dimensional (2D) mode. Two-dimensional TLC (2D-TLC) is performed by spotting the sample in the corner of a square chromatographic plate and by development in the first direction with the first eluent. After the development is completed the chromatographic plate is then removed from the developing chamber and the solvent is allowed to evaporate from the layer. The plate is rotated through 90º and then developed with the second solvent in the second direction which is perpendicular to the direction of the first development. In 2D-TLC the layer is usually of continuous composition, but two different mobile phases must be applied to obtain a better separation of the components. If these two solvent systems are of approximately the same strength but of optimally different selectivity, then the spots will be distributed over the entire plate area and in the ideal case the spot capacity of the two-dimensional system will be the product of the spot capacity of the two constituent one-dimensional systems. If the two constituent solvent systems are of the same selectivity but of different strengths, the spots will lie along a straight line; if both strength and selectivity are identical, the spots will lie along the diagonal.
Computer-aided techniques enable identification and selection of the optimum mobile phases for separation of different groups of compounds. The first report on this approach was by Guiochon and co-workers, who evaluated ten solvents of fixed composition in two-dimensional separation of nineteen dinitrophenyl amino acids chromatographed on polyamide layers (Gonnord et al., 1983). The authors introduced two equations for calculation of the separation quality – the sum of the squared distances between all the spots, D_A, and the inverse of the sum of the squared distances between all the spots, D_B. Steinbrunner et al. (Steinbrunner et al., 1986) proposed other functions for identification of the most appropriate mobile phases – the distance function DF and the inverse distance function IDF, which are the same form as D_A and D_B, respectively, but which use distances rather than the squares of distances. The planar response function PRF has been used as optimization criterion by Nurok et al. (Nurok et al., 1987). Strategies for optimizing the mobile phase in planar chromatography (including two-dimensional separation) (Nurok, 1989) and overpressured layer chromatography (including two-dimensional overpressured layer chromatography) (Nurok et al., 1997) have also been described. Another powerful tool is the use of graphical correlation plots of retention data for two chromatographic systems which differ with regard to modifiers and/or adsorbents (De Spiegeleer et al., 1987). The interpretation of plots is illustrated in **Fig. 17**. The plots on **Figure 17** indicate then directly the positions of spots on a two – dimensional chromatograms (2D-TLC). As shows **Figure 17F** the best separation of complex mixtures by 2D-TLC is possible with differentiated R_F values in both systems, then the correlation plots of retention parameters for two chromatographic systems are poor (Tuzimski & Soczewiński, 2002). Good separation can be achieved when the spots are spread over the whole of the chromatographic plate area (Tuzimski, 2004; Tuzimski & Soczewiński, 2002a-d; Tuzimski & Bartosiewicz, 2003). The largest differences were obtained by combination of normal-phase (NP) and reversed-phase (RP) systems with the same chromatographic layer, e.g., cyanopropyl (Tuzimski & Bartosiewicz, 2003; Hauck et al., 1996). An example of this type of 2D development is illustrated in **Figure 18d**.

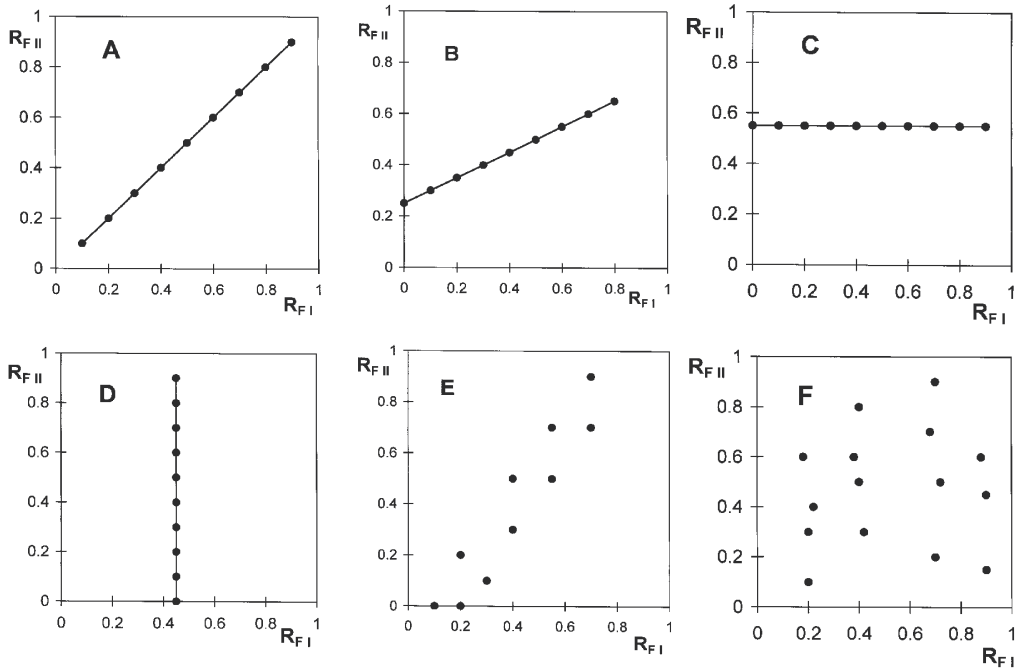


Fig. 17. Characteristic correlations $R_{F\,II}$ vs. $R_{F\,I}$ (From Tuzimski & Soczewiński 2002a, With permission.).

2.3.2.2 Two-dimensional thin-layer chromatography

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' (Hubert et al., 1988, Dzido, 2001). An example of this type of 2D development is illustrated in **Figure 18a-d**. **Figure 18d** 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 & Nyriedy, 1996) described the technique of joining two different adsorbent layers to form a single plate. Also large 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, 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 & Soczewiński, 2002a-d; Tuzimski & Bartosiewicz, 2003). Tuzimski and Soczewiński as first used bilayer Multi K plates for separation of complex mixtures (**Figure 18b**) (Tuzimski & Soczewiński, 2002a-d; Tuzimski & Bartosiewicz, 2003).

Method development for 2D-TLC of complex mixtures can be formulated as follows (Tuzimski & Soczewiński, 2002a):

- determine R_F vs. % modifier plots for polar adsorbent and nonaqueous eluents composed of heptane (or hexane) and 2-3 polar modifiers; choose compositions of eluents for optimal differentiated retention of the components (in the range 0.05 – 0.70)

- determine R_F vs. % modifier concentration plots for aqueous RP systems (octadecyl, cyanopropyl silica or other polar adsorbents) for methanol and acetonitrile modifiers and choose optimal concentration of modifier
- correlate the R_F values for NP/RP combinations and choose that corresponding to optimal spacing of spots on the plate area
- use the optimal combination of NP/RP eluents for a bilayer or monolayer plate (silica, cyanopropyl silica, etc.).

Horizontal chambers can be easily used for two-dimensional separations. The only problem seems to be the sample size. In a conventional two-dimensional separation used for analytical purposes the sample size is small. The quantity of the sample can be considerably increased when using a spray-on technique with an automatic applicator. Soczewiński and Wawrzynowicz have proposed a simple mode to enhance the size of the sample mixture with the ES horizontal chamber (Soczewiński & Wawrzynowicz, 1981).

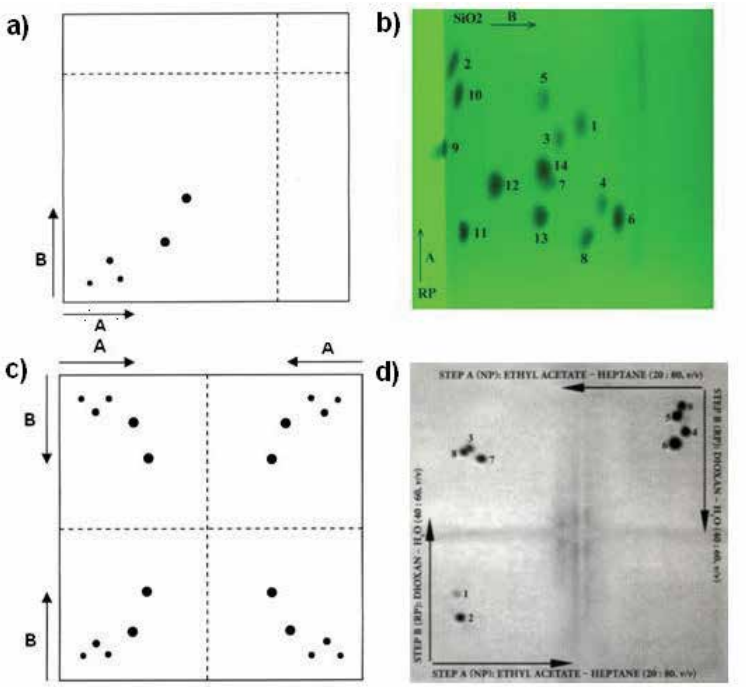


Fig. 18. Two-dimensional development, (a) schematic presentation of 2D-chromatogram (Adapted from Dzido, 2001. With permission.), (b) 2D-chromatogram of the 14-component mixture of pesticides presented as videoscan of dual-phase Multi-K CS5 plate in systems: A (first direction): methanol-water (60:40, v/v) on octadecyl silica adsorbent, B (second direction): tetrahydrofuran-*n*-heptane (20:80, v/v) on silica gel (From Tuzimski, 2002c. With permission), (c) schematic presentation of 2D- chromatogram of four samples simultaneously separated on the plate (Adapted from Dzido, 2001. With permission.), (d) 2D-chromatograms of three fractions of the mixture of nine pesticides presented as videoscan of the HPTLC plate (cyanopropyl) in systems with A (first direction): ethyl acetate-*n*-heptane (20:80, v/v), B (second direction): dioxane – water (40:60, v/v) (From Tuzimski & Soczewiński, 2004. With permission.).

2.3.2.3 Graft Thin-Layer Chromatography

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. Compounds from the first chromatographic plate after chromatogram development can be transferred to the second plate, without scraping, extraction, or re-spotting the bands by use of a strong mobile phase (Nyiredy, 2001). 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). An example of this technique is demonstrated in **Figures 19** and **20** (Tuzimski, 2007a; Tuzimski, 2005).

In graft-TLC experiments with connected adsorbent layers several mixtures can be applied as spots 1 cm from the edge of the first adsorbent, e.g., silica gel plate. Several samples can be developed at the same time in the first direction, on the first adsorbent (up to ten in case of 20 cm x 10 cm plates). After drying, the plate used in the first run is cut into 2 cm x 10 cm strips. The cut strips should have smooth edges, without irregularities resulting from partial loss of adsorbent, because such irregularities may lead to deformation of the zones during the transfer to the second adsorbent layer. If the adsorbent edge is uneven, it should be smoothed before attachment to the second adsorbent (Tuzimski, 2007a). Then individual strips are clamped to other plates and compounds are transferred. Individual strips should be connected (2 mm overlap) to 10 cm x 10 cm HPTLC plates along the longer (10-cm) side of the strip. It is essential the two the plates are in close contact, but without disintegration of the overlapping layers. To achieve this the HPTLC plates are placed between thicker glass plates pressed together with screw clamps. The transfer of analysed compounds is performed in vertical glass chamber, as the joined plates are difficult to be developed in horizontal chambers. The most important issue in graft-TLC is to choose appropriate solvent to transfer compounds from the first adsorbent to another. The choice of this solvent depends on the choice of the first and second adsorbents and character of the transferred substances (whether polar or nonpolar). MeOH is usually applied for transferring compounds from the first adsorbent to another layer (Tuzimski, 2007a). If the analyzed compounds are strongly adsorbed on the first adsorbent, the addition of organic acids, and also water, to transferring solvent, is advised. So that all sample compounds have $R_F = \approx 1.0$. If after transfer from, e.g., the silica layer the spots are spread along the 1-cm transfer distance, the second HPTLC plate can be developed to a distance of 1 cm with a strong solvent to improve their shapes. The application of narrow strip of the first adsorbent may also play the same role as the preconcentrating zone in case of multiphase (bilayer) plate. The sample components are not only separated on the first step of graft-TLC experiment, but also concentrated, and as-such developed in the second direction. The concentration is also performed during the transfer, as the strong mobile phase used in this procedure transfer the analyzed substances to another adsorbent as very thin bands. Graft thin-layer chromatographic separation (2D planar chromatography on connected layers) of three mixtures of pesticides were described (Tuzimski, 2007a; Tuzimski, 2005).

Complete separation of the components of the pesticide mixtures was also achieved by adsorbent-gradient 2D TLC when in the first, normal-phase (NP), development was

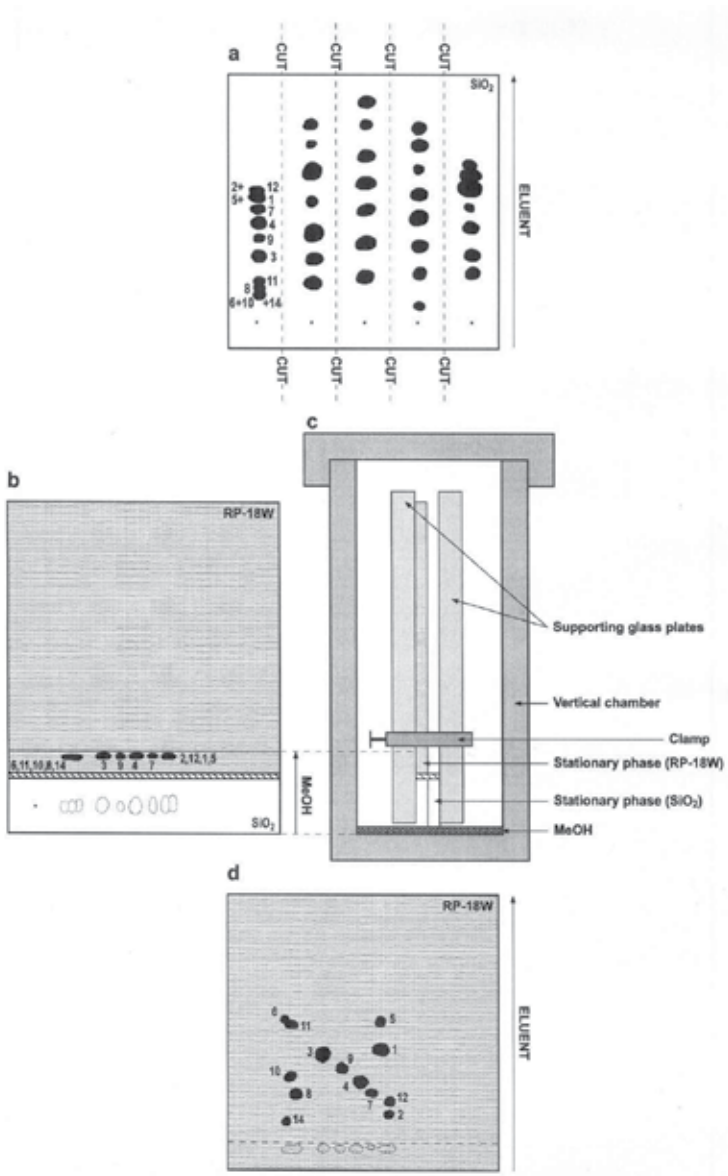


Fig. 19. Transfer of the mixture of pesticides from the first plate to the second one. (a) First development with partly separated mixtures of pesticides on silica plate. After development the silica plate was dried and cut along the dashed lines into 2 cm x 10 cm strips. (b) A narrow strip (2 cm x 10 cm) was connected (2 mm overlap – hatched area) to 10 cm x 10 cm HPTLC RP-18W plate along the longer (10 cm) side of the strip. The partly separated mixture of pesticides was transferred in a vertical chamber to the second plate using methanol as strong eluent to the distance of about 1 cm. (c) Schematic diagram of cross section of connected two adsorbents layers. (d) The HPTLC RP-18W plate was developed in the second dimension with organic - water eluent in the DS chamber (From Tuzimski, 2007a. With permission.).

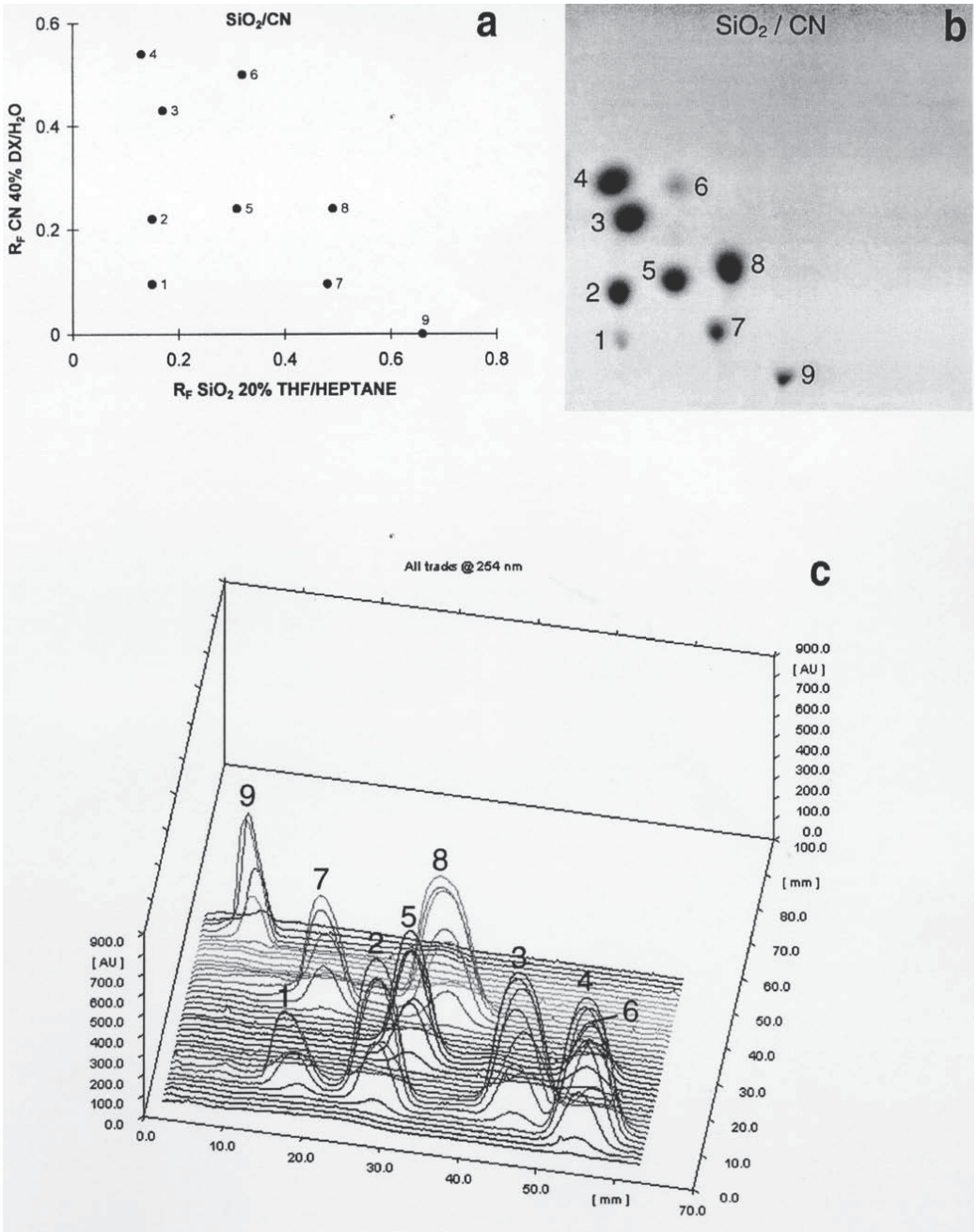


Fig. 20. (a) Correlation between normal-phase R_F values obtained with tetrahydrofuran–*n*-heptane, 20 + 80 (*v*/*v*), as mobile phase on silica and reversed-phase R_F values obtained with dioxane–water, 40 + 60 (*v*/*v*), as mobile phase on cyanopropyl layer. This pair of NP and RP systems was chosen for 2D TLC with adsorbent gradient. The videoscan (b) and densitogram (c) of the plate show the separation achieved for the nine-component pesticide mixture (From Tuzimski, 2005. With permission.).

performed, second, reversed-phase (RP), development was performed on HPTLC RP18W F_{254S} plates or HPTLC CN F_{254S} plates (**Figure 20a**). **Figure 20b** shows the videoscan and **Figure 20c** shows the densitogram; complete separation of the components of the mixture of pesticides is apparent.

2.3.2.4 Combination of Multidimensional Planar Chromatography (MDPC)

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). By the help of this new procedure 14 or 22 compounds from a complex mixtures were separated on 10 cm x 10 cm TLC and HPTLC plates (Tuzimski, 2008; Tuzimski, 2007b). In **Figure 21** an example of this procedure is presented step by step for separation of 22 compounds from complex mixtures on TLC plate (Tuzimski, 2008a).

Silica is the most popular and least expensive adsorbent used in planar chromatography, and has an excellent separation power. In the first part of experiments, solvent systems for silica stationary phase were selected to assign the investigated pesticides to groups of compounds based on the solvent classification by Snyder (Snyder, 1978) and the ‚Prisma Method' described by Nyiredy and coworkers (Dallenbach-Tölke et al., 1986; Nyiredy et al., 1988; Nyiredy et al., 1989; Nyiredy et al., 1995). For the selection of suitable mobile phases, the first experiments were carried out on TLC silica plates in unsaturated chromatographic chambers with ten solvents (diethyl ether, 2-propanol, ethanol, tetrahydrofuran, acetic acid, dichloromethane, ethyl acetate, dioxane, toluene, and chloroform) from the eight groups of Snyder's solvent-selectivity triangle for normal phase (NP) chromatography according to their properties as proton acceptors, proton donors, and their dipole interactions. The literature data (Stahl, 1967) show that the most widely applied solvent classes for NP planar chromatography are from the corners of Snyder's solvent-selectivity triangle (groups I, VII, and VIII) and from group VI (ethyl acetate, methyl ethyl ketone, dioxane, acetone, acetonitrile) where all three effects (proton acceptor, proton donor and dipole interaction) are practically equalized; the solvents from group VI have a special function in the optimization of the mobile phase (Nyiredy, 1997).

Next, the solvent strength has to be either reduced or increased, so that the R_F values of investigated compounds lie between 0.2 and 0.8. If the solvents afford good separation, other solvents from the same group were also tested. Good results (**Figure 22**) were obtained with ethyl acetate–*n*-heptane, 40:60 (v/v), as mobile phase in first direction; this separated the pesticides into five groups, the first containing compounds 1–4, the second containing 5–7, the third containing 8–12, the fourth containing 13–17, and fifth containing 18–22.

The objective of the next series of experiments of the study reported in this paper was to investigate the separation of components of the five groups of pesticides. For optimization of the mobile phases for separation of pesticides in all five groups the suitable solvents are selected by the procedure described above (Dallenbach-Tölke et al., 1986; Nyiredy et al., 1988; Nyiredy et al., 1989; Nyiredy et al., 1995). If the spots of pesticides on the plate migrate too far (R_F values of investigated compounds are above 0.7) the solvent strength must be reduced by addition of heptane (or hexane) to the mobile phase. Conversely, if the spots of compounds on the plate do not migrate far enough (R_F values of pesticides are between 0 and 0.3) the solvent strength must be increased by addition of a stronger solvent (e. g., methanol, acetic acid, water).

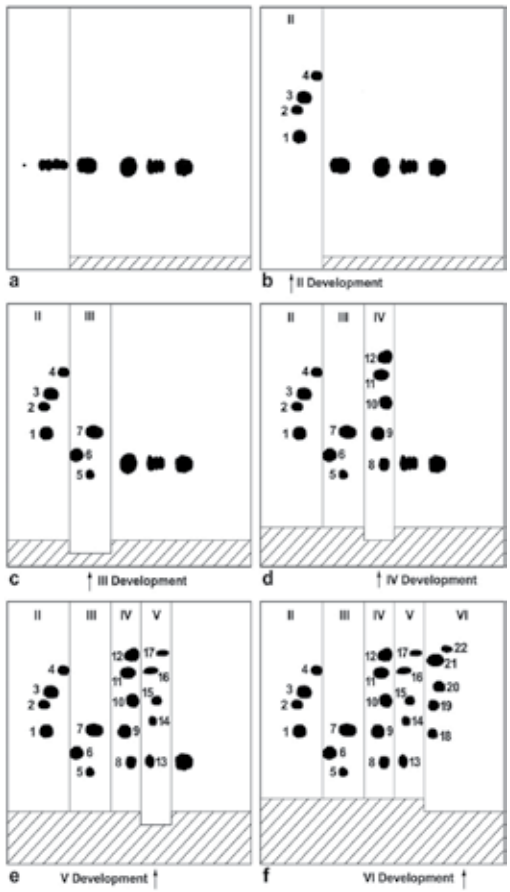


Fig. 21. Illustration of step by step selective multidimensional planar chromatographic separation. (a) The dried plate after the first separation (1st development) prepared for separation of the first group of compounds. One line (approx. 1 mm thick) is scraped in the stationary phase perpendicular to the first development, in such a way that the spot(s) of the target compounds are between the line and the edge of plate. For separation of the first group of compounds another 5-mm wide region of the silica gel layer removed from the bottom of the plate (the hatched lines indicate the stationary phase removed) so that in the next step mobile phase runs only up a narrow strip of adsorbent. (b) The dried plate after separation of the first group of pesticides (1–4) by use of acetonitrile–chloroform, 15:85 (v/v), as mobile phase in the 2nd development. (c) The prepared and dried plate after separation of the components of the second group of pesticides (5–7) by development with 100% chloroform twice over the same distance (UMD). (d) The prepared and dried plate after separation of the five components of the third group of pesticides (8–12) with nitromethane–dichloromethane, 5:95 (v/v), as mobile phase in the 4th development. (e) The prepared and dried plate after separation of the five components of next group of pesticides (13–17) with nitromethane–chloroform, 5:95 (v/v), as mobile phase in the 5th development. (f) The prepared and dried plate after separation of the five components of last group of pesticides (18–22) with toluene – *n*-heptane, 70:30 (v/v), as mobile phase in the 6th development (From Tuzimski, **2008a.** With permission.).

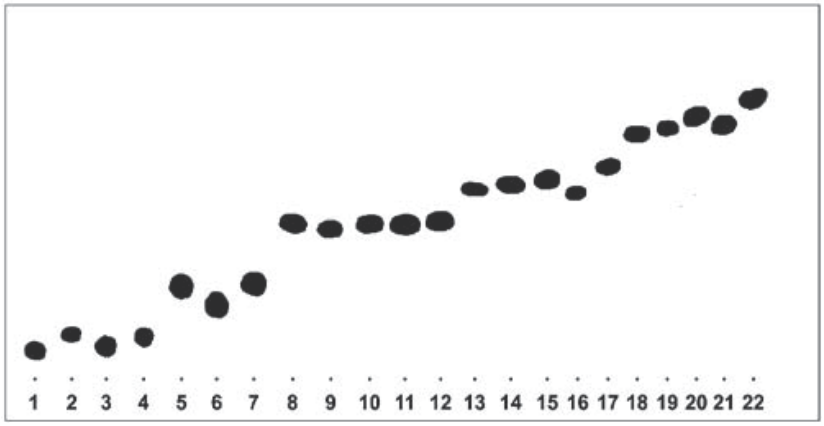


Fig. 22. Separation of 22 pesticides into five groups – compounds 1–4, 5–7, 8–12, 13–17, and 18–22 on a silica TLC plate with ethyl acetate–*n*-heptane, 40:60 (*v*/*v*), as mobile phase (From Tuzimski, **2008a.** With permission.).

After optimization of the mobile phases for separation of the components of all groups of pesticides these mobile phases were used for multidimensional planar chromatography (**Figures 21a–21f**).

Mixtures of pesticides were applied as spots, 1 cm from the bottom and 3.5 cm from the left edge of the plate. TLC plates were developed in the first dimension (step I) with ethyl acetate–n-heptane, 40:60 (v/v), as normal-phase eluent. HPTLC plates were developed in the first dimension (step I) with ethyl acetate–n-heptane, 50:50 (v/v), as normal-phase eluent. After drying in air for 20 min the plates were turned by 90° (so that the partly separated components of the complex mixture of compounds were on the start line of the next step). Next, one or two lines (approx. 1 mm wide) were scraped in the adsorbent layer perpendicular to the direction of the first development, so the spot(s) of the target compounds were between the lines. Some of the adsorbent layer (approx. 5 mm wide) must be removed, to ensure that the mobile phase of the second development (step II) develops only the spot(s) of the target compounds between the two lines. Next, all the plate and stationary phase except the part to be developed was covered by glass plates which were fixed with clamps. This procedure was repeated in subsequent steps (steps III–VI). Before each of steps III to VI a region of the adsorbent layer must again be removed from the plate to ensure that the mobile phase only develops the zone of group of compounds of interest. (The regions removed before each development are shown by the hatched lines in **Figure 21**.). The plates were developed in an unsaturated vertical chamber in MDPC experiment (**Figures 21a–21f**). To prevent the eluent from migration on adsorbent with constituents that are not aimed to be chromatographed during the particular step, the part of the plate, from which the adsorbent was removed, can be covered with lipophilic substance (wax). In this case, the plates can be developed in horizontal chamber in MDPC experiments.

The compounds from the first group (1–4) were chromatographed with acetonitrile–chloroform, 15:85 (v/v), as mobile phase (**Figure 21b**). The pesticides in the second group (5–7) were chromatographed twice with chloroform as mobile phase over the same distance (**Figure 21c**). The plate was dried for approximately 5 min between the two steps. The plate was then dried after the second separation of compounds of this group. Another portion of the stationary phase (the next 5 mm – hatched lines on **Figure 21d**) was then removed, to

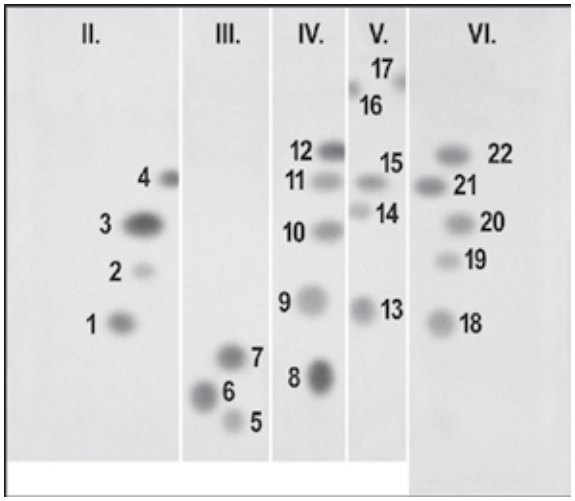


Fig. 23. Videoscan at 254 nm of the silica HPTLC plate showing separation of the 22 components of a complex mixture by developments I–VI in multidimensional planar chromatography (From Tuzimski, **2008a.** With permission.).

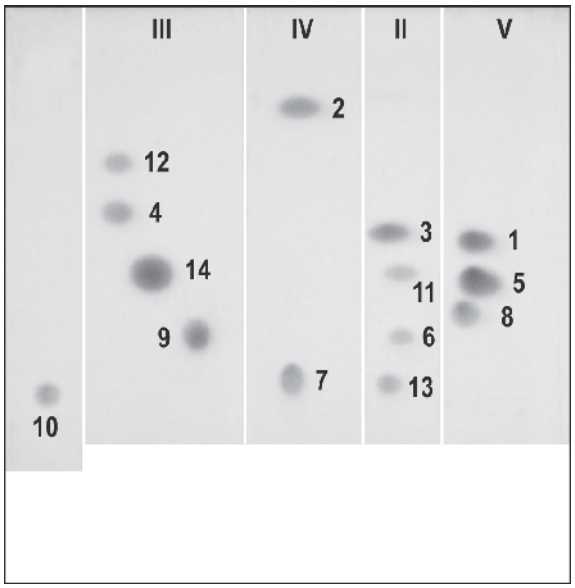


Fig. 24. Videoscan at 254 nm showing complete separation of the mixture of fourteen pesticides by multidimensional planar chromatography (MDPC) (From Tuzimski, 2007b. With permission).

ensure that the mobile phase used for development IV (**Figure 21d**) affected only the spots of the next group of compounds (8–12) between the two lines. Separation of components 13–17 with nitromethane– chloroform, 5:95 (v/v), as mobile phase in the next step on the TLC plate is depicted in **Figure 21e**. Separation of pesticides of the last group (18–22) with toluene–n-heptane, 70:30 (v/v), as mobile phase is depicted in **Figure 21f**. Separation of 22

components from a complex mixture by developments I–VI by multidimensional planar chromatography was also achieved on a silica HPTLC plate. The best results were obtained with ethyl acetate–n-heptane, 50:50 (v/v), as mobile phase in first direction, which separated the pesticides into five groups (1–4, 5–7, 8–12, 13–17, and 18–22). A videoscan of the multidimensional separation of the 22 components on a silica HPTLC plate is shown in **Figure 23** (Tuzimski, 2008a). The separation can be characterized as PC × (PC + nPC + PC + PC + PC)".
Also videoscan (**Figure 24**) was performed showing a complete separation of the next 14-component mixture of pesticides in the Ist–Vth developments by multidimensional planar chromatography [PC x (nPC + PC + PC +PC)] (Tuzimski, 2007b).

2.3.2.5 Multidimensional Planar Chromatography in combination with Diode-Array Scanning Densitometry (MDPC-DAD) and High-Performance Liquid Chromatography Coupled with Diode-Array Detection (HPLC-DAD)

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) 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). Multidimensional planar chromatography (MDPC) is performed by spotting the multicomponent mixture in the corner of a square chromatographic plate (20 cm x 20 cm) and by development in the first direction with the first eluent on the narrow zone of SiO_2 (Multi K SC5 plate) or octadecyl silica (Multi K CS5 plate). After the development is completed the chromatographic plate is then removed from the developing chamber and the solvent is allowed to evaporate from the layer. The plate is rotated through 90^o and then different solvents are used in the next steps which are perpendicular to the direction of the first development. The procedure for these steps is the easier as procedure described above and showed in **Figure 21a-f** (Tuzimski, 2010a), because in all the steps MDPC on the Multi K SC5 or CS5 plates the part to be developed was not covered by glass plates. Example MDPC procedure on Multi-K SC5 or CS5 plates are presented in **Figures 25** and **26**.
As shown in **Figure 26**, during step B the SC5 plates were developed until concentration in one band of the disturbing compounds of the extract on the border between the silica and octadecyl silica zones. This band was moved on to the second layer of adsorbent whereas only the clofentezine remained on the first layer of adsorbent and carefully separated from other compounds. During step B on CS5 the clofentezine was moved on to the second adsorbent (silica) whereas the other components of the extract remained on the first layer (octadecyl silica). These bands were concentrated on the border between the zones of adsorbents (**Figure 25**). It must be remembered that the Multi-K plates are especially useful for analysis of pesticides in multicomponent mixtures, e.g. in plant extracts, by MDPC.
In another 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 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

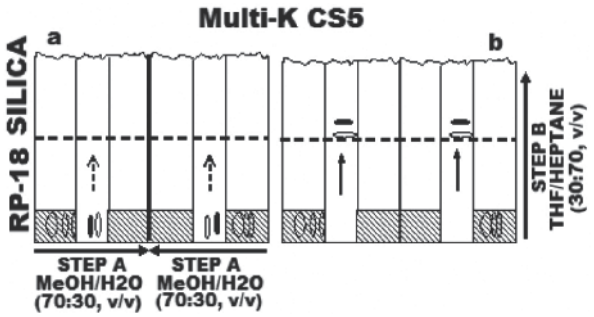


Fig. 25. Step-by-step illustration of selective multidimensional planar chromatography (MDPC) for clofentezine separation on bilayer Multi-K CS5 plate. (a) Dried plate after first separation (step A) by use of 70:30 (v/v) methanol-H_2O. Plate prepared for separation of the group of compounds with clofentezine (black band in figure) from Herba Thymi extract (left side) and extract of Herba Thymi fortified by clofentezine (right side): two lines (about 1 mm wide) are scraped in the stationary phase perpendicular to the first development, in such a way that the bands of clofentezine are between the lines. For separation of the group of compounds a 5-mm wide area of the octadecyl silica layer (RP-18) is removed from the plate (hatched lines indicate the part of stationary phase removed) such that in step B mobile phase runs only in a narrow strip of adsorbent. (b) The Multi-K CS5 plate after step B, development with 30:70 (v/v) tetrahydrofuran–*n*-heptane as mobile phase for separation of clofentezine in the Herba Thymi extract (left side) and the Herba Thymi extract fortified with clofentezine (right side). Both clofentezine bands are black in the figure (From Tuzimski, 2010a. With permission).

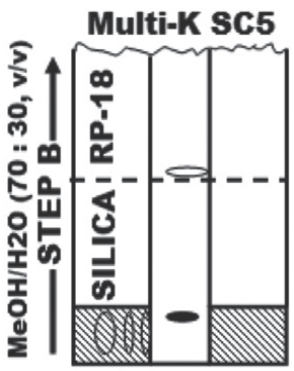


Fig. 26. Illustration of chromatogram obtained by multidimensional planar chromatography (MDPC) for clofentezine separation on bilayer Multi-K SC5 plate after step B with 70:30 (v/v) methanol-H_2O. Before step B, the Multi-K SC5 plate was prepared for separation of the group of compounds with clofentezine (black band) from Herba Thymi extract. Two lines are scraped in the stationary phase (approx. 1 mm wide) perpendicular to the first development, in such a way that the clofentezine band was between the lines. For separation of the group of compounds a 5-mm wide area of the silica gel layer is removed from the plate (hatched lines indicate the part of stationary phase removed) such that in step B mobile phase runs only in a narrow strip of adsorbent (From Tuzimski, 2010a. With permission).

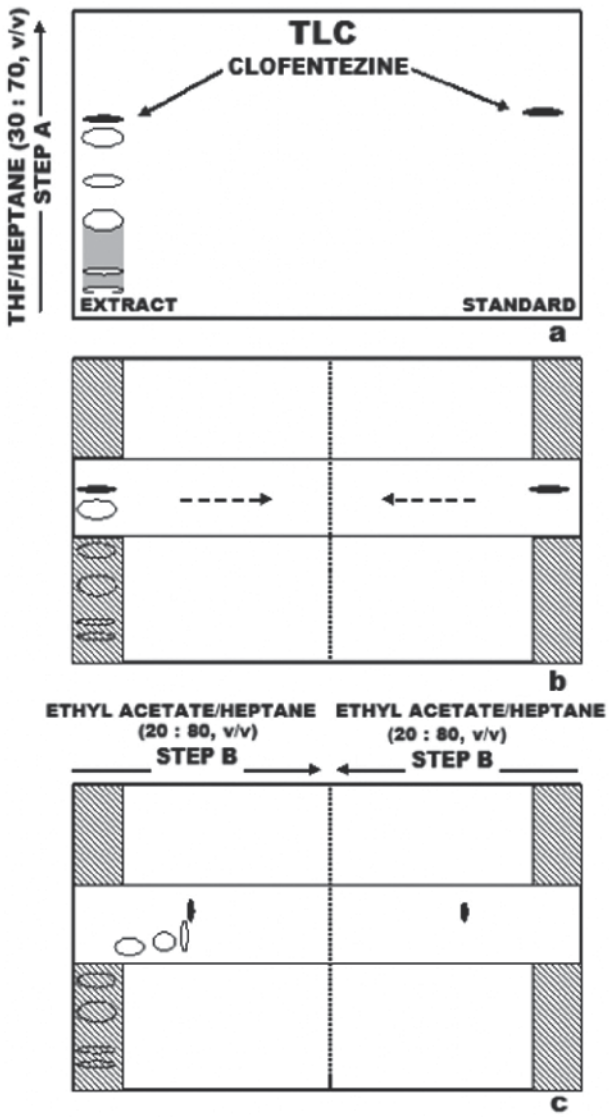


Fig. 27. Illustration of step-by-step selective multidimensional planar chromatography (MDPC) for separation of clofentezine in Herba Thymi extract. (a) Dried plate after the first separation (step A) by use of 30:70 (*v*/*v*) tetrahydrofuran–*n*-heptane. (b) Plate prepared for separation of the second group of compounds with clofentezine (black band on figure) and clofentezine standard: two lines are scraped in the stationary phase (about 1 mm wide) perpendicular to the first development, in such a way that the bands of clofentezine are between these lines. For separation of the second group of compounds a 5-mm wide area of the silica gel layer is removed from the plate (hatched lines indicate part of stationary phase removed) such that in the next stepmobile phase runs only in a narrow strip of adsorbent. (c) Dried plate after separation of the second group with clofentezine and clofentezine standard (both black bands) by use of 20:80 (v/v) ethyl acetate–n-heptane as mobile phase (step B) to half of plate from opposite sides (From Tuzimski, 2010a. With permission).

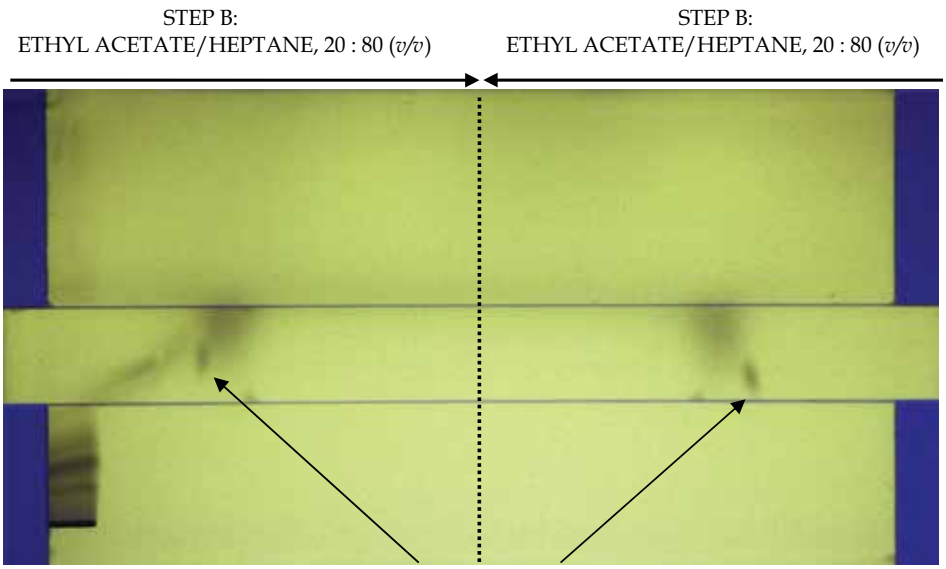


Fig. 28. Picture at 254 nm of dried plate after separation of the second group with clofentezine and standard of clofentezine by using ethyl acetate–*n*-heptane (20:80, v/v) as mobile phase (step B) to half of plate length from opposite sides (From Tuzimski, 2010b. With permission).

(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. **Figures 27 and 28** shows MDPC mode on silica layer.
MDPC combined with different modes of scanning, e.g., with diode array detection (MDPC-DAD) or mass spectrometry (MDPC-MS) enables quantitative analysis. 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., plant extracts. Comparison of contour plots obtained from the extract of Herba Thymi containing clofentezine after steps A and B are presented in **Figures 29a** and **29 b**. 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). 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) (**Figure 30**). 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. The components of two mixtures of pesticides, which were separated by 2D-TLC with adsorbent gradients of the type silica-wettable with water octadecyl silica or silica-cyanopropyl, were identified by R_F in both chromatographic systems and by comparison of UV spectra (Tuzimski, 2005).

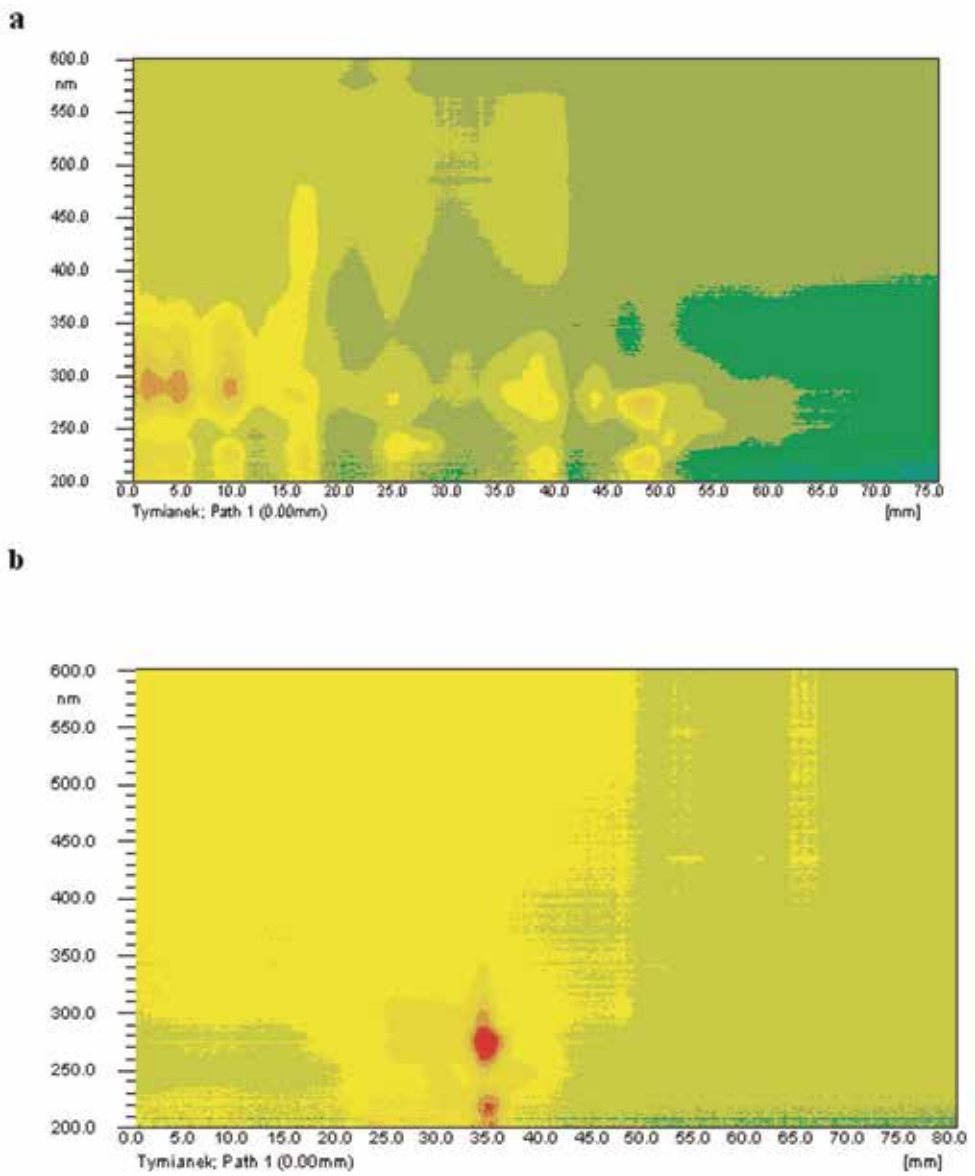


Fig. 29. Contour plots obtained from extract of Herba Thymi containing clofentezine: (a) after step A, (b) after step B (silica plate) (From Tuzimski, 2010a. With permission).

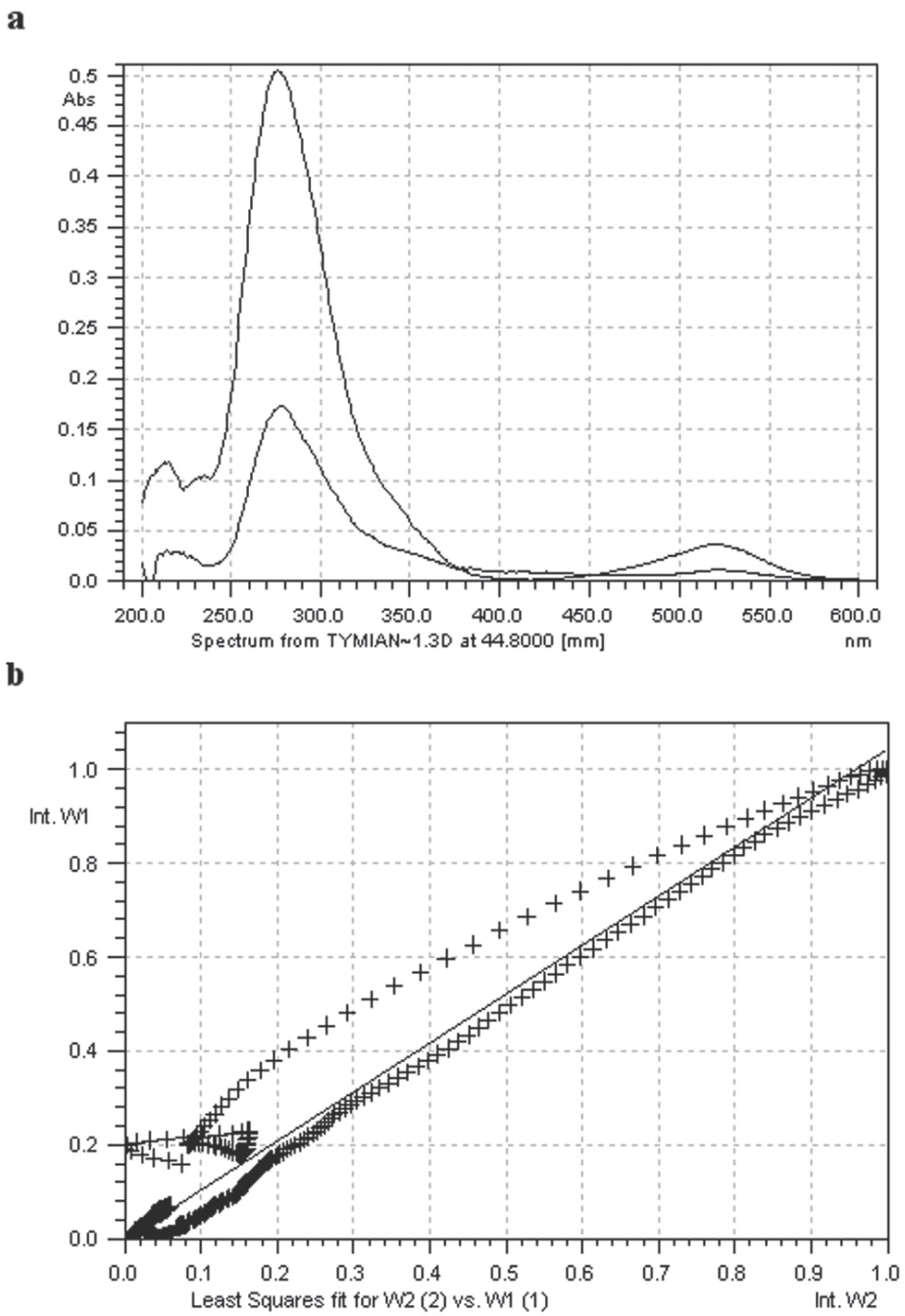


Fig. 30. (a) Comparison of UV spectrum of clofentezine found in Herba Thymi extract with UV spectrum of clofentezine standard from library. (b) Correlation curve of peak purity of spectra of clofentezine found in extract Herba Thymi and that of clofentezine standard (library) after step B. The purity index (*Pearson's r*) for the compared spectra was 0.9781 (From Tuzimski, 2010a. With permission).

Heart-cut spots of analytes from the stationary phase were also injected on e.g., a C18 column and analysed by HPLC-DAD (**Figures 31 and 32a**). Analyte identification was accomplished on the basis of the retention times of the analytes and by comparison of the UV spectrum of the reference compound in the library and the UV spectrum of the detected peak of the sample. A match equal or higher than 990 was fixed to confirm identification between both spectra for all the analytes determined (Tuzimski, 2008b,c; Tuzimski, 2009; Tuzimski & Sobczyński, 2009; Tuzimski, 2010a-c).

Figure 32c shows the purity of the pesticide peak after step B (Tuzimski, 2010a). 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 suggested 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).

Multidimensional planar chromatography can be used for identification of known and unknown compounds, and – at least equally important – for correct identification of analytes in a variety of samples, e.g., clofentezine in Herba Thymi. 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.

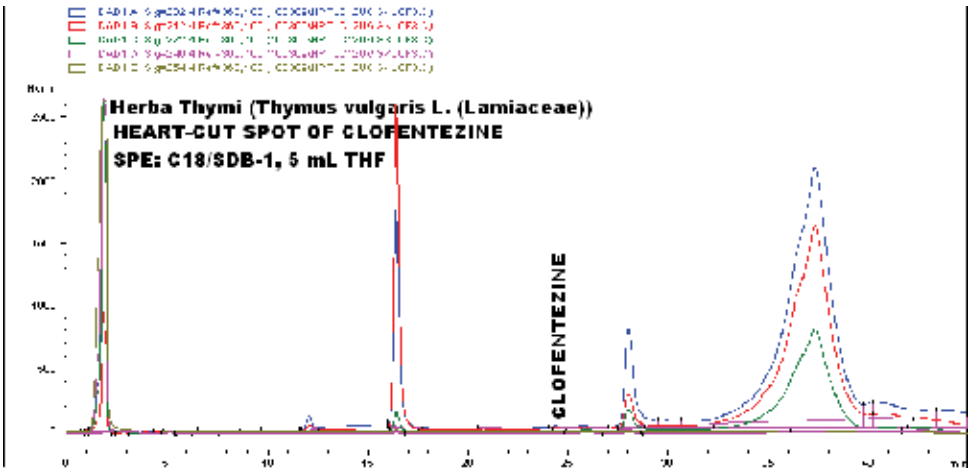
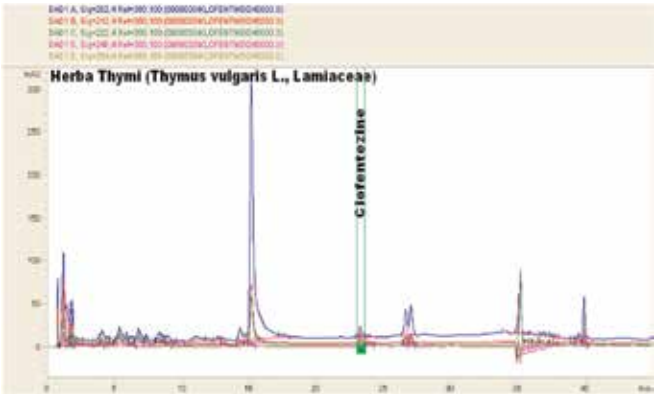
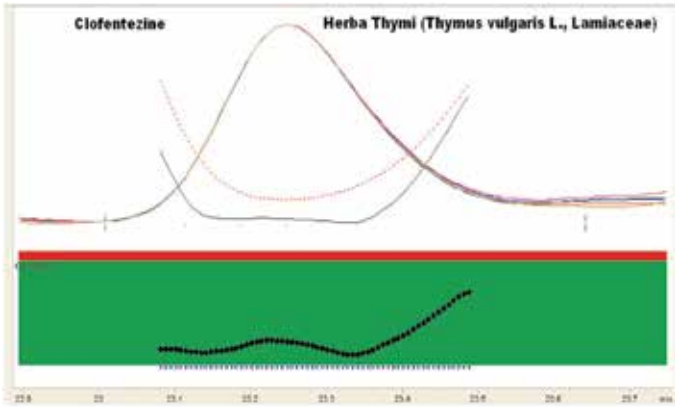


Fig. 31. Chromatogram obtained from heart-cut spot of clofentezine from extract Herba Thymi (*T. vulgaris* L., Lamiaceae) sample after SPE with C18/SDB-1 cartridge and MDPC-DAD (step B). HPLC-DAD showing detected and quantified clofentezine (From Tuzimski, 2010b. With permission).



(a)

(b)



(c)

Fig. 32. (a) Chromatogram obtained from heart-cut spot of clofentezine from extract of Herba Thymi (*T. vulgaris* L., Lamiaceae) sample after SPE with C18/SDB-1 cartridge and MDPC-DAD (step B). (b) HPLC-DAD showing detected and quantified clofentezine. (c) Purity of HPLC peak obtained for clofentezine in Herba Thymi extract after step B (From Tuzimski, 2010a. With permission).

3. Conclusions

Multidimensional chromatography techniques represent a powerful tool and alternative procedure to classical one-dimensional chromatographic methods with optimum efficiency and selectivity for the separation of the component of interest, e.g., pesticides. Multidimensional gas chromatography (MDGC) is recommendable for separation and quantitative analysis of volatile and semi-volatile analytes, while multidimensional liquid chromatography (MDLC) and multidimensional planar chromatography (MDPC) are suitable for qualitative and quantitative analysis of non-volatile pesticides from different classes. The new multidimensional methods with fast scanning detectors should enable us to detect and determine many more analytes in original samples and continue to improve modern analytical methods for better research to control the environment for persistent pesticides.

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Rapid and Easy Multiresidue Method for Determination of Pesticide Residues in Foods Using Gas or Liquid Chromatography–Tandem Mass Spectrometry

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1. Introduction

To obtain a high yield of food grains, many types of pesticides have been developed, which in turn has facilitated the prosperity of the human race and brought relief to farmers. After WWII, organophosphorus and organochlorine pesticides were extensively used worldwide. These early organophosphorus pesticides occasionally caused poisoning, while some organochlorine pesticides persisted in the environment. These facts served as a warning against an improvident use of pesticides and revealed that residual pesticides in crops should be monitored. Thus, national and local governments should monitor imported and regional foods as a policy. In Japan, all pesticides are regulated under a uniform limit (0.01 μg/g), except for a combination of foods and pesticides set under the maximum residue limits (MRLs) (Notification No. 497-499, November 29, 2005). This regulation does not require analysis of all the pesticides; however, it does necessitate pesticide residue analysis of commodities. Thus far, the chemical industry has developed more than 800 pesticides that belong to many classes such as insecticides, fungicides, nematocides, and herbicides. Each class has a different target point and physical properties and this diversity limits the coverage of a single analytical method. It is also impossible to monitor all the pesticides pertaining to one foodstuff using hundreds of methods. Thus, analytical institutes require fast and efficient multiresidue methods in order to maximize the coverage of their monitoring activities. For this reason, researchers have reported many multiresidue analytical methods (Cook, J., et al., 1999, Fillion, J., et al., 2000, Hirahara, Y., et al., 2006, Luke, M. A., et al., 1975, Ueno, E., et al., 2004). These methods were optimized to monitor multiple residues efficiently and are used routinely in quarantine stations and laboratories.
Anastassiades et al. reported a rapid approach for the analysis of pesticide residues in fruits and vegetables, named QuEChERS (quick, easy, cheap, effective, rugged and safe) method in 2003 (Anastassiades, M., et al., 2003). The main focus of this report was to shorten the analytical process during extraction and cleanup without employing expensive instruments. The characteristic points of the method are as follows: (1) shaking extraction with acetonitrile in a disposable tube, (2) one step salting out and removal of water from the

acetonitrile using anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl), (3) purification with so called "dispersive-solid phase extraction" (dispersive-SPE), in which the extract is processed by shaking with primary secondary amine (PSA) silica gel and then centrifuged to separate the PSA before analysis. Compared to ordinary multiresidue methods, this method is a remarkably rapid and easy procedure. Other researchers have reported follow-up studies of this method (Lehotay, S. J., et al., 2005, Lehotay, S. J., et al., 2005, Schenck, F. J., et al., 2008, Wang, J.&Leung, D., 2009). We also examined the QuEChERS method and found that the characteristic points of the method (step (2)) are positive aspects; however, steps (1) and (3) are negative aspects due to the weak extraction potency (shaking) and the insufficient cleanup (dispersive-SPE). To overcome these negative aspects, we developed a new powerful extraction technique with a homogenizer and with an efficient cleanup with double-layered SPE (graphite carbon black (GCB) with PSA) on the basis of the QuEChERS method (Okihashi, M., et al., 2005, Okihashi, M., et al., 2007, Takatori, S., et al., 2008). We demonstrated that this method is applicable to the multiresidue analysis in agricultural products by the combination of analysis with gas chromatography equipped with (tandem) mass spectrometry (GC-MS or GC-MS/MS) and with high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS).

For agricultural products, multiresidue methods applicable to the analysis of pesticides in processed foods are also required. In Japan (in 2007~08), serious organophosphorus pesticide poisoning occurred after people consumed dumplings imported from China. An organophosphorus pesticide, methamidophos, was detected from the leftover of the Chinese dumplings in concentrations of 3,200 ppm (Ito, T., 2009, Sumi, Y., et al., 2008). Furthermore, dichlorvos, parathion, and parathion-methyl were also detected in other packages of the Chinese dumplings. This incident necessitated the monitoring of pesticide residues in processed foods in Japan. Multiresidue methods for processed foods such as tomato purees and baby foods have been reported (Botitsi, H., et al., 2007, Cajka, T., et al., 2008, Gilbert-Lopez, B., et al., 2007, Sannino, A.&Bandini, M., 2005, 2005, Sannino, A., et al., 2003). However, the number of multiresidue methods that can be adapted for processed foods that contain high lipid ratios (more than 10% w/w) are relatively limited (Fenske, R. A., et al., 2002, Lehotay, S. J., et al., 2005, Sannino, A., et al., 1995, 1996). Lipids are a problematic component of processed foods during pesticide detection. We categorized processed foods into three categories: (i) a high lipid group (lipid content > 10% w/w), (ii) a low lipid group (lipid content ≤ 10% w/w), and (iii) a non-lipid group. Chinese dumplings fall into the high lipid group. For this group, both the sufficient extraction of pesticides within the lipids and the removal of lipids before analysis are key aspects. The multiresidue method for agricultural products described above may be applicable to the low and non-lipid groups directly or with minor modifications such as adding water to samples prior to the extraction.

As described above, the MRLs were set according to the combination of pesticides and the agricultural products. Except for some processed foods such as raisins and orange juice, the MRLs were not established for combinations of pesticides and processed foods; most of the pesticides detected in processed foods are regulated under the uniform limit (0.01 ppm/g). Thus, the uniform limit should be detectable using the multiresidue method for monitoring pesticides in processed foods. Here, we have developed a rapid and easy multiresidue method for the determination of pesticide residues in processed foods that fall in the high lipid group. The method could detect around 300 pesticides at the level of 0.01 ppm/g (Kitagawa, Y., et al., 2009, 2009, Okamoto, Y., et al., 2009).

In this chapter, we describe rapid and easy multiresidue methods for the determination of pesticide residues in foods, including agricultural products (vegetables, fruits, and cereals) and processed foods, on the basis of our recent studies (Kitagawa, Y., et al., 2009, 2009, Okamoto, Y., et al., 2009, Okihashi, M., et al., 2007, Takatori, S., et al., 2008).

2. Outline of the methods

2.1 Method for agricultural products

We extracted agricultural products weighed in disposable tubes with acetonitrile using a homogenizer. To the tube, we added NaCl and $MgSO_4$, shook it vigorously, and then centrifuged it to remove the water layer and the precipitant. The acetonitrile phase was applied onto a SPE column for purification (removing chlorophyll and fatty acids). The eluate was concentrated *in vacuo* and then reconstituted in an appropriate solvent for GC-MS, GC-MS/MS, or LC-MS/MS analysis.

2.2 Method for processed foods (high lipid containing group, Chinese dumplings, French fries, etc.)

We extracted the pesticides in homogenized processed foods with ethyl acetate in the presence of $MgSO_4$. After centrifuging, an aliquot of extract was concentrated *in vacuo*. The remainder was dissolved in hexane and then an acetonitrile/hexane partition step was conducted to remove the lipids from the extract. After centrifuging, we applied the acetonitrile phase onto a SPE column for further purification (to remove chlorophyll and fatty acids). We concentrated the eluate *in vacuo* and then reconstituted it in an appropriate solvent for GC-MS, GC-MS/MS, or LC-MS/MS analysis.

3. Experimental

3.1 Chemicals

Pesticides used in this study were analytical grade obtained from Wako (Osaka, Japan), Kanto Chemical (Tokyo, Japan), Hayashi Pure Chemical (Osaka, Japan), Riedel-de-Haën (Seelze, Germany) and Dr. Ehrenstorfer (Augsburg, Germany). Solvents and NaCl were pesticides analysis grade purchsed from Wako. Anhydrous $MgSO_4$ was the highest grade obtained from Wako. Water was prepared by Millipore system(Millipore, Bedford, MA). Other chemicals used in this study were the highest grade commercially avairable.

3.2 Apparatus

A 50 mL polypropyrene (PP) disporsal tube was purchased from Becton Dickinson (Franklin Lakes, NJ). A QS-7 conventional food processor (Toshiba, Tokyo, Japan) was used to comminute vegetable, fruit, cereal and processed foods samples. Polytron PT1200 high-speed homogenizer (KINEMATICA, Luzern, Switzerland) was used to blend sample and acetonitrile or ethyl acetate in the extraction step. A Hitachi Himac SCR 20B (Hitachi Koki, Tokyo, Japan) was utilized for centrifugation. Solvent evaporator, Iwaki REN-1000 rotary evaporators (Asahi Techno Glass, Chiba Japan), were used in this study. Double-layered cartridge column (6 mL) with 500 mg of GCB and 500mg of PSA and an octadecyl silica gel (C18) column (500 mg; 3mL) were obtained from SUPELCO (Bellefonte, PA).

3.3 Analytical systems

3.3.1 GC-MS

GCMS-QP2010 (Shimadzu, Kyoto, Japan) was used in EI (electroionization) mode. The analytical column was the Rtx-5MS (0.25 mm x 30 m, 0.25μm; Restek, Bellefonte, PA). The analysis was performed on a selected ion monitoring (SIM) mode.

3.3.2 GC-MS/MS

Quattro Micro GC (Waters/Micromass, Manchester, UK) was used in EI mode. The analytical column was the DB-5 (0.25 mm x 30 m, 0.25μm, Agilent, Santa Clara, CA). The analysis was performed on a selected reaction monitoring (SRM) mode.

3.3.3 LC-MS/MS

The LC-MS/MS system was composed of an LC system (1100 series; Agilent) and an MS/MS (API3000; Applied Biosystems, Foster City, CA) equipped with electroionization spray interface. The analytical column was Ascentis C18, 2.1 x 100 mm, 3 μm (SUPELCO). Eluents were 0.1% formic acid aquaous solution (A) and 0.1% formic acid contaning methanol (B), in whic the ratio was gradially changed as the analysis went on. The analysis was performed on an SRM mode.
The details of analytical conditions in 3.3.1~3 were omitted.

3.4 Procedure for sample preparation

3.4.1 Vegetables and fruits

We homogenized a sample (approximately 700~1,000 g) in a conventional food processor. To a 50 mL PP disposable tube containing a 10 g aliquot of the homogenized sample, we added 20 mL of acetonitrile and then homogenized it with a high-speed homogenizer for 1 min. Next, 4 g of $MgSO_4$ and 1 g of NaCl were added to the tube and it was shaken vigorously for a minute. After centrifugation, we applied 8.0 mL of the acetonitrile layer (equivalent to 4.0 g of the sample) onto a GCB/PSA, which was formerly conditioned with 30 mL of the elution solvent (acetonitrile-toluene, 3 + 1). We eluted the column with 30 mL of the elution solvent. All the eluate was concentrated *in vacuo*. For the GC-MS/MS analysis, the eluate was reconstituted in 4.0 mL of 10% acetone, containing hexane (the test solution, equivalent to 1.0 g/mL). This test solution is also applicable to the analysis via GC-MS. For the LC-MS/MS analysis, the eluate was reconstituted in 2.0 mL of methanol and then diluted four times with water before analysis (the test solution, equivalent to 0.5 g/mL) [Scheme I].

3.4.2 Cereals

We homogenized a sample (approximately 700~1,000 g) in a mill. To a 50 mL PP disposable tube containing a 5.0 g aliquot of the homogenized sample, 5.0 mL of water was added and stood for 30 min. Then, we added and homogenized 20 mL of acetonitrile with a high speed homogenizer for 1 min. We added 4 g $MgSO_4$ and 1 g NaCl to the tube and shook it vigorously for 1 min. After centrifugation, 10 mL of the acetonitrile layer (equivalent to 2.5 g of the sample) was applied onto a C18 column connecting above GCB/PSA, which was formerly conditioned with 10 mL acetonitrile and 30 mL of the elution solvent (acetonitrile-toluene, 3 + 1), respectively. We eluted the column with 10 mL acetonitrile. After removing the C18 column, the GCB/PSA column was eluted with the 30 mL of the elution solvent. The eluate was concentrated *in vacuo*. For the GC-MS/MS analysis, we reconstituted the

eluate in 2.5 mL of 10% acetone containing hexane (the test solution, equivalent to 1.0 g/mL). This test solution is also applicable to the analysis via GC-MS. For the LC-MS/MS analysis, we reconstituted the eluate in 1.25 mL of methanol and then diluted it four times with water to 5.0 mL before analysis (the test solution, equivalent to 0.5 g/mL) [Scheme II].

Sample (10 g) in a 50 mL PP disposal tube

| Add 20 mL of acetonitrile
| Homogenize (1 min)
| Add 4 g of $MgSO_4$ and 1 g of NaCl
| Shake (1 min) and centrifuge

Acetonitrile layer 8.0 mL (equivalent to 4.0 g sample)

| Load on a GCB/PSA column
| Elute with 30 mL acetonitrile–toluene (3+1)
| Collect loading and eluting fractions in a round bottom flask
| Evaporate and reconstituted in appropriate solvent

GC-MS/MS or LC-MS/MS analysis

Scheme I. Procedure of sample preparation for the determination of pesticides residues in agricultural products (for vegetables and fruits)

Sample (5.0 g) in a 50 mL PP disposal tube

| Add 5 mL of water and stand for 30 min
| Add 20 mL of acetonitrile
| Homogenize (1 min)
| Add 4 g of $MgSO_4$ and 1 g of NaCl
| Shake (1 min) and centrifuge

Acetonitrile layer 10.0 mL (equivalent to 2.5 g sample)

| Load on a C18 column connecting above a GCB/PSA column
| Elute with 10 mL of acetonitrile
| Remove the C18 column
| Elute with 30 mL acetonitrile–toluene (3+1)
| Collect loading and eluting fractions in a round bottom flask
| Evaporate and reconstituted in appropriate solvent

GC-MS/MS or LC-MS/MS analysis

Scheme II. Procedure of sample preparation for the determination of pesticides residues in agricultural products (for cereals)

3.4.3 Calibration curves and limits of quantification

For the GC-MS and GC-MS/MS analysis, we used the matrix-matched calibration curves to minimize the effects of the matrix in the test solution (Poole, C. F., 2007). The standard solutions for the matrix-matched calibration curves were prepared by mixing the serially diluted standard solutions with equivalent volumes of the doubly concentrated test solutions obtained from the same foods. We formerly determined that these foods did not contain the pesticide residues to be analyzed.

For LC-MS/MS analysis, we serially diluted the standard mixture solution with a 25% methanol aqueous solution. The limits of quantification (LOQ) of pesticides studied in this chapter were determined by LC-MS/MS and were found to be 0.01 ppm, except for flufenoxuron, lufenuron, pentoxazone, and propiconazole. The LOQs of these pesticides were 0.02 ppm.

3.4.4 Processed foods in high lipid group

We homogenized a whole sample in a distributed package (approximately 200~1,000 g) in a conventional food processor. To a 50 mL PP disposable tube containing a 5.0 g aliquot of the sample, 20 mL of ethyl acetate and 3 g of $MgSO_4$ were added to the tube and then homogenized with a high speed homogenizer for 1 min. After centrifugation, 8.0 mL of ethyl acetate supernatant (equivalent to 2.0 g of the sample) was evaporated in a round bottom flask at 40°C *in vacuo* and then dried under a nitrogen stream to remove ethyl acetate. To remove the lipid from the extract, the acetonitrile-hexane partition was conducted. Briefly, we reconstituted the extract in 10 mL hexane in a new 50 mL disposable PP tube. To the tube, we added 20 mL hexane-saturated acetonitrile and then shook it vigorously for 1 min. After centrifugation, we applied the acetonitrile layer onto the GCB/PSA, which was formerly conditioned with 30 mL of the elution solvent (acetonitrile–toluene, 3 + 1). To the remaining hexane layer in the PP tube, we added 20 mL hexane-saturated acetonitrile and then again shook it vigorously for 1 min. After centrifugation, the acetonitrile layer was applied onto the column. The column was eluted with 30 mL of elution solvent. The eluate was concentrated *in vacuo*. For GC-MS or GC-MS/MS analysis, the eluate was reconstituted in 2 mL of 10% acetone containing hexane (the test solution, equivalent to 1.0 g/mL). For LC-MS/MS analysis, we reconstituted the eluate in 2 mL of methanol (the test solution stock, equivalent to 1.0 g/mL) and then diluted it ten times with a 25% methanol aqueous solution before analysis [Scheme III].

4. Evaluation

4.1 Method for vegetables, fruits and cereals

4.1.1 Rapidity of the method

The merits of the method (saving time and solvent) are due to its simple procedure. The preparation time for the test solutions of the 12 samples was approximately 3~4.5 h for one chemist. More time for the chromatographic data analysis is necessary when increasing the number of analyzed pesticides in the method. Thus, the time saved from the sample preparation and the spare time from the chromatographic data analysis are important for the multiresidue analysis. This simple procedure would also be required to revise the method studying the applicability of new target pesticides in foods. Furthermore, this simple method does not require a long time to master the procedure and would also be effective in reducing the errors of the procedure. The ruggedness of the method derives from these points. The solvent and the amount of glassware used for the procedure are lesser than those used for the original method. Now, this method, which is used for routine monitoring of pesticides in our laboratory, would be more economic and safe than the ordinary methods.

4.1.2 Recovery tests

Recovery tests were conducted to examine the applicability of the method using GC-MS/MS (250 pesticides) and LC-MS/MS (99 pesticides). We defined an acceptable result as

the one with a recovery of about 70~120% with a relative standard deviation (RSD) ≤ 20% at both concentrations.

Sample (5.0 g) in a 50 mL PP disposal tube
Add 20 mL of ethyl acetate and 3 g of $MgSO_4$
Homogenize (1 min) and centrifuge
Ethyl acetate layer 8.0 mL (equivalent to 2.0 g sample)
Evaporate and reconstituted in 10 mL of hexane in 50 mL PP tube
Add 20 mL of acetonitrile saturated with hexane
Shake (1 min) and centrifuge
Acetonitrile layer
Hexane layer
Add 20 mL of acetonitrile
Shake (1 min) and centrifuge
Acetonitrile layer
Load on a GCB/PSA column
Elute with 30 mL of acetonitrile–toluene (3+1)
Collect loading and eluting fractions in a round bottom flask
Evaporate and reconstituted in appropriate solvent
GC-MS, GC-MS/MS or LC-MS/MS analysis

Scheme III. Procedure of sample preparation for the determination of pesticides residues in processed foods categorized as the high-lipid group

4.1.2.1 Analysis of 250 pesticides via GC-MS/MS (agricultural products)

We spiked the 250 pesticides for 3 agricultural products (bananas (Ba), carrots (Ca), and grapefruits (Gf)) at concentrations of 0.02 and 0.10 ppm. We conducted 3 trials for each test. These pesticides were classified into 3 groups as per the number of foods with acceptable results at both concentrations: A, 3; B, 2; and C, 1. Details are described below.

Group A (207 pesticides with the acceptable results in 3 agricultural products at the both concentrations):

2-Phenylphenol, Acetochlor, Aldrin, Allidochlor, Ametryn, Anilofos, Atrazine, Azinphos-methyl, Azoxystrobin, Benalaxyl, Bendiocarb, Benfluralin, Benfuresate, Benoxacor, BHC (total), Bifenthrin, Bitertanol, Bromacil, Bromophos, Bromopropylate, Bupirimate, Buprofezin, Butachlor, Butafenacil, Butamifos, Cadusafos, Cafenstrole, Carbaryl, Carbofuran, Carfentrazone-ethyl, Chlorfenvinphos, Chlorobenzilate, Chlorpropham, Chlorpyrifos, Chlorpyrifos-methyl, Chlorthal-dimethyl, Clomazone, Cloquintocet-1-methylhexyl, Cyanazine, Cyanophos, Cyflufenamid, Cyfluthrin, Cyhalofop-butyl, Cyhalothrin, Cypermethrin, Cyproconazole, Cyprodinil, DDT (total), Diazinon, Dichlofenthion, Diclobutrazol, Diclofop-methyl, Dicloran, Diethofencarb, Difenoconazole, Diflufenican, Dimepiperate, Dimethametryn, Dimethenamid, Dimethipin, Dimethylvinphos, Diofenolan, Dioxabenzofos, Diphenamid, Disulfoton, Dithiopyr, Edifenphos, Esprocarb, Ethalfluralin, Ethiofencarb, Ethion, Ethofumesate, Ethoprophos, Etobenzanid, Etoxazole, Etrimfos, Fenamiphos, Fenarimol, Fenbuconazole, Fenchlorphos, Fenitrothion, Fenobucarb, Fenothiocarb, Fenoxanil, Fenoxycarb, Fenpropimorph,

Fensulfothion, Fenthion, Fenvalerate, Fipronil, Flamprop-methyl, Fluacrypyrim, Flucythrinate, Fludioxonil, Fluquinconazole, Flusilazole, Flutolanil, Flutriafol, Fosthiazate, Fthalide, Furathiocarb, Furilazole, Halfenprox, Heptachlor, Hexazinone, Indoxacarb-MP, Iprobenfos, Iprodione, Iprovalicarb, Isazofos, Isofenphos, Isoprothiolane, Isoxathion, Kresoxim-methyl, Lactofen, Lenacil, Malathion, Mefenacet, Mepronil, Metalaxyl, Methacrifos, Methidathion, Methiocarb, Methoxychlor, Metolachlor, Metolcarb, Metominostrobin, Metribuzin, Mevinphos, Molinate, Myclobutanil, Napropamide, Nitrothal-isopropyl, Oxadiazon, Oxadixyl, Oxyfluorfen, Paclobutrazol, Parathion, Parathion-methyl, Penconazole, Pencycuron, Pendimethalin, Permethrin, Phenothrin, Phenthoate, Phorate, Phosalone, Phosphamidon, Picolinafen, Piperophos, Pirimicarb, Pirimiphos-ethyl, Pirimiphos-methyl, Pretilachlor, Procymidone, Profenofos, Promecarb, Prometryn, Propachlor, Propanil, Propaphos, Propham, Propiconazole, Propoxur, Propyzamide, Prothiofos, Pyraclofos, Pyraflufen-ethyl, Pyrazophos, Pyributicarb, Pyridaphenthion, Pyrimethanil, Pyrimidifen, Pyriminobac-methyl, Pyriproxyfen, Quinalphos, Quinoxyfen, Quintozene, Silafluofen, Simazine, Simeconazole, Simetryn, Sulprofos, Tebuconazole, Tebufenpyrad, Tecnazene, Tefluthrin, Terbacil, Terbufos, Terbutryn, Tetrachlorvinphos, Tetradifon, Thenylchlor, Thifluzamide, Thiobencarb, Thiometon, Tolclofos-methyl, Tralomethrin, Tri-allate, Triadimefon, Triazophos, Tribuphos, Triflumizole, Trifluralin, Uniconazole P, Vinclozolin, XMC

Group B (36 pesticides with the acceptable results in 2 agricultural products at the both concentrations of 0.02 and 0.1 ppm except for the agricultural product exhibited in parentheses):

Acephate (Gf), Bifenox (Gf), Bioallethrin (Gf), Bromobutide (Gf), Chlorfenapyr (Gf), Clodinafop-propargyl (Ca), Clomeprop (Gf), Cyanofenphos (Gf), Deltamethrin (Ba), Dieldrin (Ca), Dimethoate (Gf), Diphenylamine (Ba), Endrin (Gf), EPN (Ba), Fenpropathrin (Gf), Flumioxazin (Gf), Fluvalinate (Gf), Furametpyr (Gf), Heptachlor-epoxide (Ca), Hexaconazole (Gf), Isoprocarb (Ca), Methamidophos (Gf), Monocrotophos (Gf), Norflurazon (Gf), Omethoate (Gf), Phosmet (Ba), Prochloraz (Gf), Propargite (Ba), Pyridaben (Gf), Pyrifenox (Gf), Quinoclamine (Ba), Tetraconazole (Gf), Thiazopyr (Ca), Triadimenol (Gf), Trifloxystrobin (Gf), Xylylcarb (Ba)

Group C (7 pesticides with the acceptable results in 1 agricultural products at the both concentrations of 0.02 and 0.1 ppm exhibited in square brackets):

Acrinathrin [Ca], Dichlorvos [Ca], Dicofol-metabolite (4,4'-Dichlorobenzophenone) [Gf], Diphenyl [Gf], Endosulfan (α+β) [Ba], Flumiclorac-pentyl [Ca], Hexythiazox [Gf]

4.1.2.2 Analysis of 99 pesticides via LC-MS/MS (agricultural products)

We spiked the 99 pesticides for 7 agricultural products (cabbage (Cb), potatoes (Po), spinach (Sp), apples (Ap), oranges (Or), brown rice (Br), and soybeans (Sy)) at concentrations of 0.02 and 0.1 ppm. We conducted 5 trials for each test. These pesticides were classified into 6 groups as per the number of foods with acceptable results: A, 7; B, 6; C, 5; D, 4; E, 3; and F, 2. Details are described below.

Group A (47 pesticides with the acceptable results in all the 7 agricultural products):

Acetamiprid, Acetochlor, Alachlor, Atrazine, Bensulide, Bitertanol, Bromobutide, Bupirimate, Clomeprop, Cumyluron, Diethofencarb, Difenoconazole, Diflufenican, Dimethametryn, Dimethoate, Dimethomorph, Esprocarb, Fenbuconazole, Fenoxycarb, Flusilazole, Hexaconazole, Imazalil, Indanofan, Iprovalicarb, Isoxathion, Mepanipyrim, Methabenzthiazuron, Methomyl, Monocrotophos, Napropamide, Paclobutrazol,

Penconazole, Pirimicarb, Pretilachlor, Prochloraz, Propoxur, Propyzamide, Pyroquilon, Tebuconazole, Tebufenozide, Teflubenzuron, Thenylchlor, Thiacloprid, Thiobencarb, Triadimefon, Triadimenol, Triflumizole

Group B (29 pesticides with acceptable results in 6 agricultural products except for the agricultural product exhibited in parentheses):

Acephate (Or), Allethrin (Or), Azoxystrobin (Or), Buprofezin (Sy), Carbaryl (Sy), Chlorpropham (Sy), Cyanazine (Po), Cyflufenamid (Cb), Cyhalofop-butyl (Po), Daimuron (Sy), Diflubenzuron (Sy), Dimepiperate (Br), Diphenamid (Sp), Ethofumesate (Ap), Etobenzanid (Po), Fenobucarb (Po), Flufenoxuron (Sp), Hexaflumuron (Sp), Imibenconazole (Sp), Isoprocarb (Sy), Isoprothiolane (Or), Mefenacet (Sy), Metalaxyl (Cb), Methamidophos (Po), Omethoate (Po), Oxamyl (Br), Pencycuron (Or), Quizalofop-ethyl (Po), Tebufenpyrad (Sy)

Group C (10 pesticides with acceptable results in 5 agricultural products except for the agricultural products exhibited in parentheses):

Carbofuran (Sp, Ap), Fenoxaprop-ethyl (Po, Sy), Fenpropimorph (Cb, Ap), Furathiocarb (Sp, Sy), Lufenuron (Sp, Br), Pentoxazone (Br, Sy), Pyriproxyfen (Cb, Sy), Quinoclamine (Cb, Sy), Tri-allate (Cb, Sy), Trichlamide (Cb, Sy)

Group D (11 pesticides with acceptable results in 4 agricultural products except for the agricultural products exhibited in parentheses):

Bendiocarb (Ap, Br, Sy), Benfuresate (Po, Sp, Br), Cafenstrole (Or, Br, Sy), Carfentrazone-ethyl (Po, Br, Sy), Ethiofencarb (Po, Ap, Sy), Fenarimol (Cb, Or, Br), Inabenfide (Sp, Ap, Sy), Metolcarb (Cb, Po, Sy), Phenmedipham (Po, Br, Sy), Phoxim (Cb, Po, Sy), Propamocarb (Po, Br, Sy)

Group E (1 pesticide with acceptable results in 3 agricultural products exhibited in square brackets):

Molinate [Cb, Sp, Or]

Group F (1 pesticide with acceptable results in 2 agricultural products exhibited in square brackets):

Propiconazole [Cb, Sy]

4.2 Method for processed foods in high lipid group

4.2.1 Rapidity of the method

In this procedure, we used ethyl acetate for the first extraction step given its lipid solubility. The time for one chemist to prepare test solutions for 12 samples was approximately 5~6 h. For rapidity, we adopted the acetonitrile-hexane partition to remove lipids from the extract in this procedure. A chemist can conduct the procedure simultaneously for up to 12 samples. Furthermore, the collected acetonitrile layer can be applied onto the GCB/PSA column directly. Gel permeation column chromatography is an established technique to remove lipid from the extract in pesticide analysis (Gilbert-Lopez, B., et al., 2009, Sannino, A., et al., 1999). However, this technique cannot be used to analyze many samples simultaneously; for more than 12 samples, the collected fraction should be concentrated and reconstituted in an appropriate solvent for further purification. To confirm the effectiveness of the acetonitrile-hexane partition, we recorded the weight of the residues in the analysis of pesticides in Chinese dumplings. The remaining residue prior to the acetonitrile-hexane partition was 8.9%, corresponding to the original sample weight in the aliquot (2.0 g). After the acetonitrile-hexane partition, the remaining residue was less than 0.1%. Thus, the acetonitrile-hexane partition would be one of the most efficient and suitable techniques for removing lipids.

4.2.2 Recovery tests

We performed recovery tests of pesticides sensitively detectable with GC-MS or GC-MS/MS via fortification of the pesticide mixtures of the 5 processed foods (Chinese dumplings, curry, French fries, fried chicken, and fried fish) at the final concentrations of 0.02 and 0.10 ppm, respectively. We conducted 3 trials for each test and defined an acceptable result as the one with a recovery of 70~120% with a RSD ≤ 20% for both concentrations.

4.2.2.1 Analysis of 225 pesticides via GC-MS (processed foods in high lipid group)

We conducted recovery tests of 225 pesticides detectable at a concentration of 0.01 ppm by GC-MS. These pesticides were classified into 6 groups as per the number of processed foods with acceptable results: A, 5; B, 4; C, 3; D, 2; E, 1; and F, 0. Details are described below. Notations of the processed foods are as follows: D, Chinese dumplings; C, curry; P, French fries; Ck, fried chicken; and F, fried fish.

Group A (99 pesticides with the acceptable results in all the 5 processed foods):

Acetochlor, Alachlor, Ametryn, Anilofos, Atrazine, Azinphos-methyl, Azoxystrobin, Benfluralin, Benfuresate, Benoxacor, Bromacil, Bupirimate, Butafenacil, Chlorfenvinphos-*E*, Clodinafop-propargyl, Cyflufenamid, Cyhalofop-butyl, Cyprodinil, Dichlofenthion, Diflufenican, Dimethamethryn, Dioxabenzofos, Diphenylamine, Dithiopyr, Edifenphos, Esprocarb, Ethalfluralin, Ethion, Ethoprophos, Etofenprox, Etoxazole, Etrimfos, Fenoxanil, Fenthion, Flamprop-methyl, Fluacrypyrim, Fludioxonil, Flumiclorac-pentyl, Fluquinconazole, Flusilazole, Flutolanil, Fluvalinate, Furathiocarb, Hexaconazole, Iprobenfos, Isazofos, Isofenphos, Isoprocarb, Kresoxim-methyl, Mefenacet, Mepronil, Metolachlor, Metolcarb, Metribuzin, Mevinphos, Nitrothal-isopropyl, Oxadiazon, Parathion, Parathion-methyl, Penconazole, Phenthoate, Phorate, Picolinafen, Piperophos, Pirimiphos-methyl, Pretilachlor, Prometryn, Propachlor, Propanil, Propaphos, Propiconazole, Prothiofos, Pyraclofos, Pyrazophos, Pyridaben, Pyridaphenthion, Pyrimethanil, Pyrimidifen, Pyriminobac-methyl-*E*, Pyriminobac-methyl-*Z*, Qunalphos, Simazine, Simetryn, Sulprofos, Tebuconazole, Tebufenpyrad, Terbufos, Terbutryn, Tetraconazole, Thenylchlor, Thiobencarb, Thiazopyr, Tolclofos-methyl, Triadimefon, Triadimenol, Triazophos, Trifluralin, Uniconazole P, Vinclozolin, Xylylcarb

Group B (65 pesticides with the acceptable results in the 4 processed foods except for the processed food exhibited in parentheses):

Acrinathrin (D), Bendiocarb (C), β-BHC (C), γ-BHC (D), Butachlor (C), Butamifos (Ck), Cafenstrole (Ck), Carbofuran (F), Carfentrazone-ethyl, (Ck) Chlorfenvinphos-*Z* (C), Chlorobenzilate (C), Chlorpropham (Ck), Clomeprop (C), Cyanophos (Ck), p,p'-DDD (C), Diazinon (D), Diclobutrazol (Ck), Diclofop-methyl (C), Diethofencarb (C), Dimethenamid (Ck), Dimethoate (D), Dimethylvinphos (C), Diofenolan (P), EPN (F), Ethofumesate (P), Etobenzanid (P), Fenamiphos (Ck), Fenbuconazole (D), Fenitrothion (Ck), Fenobucarb (C), Flucythrinate (F), Flumioxazin (D), Furametpyr (P), Furilazole (C), Halfenprox (C), Hexazinone (P), Isoprothiolane (C), Malathion (C), Methacrifos (P), Methiocarb (C), Metominostrobin-*E* (C), Metominostrobin-*Z* (Ck), Monocrotophos (C), Myclobutanil (P), Napropamide (F), Norflurazon (P), Oxyfluorfen (Ck), Pendimethalin (Ck), 2-Phenylphenol (Ck), Phosalone (C), Primiphos-ethyl (C), Promecarb (C), Propham (C), Propoxur, (C) Pyributicarb (F), Pyriproxyfen (C), Silafluofen (Ck), Simeconazole (Ck), Tefluthrin (C), Terbacil (Ck), Tetradifon (C), Thiabendazole (Ck), Thifluzamide (D), XMC (Ck)

Group C (36 pesticides with the acceptable results in the 3 processed foods except for the processed foods exhibited in parentheses):
Acephate (P, F), δ-BHC (D, Ck), Bifenthrin (C, Ck), Bromobutide (D, C), Chlorpyrifos (C, P), Chlorpyrifos-methyl (C, P), Cyanazine (C, P), Cyanofenphos (C, Ck), Cyhalothrin (D, C), Cyproconazole (D, P), o,p'-DDT (C, Ck), p,p'-DDT (C, Ck), Dimepiperate (C, P), Diphenamid (P, Ck), Fenarimol (Ck, F), Fenchlorphos (C, P), Fenothiocarb(P, Ck), Fenpropathrin (D, F), Fensulfothion (C, P), Fipronil (C, P), Flutriafol (C, P), Iprovalicarb (D, C), Methidathion (Ck, F), Molinate (C, P), Omethoate (C, Ck), Phenothrin (C ,Ck), Primicarb (C, Ck), Procymidone (C, F), Propyzamide (D, Ck), Quinoxyfen (C, Ck), Tecnazene (C, P), Tetrachlorvinphos (C, P), Tri-allate (C, P), Tribufos (P, Ck)
Group D (18 pesticides with the acceptable results in the 2 processed foods exhibited in parentheses):
Benalaxyl [D, F], Cadusafos [P, F], Carbaryl [D, P], Clomazone [C, F], Cloquintocet-1-methylhexyl [C, F], p,p'-DDE [D, F], Dichlorvos [Ck, F], Diphenyl [Ck, F], Fthalide [Ck, F], Metalaxyl [D, F], Oxadixyl [D, P], Paclobutrazol [D, C], Pencycuron [Ck, F], Phosmet [P, F], Phosphamidon, [D, P] Profenofos [Ck, F], Pyraflufen-ethyl [Ck, F]
Group E (6 pesticides with the acceptable results in the 1 processed foods exhibited in square brackets):
Allidochlor [D], α-BHC [F], Bromophos [F], Chlorthal-dimethy,l [F], Fenoxycarb [D], Methamidophos [P]
Group F (1 pesticide without the acceptable results in all the 5 processed foods):
Fenpropimorph

4.2.2.2 Analysis of 258 pesticides via GC-MS/MS (processed foods in high lipid group)

On the chromatograms obtained via GC-MS analysis, some pesticides were interfered from the matrix derived from the foods. A GC-MS/MS is one of the most useful tools to overcome the interference on these chromatograms. As a relevant example, Figure 1 shows the chromatograms of methidathion fortified in the fried fish at a concentration of 0.02 ppm obtained via GC-MS and GC-MS/MS (Kitagawa, Y., et al., 2009, 2009). On the GC-MS chromatogram, methidathion could not be detected with either of the selected ion monitoring channels because of the interference. On the other hand, methidathion could be clearly detected with quantitative accuracy on the GC-MS/MS chromatogram. The improvement in the signal to noise ratio on the chromatogram (i.e. sensitivity) was due to the use of GC-MS/MS, given its high selectivity in monitoring pesticides. The GC-MS/MS also expanded the pesticides detectable at a concentration of 0.01 ppm. We conducted recovery tests of 258 pesticides detectable at this concentration via GC-MS/MS. The pesticides with asterisks were examined only with GC-MS/MS in this section. These pesticides were classified into 6 groups (A~F) as described above. The percentage of pesticides classified in group "A" increased from 44.0% with GC-MS to 71.3% using GC-MS/MS. The sensitivity and selectivity of GC-MS/MS would be helpful for the determination of pesticides in foods with interference, such as processed foods classified in the high lipid group. The relevant details are as follows.
Group A (184 pesticides with the acceptable results in all the 5 processed foods):
Acetochlor, Alachlor, Ametryn, Anilofos, Azinphos-methyl, Azoxystrobin, Benalaxyl, Benfluralin, Benfuresate, Benoxacor, β-BHC, δ-BHC, γ-BHC, Bifenox, Bifenthrin, Bitertanol, Bromacil, Bromobutide, Bromophos, Bromopropylate, Bupirimate, Buprofezin, Butafenacil,

Butamifos, Cadusafos, Cafenstrole, Carbaryl, Carbofuran, Carfentrazone-ethyl, Chlorfenapyr, α-Chlorfenvinphos, β-Chlorfenvinphos, Chlorobenzilate, Chlorpropham, Chlorpyrifos, Chlorpyrifos-methyl, Chlorthal-dimethyl, Clomazone, Clomeprop, Cloquintocet-1-methylhexyl, Cyanazine, Cyanophos, Cyflufenamid, Cyfluthrin, Cyhalofop-butyl, Cyhalothrin, Cypermethrin, Cyproconazole, Cyprodinil, p,p'-DDD, p,p'-DDT, Diazinon, Dichlofenthion, Diclobutrazol, Dicloran, Dieldrin, Diethofencarb, Diflufenican, Dimethametryn, Dimethenamid, Dimethylvinphos, Diofenolan, Dioxabenzofos, Diphenamid, Diphenylamine, Dithiopyr, Edifenphos, α-Endosulfan, β-Endosulfan, EPN, Esprocarb, Ethalfluralin, Ethion, Ethofumesate, Ethoprophos, Etobenzanid, Etofenprox, Etoxazole, Etrimfos, Fenarimol, Fenbuconazole, Fenchlorphos, Fenitrothion, Fenothiocarb, Fenoxanil, Fenpropathrin, Fenvalerate, Fipronil, Flamprop-methyl, Fluacrypyrim, Flucythrinate, Fludioxonil, Flumioxazin, Fluquinconazole, Flusilazole, Flutolanil, Fluvalinate, Fosthiazate, Fthalide, Furametpyr, Furathiocarb, Halfenprox, Iprobenfos, Iprovalicarb, Isofenphos, Mefenacet, Mepronil, Metalaxyl, Methidathion, Methiocarb, Methoxychlor, Metolachlor, Metominostrobin-E, Metominostrobin-Z, Metribuzin, Monocrotophos, Myclobutanil, Napropamide, Nitrothal-isopropyl, Omethoate, Oxadiazon, Oxadixyl, Oxyfluorfen, Paclobutrazol, Parathion, Parathion-methyl, Penconazole, Pencycuron, Pendimethalin, Permethrin, Phenothrin, Phenthoate, 2-Phenylphenol, Phosalone, Phosphamidon, Piperophos, Pirimicarb, Pirimiphos-ethyl, Pirimiphos-methyl, Pretilachlor, Procymidone, Profenofos, Promecarb, Prometryn, Propachlor, Propanil, Propoxur, Propyzamide, Prothiofos, Pyraclofos, Pyraflufen-ethyl, Pyrazophos, Pyributicarb, Pyridaphenthion, Pyrifenox-E, Pyrimethanil, Pyriminobac-methyl-E, Pyriminobac-methyl-Z, Quinalphos, Quinoxyfen, Silafluofen, Simazine, Simeconazole, Simetryn, Tebuconazole, Tebufenpyrad, Tefluthrin, Terbutryn, Tetrachlorvinphos, Tetraconazole, Thenylchlor, Thifluzamide, Thiobencarb, Tolclofos-methyl, Triadimefon, Triadimenol, Tri-allate, Triazophos, Tribufos, Trifloxystrobin, Triflumizole, Trifluralin, Uniconazole P, XMC, Xylylcarb

Group B (39 pesticides with the acceptable results in the 4 processed foods except for the processed food exhibited in parentheses):

Bendiocarb (C), α-BHC (C), Butachlor (D), Clodinafop-propargyl (C), p,p'-DDE (C), o,p'-DDT (C), Diclofop-methyl (D), Difenoconazole (C), Dimethoate (D), Fenobucarb (C), Fensulfothion (C), Flumiclorac-pentyl (C), Furilazole (Ck), Heptachlor (C), Heptachlor-epoxide (D), Hexaconazole (Ck), Indoxacarb-MP (C), Iprodione (P), Isazofos (C), Isoprocarb (C), Isoprothiolane (D), Isoxathion (D), Kresoxim-methyl (C), Lactofen (D), Lenacil (P), Malathion (D), Metolcarb (P), Mevinphos (P), Norflurazon (P), Picolinafen (C), Prochloraz (D), Propiconazole (F), Pyridaben (Ck), Pyrimidifen (C), Quintozene (C), Terbacil (C), Terbufos (P), Vinclozolin (C)

Group C (18 pesticides with the acceptable results in the 3 processed foods except for the processed foods exhibited in parentheses):

Atrazine (D, C), Bioallethrin (C, Ck), Cyanofenphos (D, F), Deltamethrin (C, Ck), Dimepiperate (C, P), Endrin (C, P), Fenoxycarb (D, Ck), Fenthion (P, Ck), Flutriafol (P, F), Hexazinone (P, F), Methacrifos (C, P), Molinate (C, P), Propargite (D, Ck), Pyrifenox-Z (C, P), Pyriproxyfen (D, F), Tecnazene (C, P), Tetradifon (D, Ck), Tralomethrin (D, Ck)

Group D (7 pesticides with the acceptable results in the 2 processed foods exhibited in square brackets):

Acrinathrin [D, P], Dicofol [D, F], Fenamiphos [D, Ck], Propaphos [D, F], Propham [P, Ck], Sulprofos [D, C], Thiazopyr [C, Ck]

Group E (6 pesticides with the acceptable results in the 1 processed foods exhibited in square brackets):
Acephate [Ck], Allidochlor [F], Methamidophos [F], Phorate [D], Phosmet [F], Thiabendazole [D]
Group F (4 pesticides without the acceptable results in all the 5 processed foods):
Aldrin, Dichlorvos, Diphenyl, Fenpropimorph

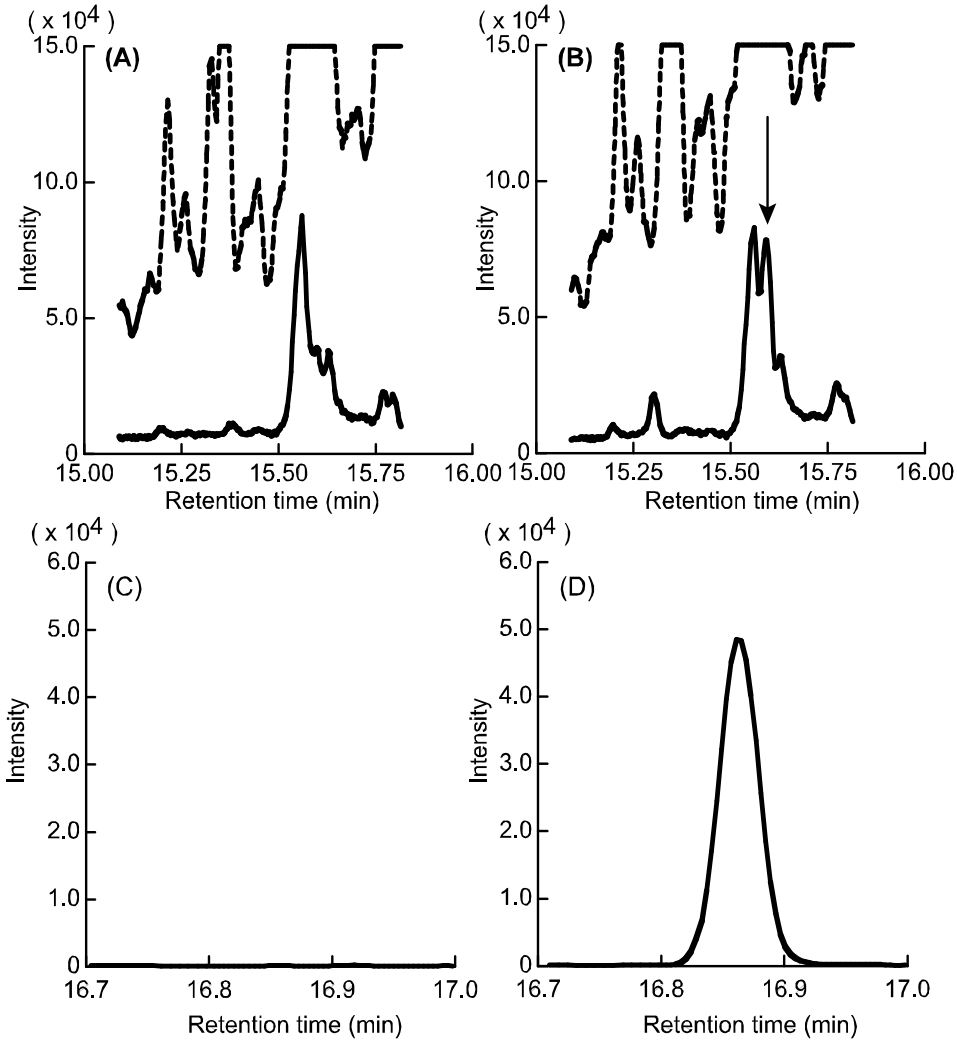

Fig. 1. SIM [(A) and (B)] and SRM [(C) and (D)] chromatograms of methidathion (0.02 ppm) in the test solution obtained from the recovery test of fried fish. (A) and (C), non-fortified fried fish; (B) and (D), fortified fried fish. The broken and solid lines in (A) and (B) are monitoring at m/z's of 145 and 85, respectively. The solid lines in (C) and (D) are monitoring the transition from 145 to 85. An arrow in (B) indicates the retention time of methidathion. The retention time of methidathion in SIM and SRM were 15.60 and 16.87, respectively. (The time programs of the GC oven temperature were not the same in these experiments)

4.2.2.3 Analysis of 99 pesticides via LC-MS/MS (Chinese dumplings)

The 99 pesticides detectable via LC-MS/MS were fortified to the Chinese dumplings at the concentrations of 0.02 and 0.10 ppm, respectively. We conducted 5 trials for each test. The pesticides were categorized into 4 groups on the basis of the results.
Group A (72 pesticides exhibiting the acceptable results at the both concentrations):
Acetochlor, Allethrin, Atrazine, Azoxystrobin, Benfuresate, Bensulide, Bitertanol, Bromobutide, Bupirimate, Buprofezin, Cafenstrole, Carfentrazone-ethyl, Carbaryl, Carbofuran, Chlorpropham, Cyanazine, Cyhalofop-butyl, Daimuron, Diethofencarb, Difenoconazole, Diflubenzuron, Diflufenican, Dimepiperate, Dimethametryn, Dimethoate, Dimethomorph, Diphenamid, Ethofumesate, Etobenzanid, Fenarimol, Fenbuconazole, Fenoxycarb, Fenoxaprop-ethyl, Flusilazole, Furathiocarb, Hexaconazole, Hexaflumuron, Imibenconazole, Inabenfide, Indanofan, Iprovalicarb, Isoprocarb, Isoprothiolane, Isoxathion, Mefenacet, Mepanipyrim, Metalaxyl, Methabenzthiazuron, Methomyl, Metolcarb, Monocrotophos, Napropamide, Omethoate, Paclobutrazol, Penconazole, Pencycuron, Pirimicarb, Pretilachlor, Prochloraz, Propoxur, Pyriproxyfen, Pyroquilon, Quizalofop-ethyl, Tebuconazole, Tebufenozide, Teflubenzuron, Thenylchlor, Thiacloprid, Thiobencarb, Tri-allate, Triadimefon, Triadimenol
Group B (15 pesticides exhibiting the acceptable results at only 0.10 ppm):
Acetamiprid, Acephate, Alachlor, Clomeprop, Cyflufenamid, Fenobucarb, Flufenoxuron, Imazalil, Lufenuron, Pentoxazone, Phoxim, Propyzamide, Quinoclamine, Tebufenpyrad, Triflumizole
Group C (4 pesticides exhibiting the semi-acceptable recovery results, 50~69% or 120~150%, at least at the concentrations 0.10 ppm with RSD ≤ 20%):
Cumyluron, Esprocarb, Methamidophos, Oxamyl
Group D (8 pesticides could not be categorized as A~C):
Bendiocarb, Ethiofencarb, Fenpropimorph, Molinate, Phenmedipham, Propamocarb, Propiconazole, Trichlamide

4.2.3 Case of a pesticide detected with our method

The method should be useful for analyzing pesticide residues in foods with complaints, such as odors derived from uncertain chemicals. As an example, we present a case in which we successfully detected the pesticide, phenothrin, from a suspected consumers' food (omelets in catering lunch boxes) at a concentration of 0.06 ppm in a half-day period. In this case, the pesticide, which was used to sanitize the catering kitchen, migrated into the refrigerator due to a faulty door and contaminated the omelets inside. To analyze the pesticide residues in foods in cases such as this, rapidity is one of the most pivotal aspects. Thus, governments and food industries should develop rapid methods to analyze pesticide residues in foods as a part of crisis management.

4.3 Method for processed foods in non- and low lipid groups (under study)

We are currently studying methods to determine the presence and quantities of pesticide residues in processed food in the low and non-lipid groups, such as dried fruits, marmalade, pickles (including soured vegetables and fruits), and seasonings. In this method, the process for removing lipids is not necessary. For the sake of efficiency, a method should be developed with minimum modification of the method described in 3.4.1 to determine pesticides in agricultural products. We conducted pilot studies for the development of such

a method and found that dried fruits and marmalades were not miscible with acetonitrile in the extraction step. This problem was successfully overcome by adding an equal weight of water to the sample and allowing it to stand for 30 min prior to the extraction with acetonitrile. We are currently validating the method in our laboratory. Details will be published later.

Homogenized sample 5.0 g in a 50 mL PP disposal tube
- Add 5 mL of water and stand for 30 min
- Add 20 mL of acetonitrile
- Homogenize (1 min)
- Add 4 g of $MgSO_4$ and 1 g of NaCl
- Shake (1 min) and centrifuge

Acetonitrile layer 16 mL (equivalent to 4.0 g sample)
- Load on a GCB/PSA column
- Elute with 30 mL acetonitrile–toluene (3+1)
- Collect loading and eluting fractions in a round bottom flask
- Evaporate and reconstituted in appropriate solvent

GC-MS (/MS) or LC-MS/MS analysis

Scheme IV. Procedure of sample preparation for the determination of pesticides residues in processed foods categorized as the non or low-lipid group.

	Foods			
Category	Agricultural Products		Processed Food	
Group	Vegetables and fruits	Cereals	High-lipid	Non or Low-lipid
Water #	No	Yes	No	Yes
Extraction ##	AcCN	AcCN	EtOAc	AcCN
Cleanup $	GCB/PSA	C18 GCB/PSA	AcCN/Hex GCB/PSA	GCB/PSA
Time (h) $$	3~4	3.5~4.5	5~6	3.5~4.5
Analysis	GC-MS, GC-MS/MS and LC-MS/MS			
Scheme	I	II	III	IV

#: Add water before extraction.
##: Extraction solvents; acetonitrile and ethyl acetate are abbreviated to AcCN and EtOAc, respectively.
$: Using columns and extraction procedure; GCB/PSA, GCB, and PSA double layer column; C18, octadecylsilyl column; AcCN/Hex, acetonitrile-hexane partition.
$$: Time for preparation of 12 samples by a chemist.

Table 1. Summary of the analytical methods of pesticides in foods.

5. Conclusion

"Rapid and easy" multiresidue methods for the determination of pesticide residues in foods have been developed. Table 1 summarizes these methods. The methods are based on a simple extraction with organic solvents, a purification with SPE cleanup, and determinations with GC-MS, GC-MS/MS, and LC-MS/MS. The proposed methods

exhibited good sensitivity and recovery and allowed for rapid analysis. For agricultural products, a single chemist could prepare test solutions from 12 corresponding homogenized samples within 4.5 h. For processed foods, a single chemist could prepare test solutions for 12 corresponding homogenized samples within 6 h. Our method does not require special techniques in sample preparation. The characteristic points of the methods, "rapid and easy," would induce substantial benefits: (a) reduction of time and costs for sample preparation, (b) reduction of time for mastering the operations, and (c) reduction of the errors within the procedures. These reductions would produce more time and money to simultaneously analyze more pesticides with better performance and to test the adaptation of new pesticides to this method. The methods described here have a high potential covering a wide range of pesticides. Thus, they would be applicable to various foods and ideally suited for use in regulatory laboratories.

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9

Organochlorine Pesticides in Human Serum

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1. Introduction

Organochlorine pesticides are a group of chlorinated compounds that persist in the environment. They were used mainly in agriculture and for pest control in cities for several decades. Their widespread use has resulted in their ubiquitousness in various environments and bioaccumulation throughout the food chain. Although many organochlorine pesticides are banned, their accumulation can have adverse effects on human health and the environment. In this chapter, we emphasize the organochlorine pesticides listed by the Stockholm Convention on persistent organic pollutants: aldrin, chlordanes, dieldrin, endrin, heptachlor, heptachlor epoxide, hexachlorobenzene (HCB), mirex, dichlorodiphenyltrichloroethanes (DDTs), toxaphene, and hexachlorcyclohexanes (HCHs).
As these organochlorine pesticides have similar physicochemical properties, they can be analyzed by using the same analytical procedures, which are composed of extraction, cleanup, and selective and sensitive detection via gas chromatography with an electron capture detector, mass spectrometry, and high resolution mass spectrometry. The application of gas chromatography and high resolution mass spectrometry with an isotope dilution method provides highly accurate and reliable data. We describe a comprehensive extraction and purification method for analysis of target organochlorine pesticides and a measurement method using several equipments later in this chapter.
Many studies have revealed that the general population (including neonates), who lack occupational exposure to organochlorine pesticides, is exposed to background environmental levels of these chemicals. Among the organochlorine pesticides investigated, *p,p'*-DDE, β-HCH, hexachlorobenzene, *p,p'*-DDT, oxychlordane, and *trans*-nonachlor were frequently detected in human serum which are comparatively convenient human biological specimens obtained from all individuals. Epidemiologic research has revealed that these chemicals are associated with several health disorders. Therefore, we conclude this chapter with a summary and critical review on the status of organochlorine pesticides in serum of the general human population.

2. Characteristics of organochlorine pesticides

Organochlorine pesticides have similar chemical structures, showing chlorine-substituted aliphatic or aromatic cyclic rings. Because of the structural similarities, these pesticides share certain physicochemical characteristics such as persistence, toxicity, bioaccumulation, and long-range transport potential. As a result, the Stockholm Convention considered some organochlorine pesticides as environmental hazards and listed them as persistent organic

pollutants. The Convention is global treaty to protect human health and the environment from persistent organic pollutants.

Organochlorine pesticides persist in the environment. Persistency is defined as a half-life greater than two months in water or six months in soil and sediment. These chemicals are difficult to degrade into less hazardous substances in the environment. They are lipophilic compounds that tend to bioaccumulate in fatty tissues through the food chain. Bioaccumulation of organochlorine pesticides is defined as a log K_{OW} value greater than five or bioaccumulation factor in aquatic species greater than 5000. These pesticides are water insoluble and semivolatile, enabling their entry in the atmosphere and transport over long distances globally, mainly by air mass movements. They can reach polar or high mountainous regions and are effectively deposited in cold regions by snow through the phenomenon of cold condensation and global distillation (Wania and Mackay, 1995). Shen and Wania (2005) derived and compiled select physicochemical properties, including vapor pressure, water solubility, Henry's law constant (*H*), octanol–water partition coefficient (K_{OW}), and octanol–air partition coefficient (K_{OA}) of major organochlorine pesticides (Shen and Wania, 2005). These properties have been used to screen the many chemicals for persistent organic pollutants criteria (Muir and Howard, 2006).

2.1 Dichlorodiphenyltrichloroethane

Dichlorodiphenyltrichloroethane (DDT) is the common name of 1,1,1-trichloro-2,2-di-(4-chlorophenyl)ethane. Technical-grade DDT is a mixture of up to 14 compounds (Fig. 1). The active ingredient is *p,p'*-DDT (65–80%). The other compounds include 15–21% of *o,p'*-DDT, up to 4% of *p,p'*-DDD and 1-(*p*-chlorophenyl)-2,2,2-trichloroethanol, and traces of *o,o'*-DDT and bis(*p*-chlorophenyl)sulfone. Othmar Zeidler, an Austrian scientist, first synthesized DDT in 1874. Paul Hermann Müller, a Swiss chemist who received the Nobel Prize in 1948 by the discovery of its insecticidal properties in 1939; it was widely used as an agricultural insecticide after the war. DDT easily degrades into dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE), which are more persistent than the parent compound. The half-life of *p,p'*-DDE in humans has been estimated as more than 7 years

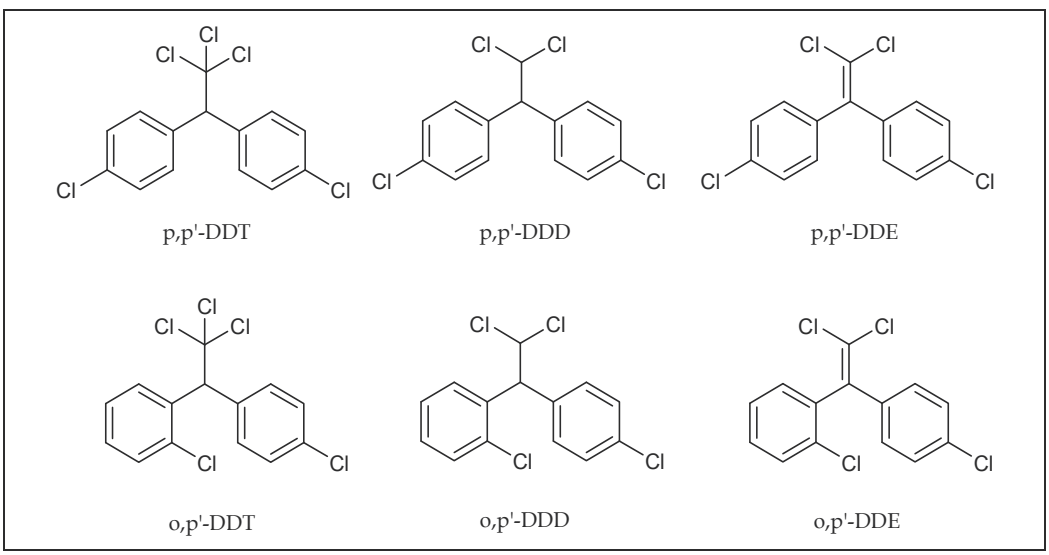


Fig. 1. Structures of dichlorodiphenyltrichloroethane analogues

(Axmon and Rignell-Hydbom, 2006). In the 1970s and 1980s, most countries banned the agricultural use of DDT. It was restricted in the United States in 1972 and finally banned in 1979. Agricultural use continues in some countries, and developing countries use about 4000–5000 tonnes annually for vector control applications.

2.2 Hexachlorcyclohexanes

Hexachlorcyclohexanes (HCHs) are broad-spectrum insecticides used on fruits, vegetables, and forest crops. They are available as two commercial formulations. Technical-grade HCH is a mixture of isomers containing mostly 64% α-HCH, 10% β-HCH, 13% γ-HCH, 9% δ-HCH, and 1% ε-HCH (Fig. 2). The other commercial formulation contains more than 99% γ-HCH (lindane). Micheal Faraday discovered HCHs in 1825 and Bender determined their insecticidal properties in 1935. Slade showed that γ-HCH was the only active insecticide of technical-grade HCH in 1942. Technical-grade HCH has been commercially produced since 1947. China and India, the two main users of technical-grade HCH globally, stopped its agricultural use in 1983 and 1990, respectively. The half-life of β-HCH in blood is 7 years, whereas γ-HCH has a half-life of only 20 hours.

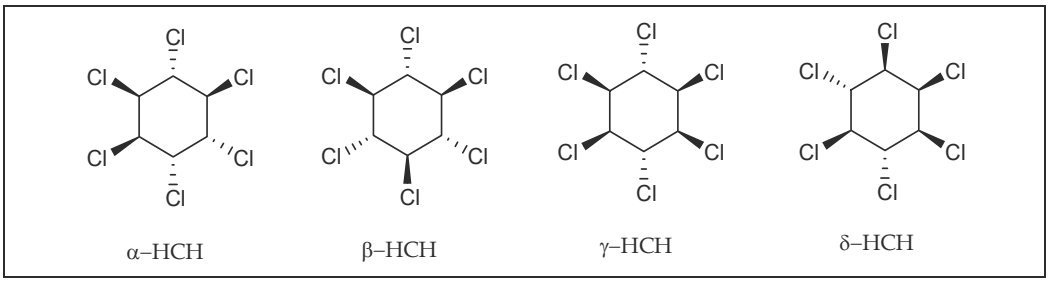


Fig. 2. Structures of hexachlorcyclohexane isomers

2.3 Chlordane

Chlordane was used as a contact insecticide for agricultural crops and lawns, and for termite control in buildings. It has been commercially produced since 1947. Technical-grade chlordane is a mixture of at least 23 compounds and typically consists of 15% *cis*-chlordane, 15% *trans*-chlordane, 9.7% *trans*-nonachlor, 3.9% heptachlor, 3.8% *cis*-nonachlor, other chlorinated hydrocarbons, and by-products (Fig. 3). Nonachlor is an impurityies of technical chlordane. Oxychlordane is an oxidized form of chlordane. In the United States, the use of chlordane on food crops was ceased in 1978 and all uses were banned after 1988.

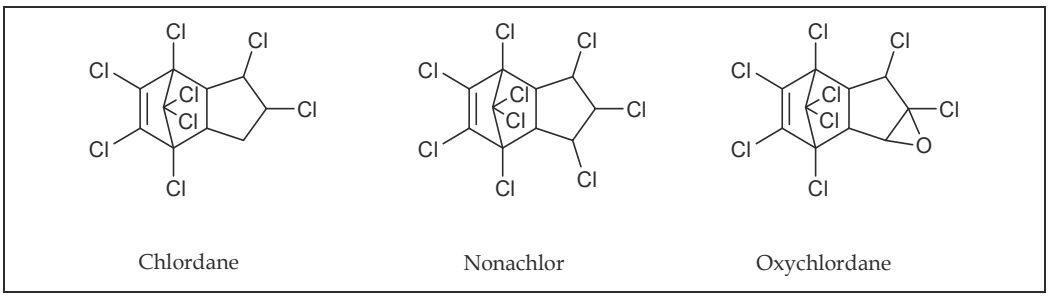


Fig. 3. Structures of chlordane, nonachlor, and oxychlordane

2.4 Heptachlor and heptachlor epoxide

The chemical structure of heptachlor is similar to that of chlordane (Fig. 4). Heptachlor has been used as an insecticide to control fire ants. It is rapidly oxidized by both photochemical and biological processes to heptachlor epoxide, which is an oxidation product of heptachlor. Thus, heptachlor epoxide appears after the use of heptachlor. Heptachlor use was voluntarily discontinued in 1987 in the United States.

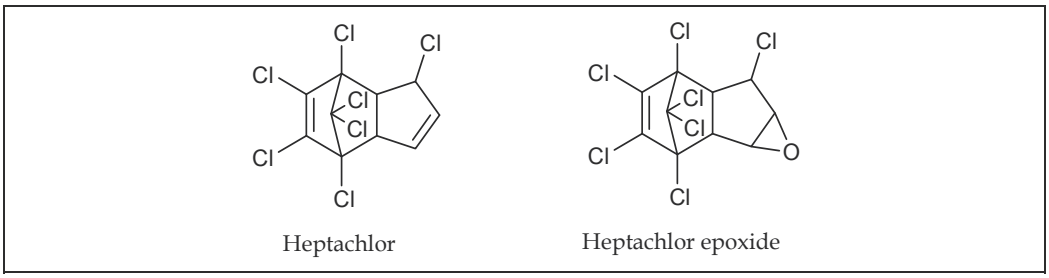


Fig. 4. Structures of heptachlor and heptachlor epoxide

2.5 Aldrin, dieldrin, and endrin

Aldrin, dieldrin, and endrin were used as insecticides from the 1950s to the mid-1970s. They have a similar structure (Fig. 5). Aldrin was first synthesized in 1948 and commercially manufactured in 1950. It is the common name of 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-exo-1,4-endo-5,8-dimethanonapthalene, and technical-grade aldrin contains 90% of aldrin. Dieldrin, a pesticide product, is the oxygenated metabolite of aldrin. Aldrin easily degrades into dieldrin and is therefore rarely detected in the environment. Endrin was introduced in 1951 and primarily used as a cotton insecticide. It is a stereoisomer of dieldrin and is rapidly metabolized in the environment. Endrin aldehyde and endrin ketone are its degradation products. In most countries, aldrin, dieldrin, and endrin are banned for agricultural use and severely restricted for nonagricultural applications. Agricultural use of these chemicals was banned in 1970 and all uses were banned in 1987 in the United States.

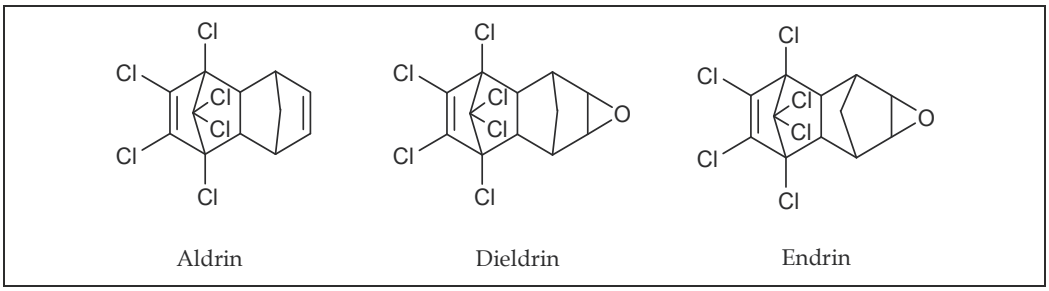
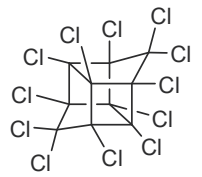


Fig. 5. Structures of aldrin, dieldrin, and endrin

2.6 Mirex

Mirex was synthesized in 1946 and has been used as a pesticide since 1955. It is produced by the dimerization of hexachlorocyclopentadiene in the presence of aluminum chloride (Fig. 6). Mirex was used not only as an insecticide to control fire ants but also as a flame-retardant

in plastic, rubber, paint, paper, and electrical goods. Hooker Chemical developed mirex as a chlorinated flame-retardant under the trade name Dechlorane. Its use as a pesticide was banned in the United States in 1978. Dechlorane was replaced by Dechlorane Plus in 1972.

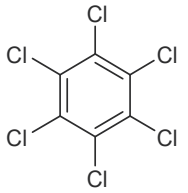


Mirex

Fig. 6. Structure of mirex

2.7 Hexachlorobenzene

Hexachlorobenzene (HCB) was introduced in 1945 as a fungicide for seed treatment (Fig. 7). It is formed as a by-product or an impurity during chemical and pesticide manufacture, and unintentionally produced by incomplete combustion in municipal solid waste incinerators and industrial processes. Most countries have banned or severely restricted its use as a pesticide. Hexachlorobenzene was banned in the United States in 1984.



Hexachlorbenzene

Fig. 7. Structure of hexachlorobenzene

3. Methods for analysis of organochlorine pesticides in human serum

3.1 Sample preparation of serum for analysis

The literature describes several preparation methods to measure organochlorine pesticides in human serum (Barr et al., 2003; Sandau et al., 2003; Focant et al., 2004; Conka et al., 2005; Ramos et al., 2007). The standard method consists of three steps: denaturation, extraction, and cleanup.

Either organic solvents (methanol, propanol, or acetonitrile) or acids (acetic acid, formic acid, and hydrochloric acid) are used for denaturation of the serum protein. As formic acid does not destroy acid-sensitive pesticides such as cyclodienes, it is widely used for denaturation (Sandau et al., 2003). The serum sample can also be denaturated with hydrochloric acid and 2-propanol (Hovander et al., 2000).

Serum samples are extracted by using liquid liquid extraction or solid-phase extraction. The conventional method is liquid liquid extraction with an organic solvent such as hexane;

however, this method is laborious and time consuming, and thus unsuitable for large-scale biomonitoring in epidemiologic studies. On the other hand, solid-phase extraction is a simple and efficient method. Commonly, a C_{18} solid-phase extraction cartridge is used for organochlorine pesticide extraction because the reverse-phase sorbent retains most organic analytes from aquatic matrices. C_{18} (octadecyl) is bonded to the silica surface to provide nonpolar interactions with the analytes. The Oasis HLB cartridge, a polymeric water-wettable reverse-phase sorbent, can be used for organochlorine pesticide extraction without additional lipid cleanup steps (Sundberg et al., 2006). Each of these sorbents exhibits unique properties of retention and selectivity for organochlorine compounds.

The extract is purified by using several kinds of cleanup methods to remove interferences such as lipids. These methods include conventional glass columns or solid-phase extraction cartridges with neutral silica, florisil, and acid silica. For additional cleanup steps, sulfuric acid treatment, gel permeation chromatography, and active carbon treatment can be added according to interferences. Of the organochlorine pesticides discussed here, heptachlor epoxide, aldrin, dieldrin, and endrin are sensitive to concentrated sulfuric acid. To analyze acid-sensitive pesticides, sulfuric acid treatment and sulfuric acid silica should be avoided during the cleanup steps (Goni et al., 2007). Extraction and cleanup steps with an adequate solid-phase extraction cartridge have advantages such as simplicity, ease of automation, and high throughput.

The typical analytical methods for serum analysis recommended by the Centers for Disease Control and Prevention (CDC) are followed, with minor modifications (Barr et al., 2003; CDC, 2003; Kang et al., 2008). In brief, serum samples (1–2 mL) are spiked with ^{13}C-labeled cleanup standards of organochlorine pesticides (ES-5349; Cambridge Isotope Laboratories, USA) and allowed to equilibrate. The samples are denatured and diluted with an equal amount of formic acid and water. The mixtures are remixed to ensure homogeneity and then loaded into preconditioned C_{18} solid-phase extraction cartridges. Each cartridge is dried and then eluted with 16 mL hexane, followed by concentration of the eluate to less than 6 mL. The eluate is applied to a silica gel/florisil solid-phase extraction cartridge and then eluted with 12 mL hexane followed by 12 mL dichloromethane/hexane (1:1, v/v). The cleaned extracts are concentrated with a gentle stream of nitrogen and transferred to vials. The solvents are evaporated at room temperature with dodecane. Before instrumental analysis, the samples are reconstituted with ^{13}C-labeled recovery standards (EC-5350; Cambridge Isotope Laboratories).

3.2 Instrumental analysis

After serum sample preparation, the cleaned extracts are analyzed by gas chromatography with several detectors including an electron capture detector, mass spectrometry, and high resolution mass spectrometry. An electron capture detector is one of the most sensitive detectors to measure halogenated compounds. Gas chromatography with an electron capture detector is employed because of the low cost and ease of operation as well as high sensitivity to organochlorine pesticides. However, this technique cannot differentiate co-eluted compounds. A DB-5 capillary column (30 m height × 0.25 mm inner diameter × 0.25 μm film thickness) is widely used to identify and quantify organochlorine pesticides in human serum. Some pesticides cannot be completely separated in these column conditions. The usual co-eluted organochlorine pesticides are heptachlor epoxide and oxychlordane (Fig. 8).

Gas chromatography with mass spectrometry can resolve the problem of co-elution and avoid misidentification of analytes if operated in the selected ion monitoring mode.

However, the current serum concentrations of some organochlorine pesticides in the general population are too low for quantification by gas chromatography with an electron capture detector or gas chromatography with mass spectrometry. Mass spectrometry allows quantification by isotope dilution. Gas chromatography–high resolution mass spectrometric analysis with an isotope dilution method enables better identification and quantification than the other methods mentioned here. Chemically, the analytes and ^{13}C-labeled analogues behave identically. However, they are distinguishable by their mass differences, allowing complete and automatic recovery correction for each analyte in each sample (Barr et al., 2003). To increase the sensitivity, the selected ion monitoring mode is also used during gas chromatography–high resolution mass spectrometric analysis.

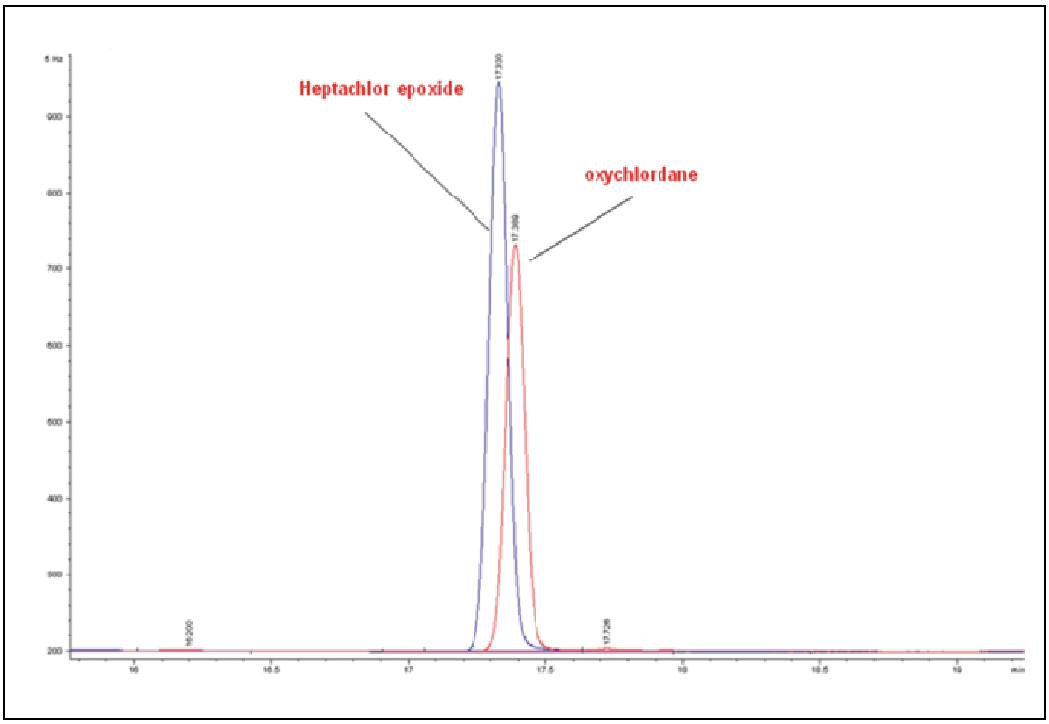


Fig. 8. Overlapping chromatograms of heptachlor epoxide and oxychlordane by gas chromatography with an electron capture detector

The state-of-the-art analytical method for organochlorine pesticides is isotope dilution gas chromatography–high resolution mass spectrometric quantification. This method is mandatory for PCDD/Fs and coplanar PCB analysis to achieve high sensitivity and precise quantification at low environmental levels. The Centers for Disease Control and Prevention have developed an isotope dilution gas chromatography–high resolution mass spectrometric method for quantification of organochlorines pesticides in human serum samples (CDC, 2003). The isotope-labeled pesticide standards and isotope dilution method support gas chromatography–high resolution mass spectrometric analysis (Kumar et al., 2005). For quantification of 22 persistent organochlorine pesticides, ^{13}C-labeled cleanup internal standards, recovery standards, and calibration standards have been commercially available at Cambridge Isotope Laboratories since 2006 (Kang et al., 2008).

Group	Compound	Monitor	Ion 1	Monitor	Ion 2
Group 1 (15.0-22.3)	HCH	216.9145	$[M]^+$	218.9155	$[M+2]^+$
	$^{13}C_6$-HCH	222.9347	$[M]^+$	224.9117	$[M+2]^+$
	HCB	283.8102	$[M+2]^+$	285.8072	$[M+4]^+$
	$^{13}C_6$-HCB	289.8303	$[M+2]^+$	291.8273	$[M+4]^+$
	Heptachlor	271.8102	$[M+2]^+$	273.8072	$[M+4]^+$
	$^{13}C_{10}$-Heptachlor	276.8269	$[M+2]^+$	278.8240	$[M+4]^+$
	$^{13}C_{12}$-CB-15	234.0406		226.9287	
Group 2 (22.3-23.5)	Aldrin	262.8570	$[M+2]^+$	264.8541	$[M+4]^+$
	^{13}C-Aldrin	269.8805	$[M+2]^+$	271.8775	$[M+4]^+$
	Oxychlordane	386.8053	$[M+2]^+$	388.8024	$[M+4]^+$
	$^{13}C_{10}$-Oxychlordane	396.8388	$[M+2]^+$	398.8358	$[M+4]^+$
	Heptachlor Epoxide	352.8442	$[M+2]^+$	354.8413	$[M+4]^+$
	$^{13}C_{10}$-Heptachlor Epoxide	362.8778	$[M+2]^+$	364.8748	$[M+4]^+$
	$^{13}C_{12}$-CB-70	301.9626		303.9597	
Group 3 (23.5-24.25)	o,p′-DDE	246.0003	$[M]^+$	247.9975	$[M+2]^+$
	$^{13}C_{12}$-o,p′-DDE	258.0406	$[M]^+$	260.0376	$[M+2]^+$
	Chlordane (trans, cis)	372.8260	$[M+2]^+$	374.8231	$[M+4]^+$
	$^{13}C_{10}$-trans-Chlordane	382.8595	$[M+2]^+$	384.8566	$[M+4]^+$
	trans-Nonachlor	406.7870	$[M+2]^+$	408.7841	$[M+4]^+$
	$^{13}C_{10}$-trans-Nonachlor	416.8205	$[M+2]^+$	418.8175	$[M+4]^+$
Group 4 (24.25-25.0)	o,p′-DDD	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	$^{13}C_{12}$-o,p′-DDD	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	p,p′-DDE	246.0003	$[M]^+$	247.9975	$[M+2]^+$
	$^{13}C_{12}$-p,p′-DDE	258.0406	$[M]^+$	260.0376	$[M+2]^+$
	Dieldrin	262.8570	$[M+2]^+$	264.8541	$[M+4]^+$
	$^{13}C_{12}$-Dieldrin	269.8805	$[M+2]^+$	271.8775	$[M+4]^+$
Group 5 (25.0-26.0)	Endrin	262.8570	$[M+2]^+$	264.8541	$[M+4]^+$
	$^{13}C_{12}$-Endrin	269.8805	$[M+2]^+$	271.8775	$[M+4]^+$
	p,p′-DDD	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	$^{13}C_{12}$-p,p′-DDD	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	o,p′-DDT	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	$^{13}C_{12}$-o,p′-DDT	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	cis-Nonachlor	406.7870	$[M+2]^+$	408.7841	$[M+4]^+$
	$^{13}C_{10}$-cis -Nonachlor	416.8205	$[M+2]^+$	418.8175	$[M+4]^+$
Group 6 (26.0-33.0)	p,p′-DDT	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	$^{13}C_{12}$-p,p′-DDT	235.0081	$[M]^+$	237.0053	$[M+2]^+$
	Mirex	271.8102	$[M]^+$	273.8072	$[M+2]^+$
	$^{13}C_{10}$-Mirex	276.8269	$[M]^+$	278.8240	$[M+2]^+$

Table 1. Accurate mass for selected monitoring ions, grouping retention times, and fragments

The conventional gas chromatography–high resolution mass spectrometric conditions are as follows. The separation of organochlorine pesticides is carried out by using a DB-5MS capillary column (60 m height × 0.25 mm inner diameter × 0.25 μm film thickness). A splitless injection (1 μL) is used with an injector temperature of 260 °C. The oven is programmed from 100 °C (5 min) to 280 °C with a ramp rate of 10 °C/min and then held at 280 °C for 10 min. The transfer line and source temperatures are 280 °C in the electron impact mode. The resolution is maintained over 10,000 (10% valley definition). The retention time is divided to collect data on 22 organochlorine pesticides in six groups. The accurate mass of the selected monitoring ions, grouping retention times, and fragments are shown in Table 1. The typical chromatograms of HCHs and DDEs in human serum are presented in Fig. 9.

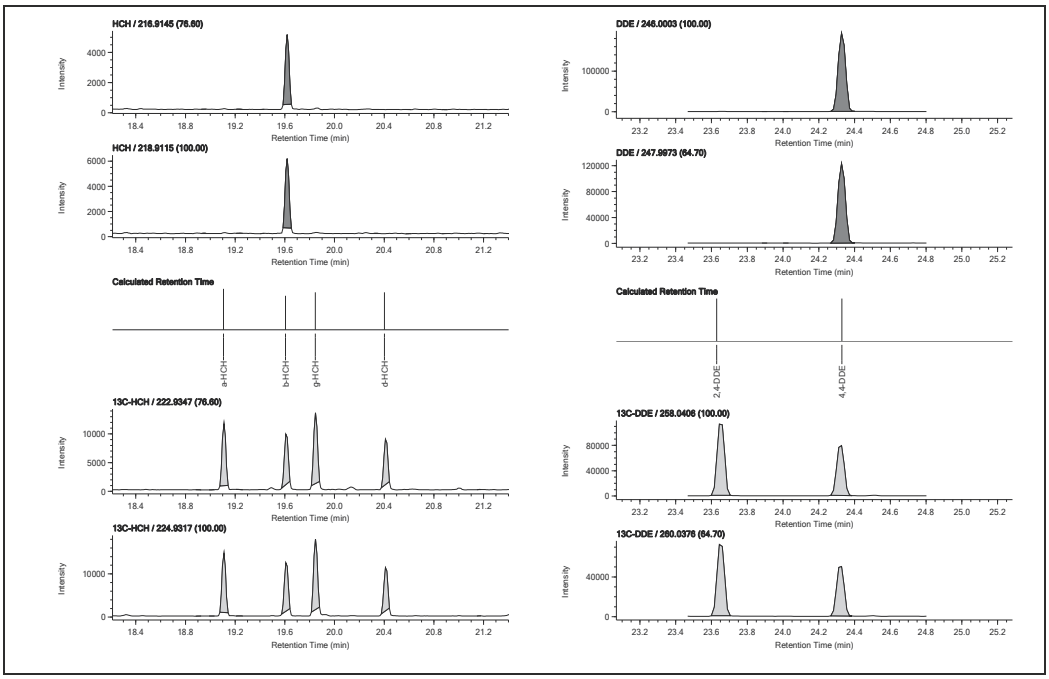


Fig. 9. Gas chromatography–high resolution mass spectrometric chromatograms of hexachlorcyclohexanes and dichlorodiphenyldichloroethylenes in human serum

4. Organochlorine pesticides in human serum

4.1 Organochlorine pesticides in the general population

Everybody has organochlorine pesticides in their body. Irrespective of age, gender, socioeconomic status, and country, these pesticides and their metabolites are detectable in blood or tissue, although the exposure levels differ according to various factors. Reported data on the levels of organochlorine pesticides in human serum are summarized in Table 2. It is difficult to compare these results directly because of the different periods of survey, ranging from 1959 to 2006. However, the serum levels of these chemicals in the general population have been decreasing over time, after the periods of their peak production and use. The most abundant residue in the general population is *p*,*p*′-DDE (Table 2); its median

serum concentration is much higher in the Chinese general population than in the United States, European, and other Asian populations in the same sampling year. The serum concentrations of these pesticides show regional differences because of regional variations in their use and different times of discontinuation.
Human exposure to organochlorine pesticides is primarily through the diet. The dietary main source is fatty food, such as meat, fish, poultry, and dairy products. Continuous exposure primarily occurs through the food supply, even if the produced foods within a country do not contain detectable residues, because food imported from other countries that still use organochlorine pesticides may be a source of human exposure. Exposure via inhalation of the ambient air is thought to be insignificant compared with dietary intake. Prenatal exposure to organochlorine pesticides is possible via the placenta. Breast feeding is the major source of infant exposure.

4.2 Levels of organochlorine pesticides in the North American population

The National Health and Nutrition Examination Survey (NHANES), conducted by the CDC's National Center for Health Statistics, is a program of studies designed to assess the health and nutritional status of adults and children in the United States. Of the part of the NHANES, organochlorine pesticides were measured in the 1999–2000, 2001–2002, and 2003–2004 surveys in a subsample of participants aged 20 years and older, who were selected as the representative sample of the United States population. The abundant organochlorine pesticides in serum were *p,p'*-DDE, *trans*-nonachlor, oxychlordane, HCB, and β-HCH. In the NHANES 1999–2000 and 2001–2002, the median serum HCB concentrations were below the detection limit, and in the NHANES 2003–2004 survey, the median serum concentration was 14.9 ng/g lipid. The median concentration of *p,p'*-DDE ranged from 203 to 250 ng/g lipid. The serum concentration of this compound was higher in Mexican-Americans than in non-Hispanic blacks and whites. The median serum concentrations of *trans*-nonachlor ranged from 17.8 to 14.8 ng/g lipid between 1999 and 2004. The median concentrations of oxychlordane in 2001–2002 and 2003–2004 were 11.1 and 12.3 ng/g lipid, respectively. The median concentrations of *p,p'*-DDT and *o,p'*-DDT were below the detection limit in all these surveys. The median concentrations of aldrin, dieldrin, endrin, β-HCH, γ-HCH, heptachlor epoxide, and mirex were also below the limit of detection. The serum concentrations of HCB, β-HCH, *p,p'*-DDE, oxychlordane, and *trans*-nonachlor increased with increasing age and were significantly higher in the 60 years and older age group (CDC, 2005; Patterson et al., 2009).
Because the general population was exposed to these pesticides by eating contaminated fatty food such as fish, an Inuit population in Greenland showed high levels of exposure (Rusiecki et al., 2008). Serum samples were collected from 70 Greenlandic Inuit (61 men, 9 women) under the Arctic Monitoring and Assessment Program (AMAP) in 1997–1998. Their age of the subject ranged from 19 to 67 years. The median concentrations of *p,p'*-DDT, *p,p'*-DDE, β-HCH, HCB, *trans*-chlordane, oxychlordane, and mirex were 25.1, 1268, 40.0, 239, 883, 249, and 38.9 ng/g lipid, respectively. Compared with the other populations and three National Health and Nutrition Examination Survey results, the serum organochlorine pesticide levels in Greenland Inuit are relatively high and the *p,p'*-DDE and β-HCH levels rank among the highest worldwide.

The National Health and Nutrition Examination Survey biomonitoring study show the current levels of organochlorine pesticides in the general population of the United States, while a longitudinal cohort study reveal the past exposure levels. The Child Health and Development Studies (CHDS) established a longitudinal cohort of 20,754 pregnant women enrolled in the San Francisco Bay Area Kaiser Foundation Health plan between 1959 and 1967 (Bhatia et al., 2005). During this period, DDT was produced and used in the United States (banned since 1972). Of the cohort, 283 maternal serum samples were randomly selected for DDT and DDE analysis. The median serum concentrations of *p,p'*-DDE and *p,p'*-DDT in maternal serum were 5200 and 1400 ng/g lipid, respectively. James et al. (2002) measured the serum concentrations of *p,p'*-DDT, *p,p'*-DDE, *o,p'*-DDT, *o,p'*-DDE, and heptachlor epoxide in pregnant women participating in the Child Health and Development Study (CHDS), conducted during 1963–1967, which was the time of peak usage and production of these organochlorine pesticides (James et al., 2002). The median concentrations of *p,p'*-DDE and *p,p'*-DDT were 5878 and 1611 ng/g lipid, respectively. The serum from the women of this cohort showed higher organochlorine pesticide concentrations than those of the recent general population samples. The serum concentration of *p,p'*-DDE and *p,p'*-DDT in the United States dramatically decreased over the period between 1950s and 2000s. The concentrations of *p,p'*-DDE were comparable in the Child Health and Development Study and in Chinese women from Shanghai in 1996–1998, whereas the concentration of *p,p'*-DDT was much higher than that in the Chinese women (Lee et al., 2007b).

4.3 Levels of organochlorine pesticides in the general Asian population

4.3.1 China

Currently, the general Chinese population shows one of the highest organochlorine pesticide residues in their body, indicating recent heavy use of these pesticides. The serum samples of 250 Chinese women from Shanghai were collected in 1996–1998 as part of a population-based case–control study on breast cancer (Lee et al., 2007b). The median serum concentrations of β-HCH, *p,p'*-DDT, *p,p'*-DDE, and HCB, measured by gas chromatography with an electron capture detector, were 5065, 309, 7635, and 62.7 ng/g lipid, respectively. The serum concentrations of β-HCH and *p,p'*-DDE among these women were much higher than those observed in other general populations in the same year. To assess the exposure to persistent organic pollutants in south China, serum samples were collected from the inhabitants of Guiyu and Haojiang, Guangdon Province, in 2005 and analyzed for 12 organochlorine pesticides by using gas chromatography and mass spectrometry in electron impact and selected ion monitoring modes (Bi et al., 2007). The *p,p'*-DDE concentrations ranged from 81 to 1500 ng/g lipid (median concentration of 540 ng/g lipid) in Guiyu and from 320 to 3900 ng/g lipid (median concentration of 1800 ng/g lipid) in Haojiang. The median HCH and DDT concentrations in Haojiang were three times higher than those in Guiyu although these cities are only about 50 km apart; the main industry of Guiyu is e-waste recycling and that of Haojiang is fishing. Guiyu showed one of the highest serum PBDE concentrations in the world. The general Chinese population has the highest concentrations of *p,p'*-DDE, *p,p'*-DDT, and β-HCH in their serum, suggesting that this population was heavily exposed to these pesticides, despite the fact that DDTs and HCHs have been banned in China.

4.3.2 Japan

The background serum levels of organochlorine compounds were evaluated by determination of 8 PCDDs, 10 PCDDFs, 40 PCBs, and 13 organochlorine pesticides in Japanese women of reproductive age (Tsukino et al., 2006). The serum samples were obtained from of 80 Japanese women aged 26–43 years and then analyzed by isotope dilution gas chromatography and high resolution mass spectrometry at the CDC in the United States. The frequently detected organochlorine pesticides were *p,p'*-DDE (median concentration of 221 ng/g lipid) followed by β-HCH, *trans*-nonachlor, and oxychlordane. The median concentrations of HCB, γ-HCH, heptachlor epoxide, dieldrin, *o,p'*-DDT, *p,p'*-DDT, and mirex were below the limit of detection. The results of this study firstly revealed the median serum concentration of *p,p'*-DDE in the general Japanese population. This concentration in serum was significantly and positively associated with fish intake. The geographic variation of serum organochlorine pesticide concentrations in Japan was studied by using serum samples obtained from the general population living in three locations (Miyako, Saku, and Nagono) in 1999 (Minh et al., 2006). The plasma samples were prepared by liquid liquid extraction, lipids were removed by a gel permeation chromatography and florisil column, and organochlorine pesticides were measured by gas chromatography with an electron capture detector. There were no significant regional differences in the concentrations of HCHs, DDTs, and HCB. On the other hand, chlordane compounds showed apparent geographic differences. Miyako had higher serum concentrations of chlordanes, suggesting historic use of chlordane pesticides for termite control.

Fukata et al. (2005) assessed fetal exposure to chlorine contaminants. In their study, 32 maternal serum and umbilical cord serum samples were collected from pregnant women who lived in Chiba and Yamanashi, near Tokyo, Japan in 2002–2003 (Fukata et al., 2005). Nineteen organochlorine pesticides were measured by using gas chromatography and mass spectrometry after liquid liquid extraction and a florisil cartridge cleanup. The highest concentrations found in maternal serum were *p,p'*-DDE (median concentration of 93 ng/g lipid), which was the lowest level of this compound among other studies on the general Japan population. The median concentrations of HCB, HCHs, *p,p'*-DDT, oxychlordane, and *trans*-nonchlor were 16, 26, 2.4, 1.2, and 7.0 ng/g lipid, respectively, with a detection rate over 80%. The median concentrations of *p,p'*-DDE and HCB in maternal serum were higher than those in cord serum. This study found a strong correlation between maternal serum and cord serum for some organochlorine pesticides such as HCB, HCHs, and heptachlor epoxide, which showed a correlation coefficient over 0.72; in particular, maternal serum showed higher organochlorine pesticide concentrations than cord serum.

Masuda et al. (2005) collected 152 blood samples from residents aged 20–60 years in Fukuoka in 1999 (Masuda et al., 2005). The serum samples were extracted with acetone/hexane and the lipid was removed by gel permeation chromatography. The samples were analyzed for HCB, *p,p'*-DDE, *p,p'*-DDT, *p,p'*-DDD, β-HCH , dieldrin, *trans*-nonachlor, *cis*-nonachlor, heptachlor epoxide, *trans*-chlordane, *cis*-chlordane, and oxychlordane by using gas chromatography–high resolution mass spectrometry. ^{13}C-labeled internal standards for organochlorine pesticides were not used in this study. All the pesticides analyzed were detected in serum samples. The median serum concentrations of HCB, β-HCH, dieldrin, *p,p'*-DDE, *p,p'*-DDT, *trans*-nonachlor, and oxychlordane were over 10 ng/g lipid, whereas those of heptachlor epoxide, *p,p'*-DDD, *trans*-chlordane, *cis*-chlordane,

and *cis*-nonachlor were lower than 10 ng/g lipid. The most abundant compound was *p,p'*-DDE (median concentration of 312 ng/g lipid) followed by β-HCH (median concentration of 280 ng/g lipid), which was the highest levels in Table 2, except for the Chinese samples from Shanghai.

Takasuka et al. (2004a) studied biological elimination of persistent organic pollutants in humans via intake of fermented brown rice with *Aspergillus oryzae* (Takasuga et al., 2004a). After two years of consumption, those who ate the fermented brown rice had greater elimination of PCDD/Fs than those who did not, although the former group showed slightly higher serum PBDE concentrations (Takasuga et al., 2004b). In 18 serum samples from nine couples aged 37–48 years in Japan, selected organochlorine pesticides were identified by gas chromatography–high resolution mass spectrometry to assess the impact of intake of fermented brown rice with *Aspergillus oryzae* on biological elimination of organochlorine pesticides (Takasuga et al., 2006). This paper reported total DDT, total HCH, total chlordane, and HCB concentrations of 230, 53, 36.5, and 13.5 ng/g lipid, respectively. Those who ate the fermented brown rice (7.5–10.5 g after their meal) for two years did not show reduced serum concentrations of DDTs, HCHs, and chlordanes.

4.3.3 Korea

Kang et al. (2008) reported the first investigation on the levels of organochlorine pesticides in human serum samples from urban areas in Korea (Kang et al., 2008). The serum samples were obtained from 40 subjects who participated in the Health Assessment Study of Seoul Citizens related to municipal solid waste incinerators and lived in three areas (Kangnam, Nowon, and Yangchun) in Seoul. The participants consisted of 20 males and 20 females (age range 27-58 years, mean age=45). The serum samples were extracted on C_{18} solid-phase extraction cartridges and then applied to silica gel/florisil solid-phase extraction cartridges for cleanup. The concentrations of organochlorine pesticides and PCBs were measured by isotope dilution gas chromatography–high resolution mass spectrometry, which gave accurate and precise data for investigations of trend and international comparisons. Among the 22 investigated organochlorine pesticides, *p,p'*-DDE, β-HCH , *p,p'*-DDT, HCB, and *trans*-nonachlor were frequently detected in all samples. The most abundant pesticide was *p,p'*-DDE, having a median concentration of 224 ng/g lipid. The median serum concentrations of β-HCH, *p,p'*-DDT, and HCB were higher than 10 ng/g lipid, whereas *trans*-nonachlor, heptachlor epoxide, oxychlordane, and *p,p'*-DDD had median concentrations below 10 ng/g lipid. The correlation coefficients between PCBs and organochlorine pesticides ranged from 0.365 to 0.906, with the highest correlation found between PCB153 and *trans*-nonachlor (r = 0.906). The organochlorine pesticides were also positively correlated to each other. Strong correlations between serum concentrations of organochlorine pollutants suggest that humans are exposed to PCBs and organochlorine pesticides via similar routes, because they were both widely used in the same period. In studies conducted within the Chinese population, extremely high concentrations of organochlorine pesticides contrasted with very low concentrations of PCBs (Lee et al., 2007b). A community-based health survey was performed in 2006 in Uljin (Son et al., 2010). Uljin is a geographically small county located on the east coast of Korea. Among 1007 participants, 40 subjects with diabetes and 40 controls matched for sex and age were selected. The most abundant organochlorine pesticide was *p,p'*-DDE,

followed by β-HCH, *p,p'*-DDT, *trans*-nonachlor, HCB, oxychlordane, and heptachlor epoxide among the 22 organochlorine pesticides analyzed. The total organochlorine pesticide concentrations ranged from 38.8 to 4598 ng/g lipid, with the mean and median concentrations of total organochlorine pesticides being 638 and 483 ng/g lipid, respectively. In addition, when the concentratons of organochlorine pesticides were compared between Seoul and Uljin, regional differences were found.

4.3.4 New Zealand

The serum samples were obtained from the New Zealand population aged 15 years and older during 1996–1997 (Bates et al., 2004). This is the first persistent organochlorine biomonitoring study in the adult population of an entire country, and the 60 serum samples were pooled according to stratification criteria by using 1834 individual serum samples. The aim of the study was to estimate the baseline concentration of organochlorine pesticides in serum among the general New Zealand population. Organochlorine pesticides were extracted by using a C_{18} solid-phase extraction cartridge and purified by a florisil cartridge. Target analytes were quantified by isotope dilution gas chlormatography–high resolution mass spectrometry operating in the selected ion monitoring mode. The frequently detected pesticides were *p,p'*-DDE, β-HCH, dieldrin, *p,p'*-DDT, HCB, and *trans*-nonachlor, but aldrin, endrin, heptachlor epoxide, oxychlordane, and mirex were not detected in the pooled serum. The median concentrations of *p,p'*-DDE and β-HCH were 919 and 10.7 ng/g lipid, respectively. The *p,p'*-DDE concentration showed an increasing trend from the north to the south regions, reflecting historical patterns of DDT use. In this study, dieldrin was frequently detected, with a median concentration of 11.5 ng/g lipid. The use of DDT and dieldrin in agriculture was banned in the 1970s. The serum concentrations of pesticides increased with age. The concentrations of *p,p'*-DDE, β-HCH, and dieldrin were significantly higher in the group aged over 50 years than in the younger age group. The median *p,p'*-DDE concentration of these subjects was lower than that of subjects from China (Bi et al., 2007), Romania (Dirtu et al., 2006), and Slovakia (Petrik et al., 2006). However, the median concentration of this compound in the New Zealand population was higher than that in subjects from Korea (Kang et al., 2008), Japan (Tsukino et al., 2006), the United Kingdom (Thomas et al., 2006), and the United States (CDC, 2005).

4.4 Levels in the European population

4.4.1 Sweden

In a population-based case–control study of organochlorines and endometrial cancer risk, the serum concentrations of five organochlorine pesticides in elderly women in Sweden were reported to evaluate associations between serum concentrations of organochlorines and lifestyle or medical characteristics of Swedish women (Glynn et al., 2003). The 205 women aged 50-74 years old were participated as controls from 12 Swedish counties on the coasts of the Gulf of Bothnia, the Baltic Sea, and the largest Swedish lakes in 1996-1997. The frequently detected organochlorine pesticides, *p,p'*-DDE, HCB, β-HCH, and *trans*-nonachlor, and oxychlordane, were analyzed on gas chromatography with an electron capture detector. The most abundant pesticide was *p,p'*-DDE with the median concentration of 497 ng/g lipid. The other pesticides had median concentrations below 10 ng/g lipids. Correlation analysis

showed that the concentrations of chlorinated pesticides and their metabolites were positively correlated with each other (r = 0.48-0.89). High correlation was found between oxychlordane and *trans*-nonachlor. Age was a significant determinant of serum concentration of chlorinated pesticides in this study. Glynn et al. (2007) also reported another serum concentration of the general Swedish population (Glynn et al., 2007). The serum of 323 pregnant primiparous women living in Uppsala county (age 18-41 years) sampled in 1996-1999. Organochlorine pesticides and their metabolites were analysed by gas chromatography with an electron capture detector. Concentrations of α-HCH, γ-HCH, oxychlordane, *o,p'*-DDT, *o,p'*-DDT, *p,p'*-DDT, and *p,p'*-DDD were below the limit of quantification, indicating no recent exposure to technical mixtures of these pesticides. Median concentrations of β-HCH, HCB, *trans*-nonachlor and *p,p'*- DDE were 9, 23, 5, and 88 ng/g lipid, respectively. Compared to previous study, median concentration of *p,p'*-DDE was extremely different. It could be explained that different consumption patterns of fish. More than half of the women reported they did not eat fish from the Baltic Sea in this study. Regression analysis showed that women born in the Nordic region had higher concentrations of β-HCH and *p,p'*-DDE compared to the non-Nordic region. Concentrations of β-HCH, HCB, *trans*-nonachlor and *p,p'*-DDE were also increased with increasing age and positively associated with consumption of fatty fish during adolescence.

4.4.2 United Kingdom

The serum was collected from 154 volunteers aged 22 to 80 from 13 UK cities and towns in 2003 (Thomas et al., 2006). Because the serum extract was cleaned using concentrated sulfuric acid, 12 organochlorine pesticides were analyzed by gas chromatography and mass spectrometry (*trans*-chlordane and *cis*-chlordane, HCB, six DDT analogues, α-HCH, β-HCH, and γ-HCH). The median concentrations of HCB, β-HCH, and *p,p'*-DDE were 11, 12, and 100 ng/g lipid, respectively. The concentration of *p,p'*-DDE showed relatively wide range from 15 to 2600 ng/g lipid. The median concentrations of *p,p'*-DDE in UK was lower than those found in Belgium (Covaci et al., 2002; Koppen et al., 2002), the United States (CDC, 2005), and similar to Sweden (Glynn et al., 2003). β-HCH and *p,p'*-DDE concentration also showed that significant and positive correlation with age.

4.4.3 Spain

The 682 serum samples of the Spanish population from the Canary Islands was collected in 1998 (Zumbado et al., 2005). The study subjects aged between 6 and 75 years and lived in the Canary Islands, where are extensive farming areas and compose of seven islands. Investigated organochlorine pesticides were determined by gas chromatography with an electron capture detector. Of the DDT analogues, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, *o,p'*-DDE, o,p'-DDD, and *o,p'*-DDT were detected in the serum samples. Even technical DDT prohibited nowadays, *o,p'*-DDT and *p,p'*-DDT were frequently present in 40% of the samples. The median concentrations of *p,p'*-DDE was 118 ng/g lipid, while the total DDT serum concentration was 380 ng/g lipid. Of the seven islands (Gran Canaria, Lanzarote, Fuerteventura, Tenerife, La Palma, La Gomera, and El Hierro), median serum concentrations of *o,p'*-DDT and *p,p'*-DDT from Gran Canaria showed 250 and 233 ng/g lipid, respectively, while the median concentrations of those compounds from the other

regions were not detected over limit of the detection. However, serum concentration of *p,p'*-DDE showed between 94 and 140 ng/g lipid, which is consistent according to the islands. Luzardo et al. (2006) reported the serum concentrations of lindane and cyclodienes (aldrin, dieldrin and endrin) in the young population of the Canary Islands (Luzardo et al., 2006). Lindane, aldrin, and endrin were detected with the detection frequency of over 50% of the samples and the median concentrations were 69,9, 54.5, and 34.9 ng/g lipid, respectively. Endrin was the most frequently detected cyclodienes (72%), and dieldrin has 27% of detection rate in this population. Even this study reported for the first time the presence of cyclodiene and lindane in a Spanish population, lindane, aldrin, and endrin were not commonly detected pesticides in other previous studies. The serum of 72 women living in El Ejido and Granada in Spain were collected and the subjects aged from 18 to 35 years (Jimenez Torres et al., 2006). At the same time their adipose tissue and umbilical cord blood were also collected during giving birth by caesarean section to establish a correlation in organochlorine compounds between these biological compartments. The serum mean concentrations of lindane and HCB were 1.3 and 20.1 ng/mL serum. The serum mean concentrations of *p,p'*-DDE, *p,p'*-DDD, and *p,p'*-DDT were 31.9, 44.5, and 10.5 ng/ml serum, respectively. The most abundant contaminated pesticides were endosulfan II, followed by *p,p'*-DDE. The concentration of *p,p'*-DDE in between maternal serum and umbilical cord serum was significantly different ($p<0.001$). Of the EPIC (European Prospective Investigation into Cancer and Nutrition) Spain cohort, serum samples consisted of 953 subjects aged 35-64 years old from five Spanish regions during 1992-1996 (Jakszyn et al., 2009). The serum were analyzed using gas chromatography with an electron capture detector to quantify β-HCH, HCB, *p,p'*-DDE, and *p,p'*-DDT. The median concentrations of β-HCH, HCB, *p,p'*-DDE were 221, 462, and 857 ng/g lipid, respectively. Increasing level of *p,p'*-DDE and β-HCH to southern region in the Mediterranean coast at the south of Spin, while level of HCB increasing to the north region. After adjusting by age, gender, region, body mass index (BMI), and sampling year, serum concentrations of organochlorine pesticides were not associated with any dietary consumption patterns.

4.4.4 Denmark

The Faroese birth cohort consisted of 1022 children in the Faroese islands during 1986-1987, which have been carried to characterize the adverse effects of seafood contamination and assess the prenatal exposure of the contaminants (Barr et al., 2006). Serum samples were analyzed for PCBs and *p,p'*-DDE at delivrating time and age 14 years. The median serum concentration of *p,p'*-DDE for the cord blood was 71 ng/g lipid, and for 14 years old children was 467 ng/g lipid. The concentrations of *p,p'*-DDE in umbilical cord and children at age 14 showed significantly different concentrations, which were increased by age. The correlation analysis by the pearson correlation coefficients showed that the *p,p'*-DDE was highly correlated with highly chlorinated PCB congener, PCB 180.

4.4.5 Belgium

Of the Flemish Environment and Health Study (FLEHS), serum samples were obtained from 200 Flemish women living in Antwerp (urban area) and Peer (rural area) in 1999 (Koppen et al., 2002). The 47 pooled serum samples were made by mixing of individual serum sample.

The pooling categorized by ranking the women in the order of four criteria; decreasing daily intake of meat and fish, decreasing daily intake of eggs and milk, increasing total number of weeks of lactation and increasing BMI. The pooled serum samples were spiked with internal standard and added with formic acid. The mixtures were subjected to extraction using solid-phase extraction cartridge, followed by cleanup on acidified silica gel cartridge. HCB, *p,p'*-DDT, *p,p'*-DDE, and γ-HCH were measured using gas chromatography and mass spectrometry. The median concentrations of HCB, *p,p'*-DDT, *p,p'*-DDE, and γ-HCH were 109.9, 2.6, 871.3 and 5.7 ng/g lipid, respectively. Concentrations of *p,p'*-DDE, and γ-HCH were higher in rural region (Peer). As the use of DDT has been banned more than 30 years ago, concentrations of *p,p'*-DDT were significantly higher in Peer. HCB was significantly higher in urban area (Antwerp). This study reported γ-HCH concentration in serum samples.

5. Effects of organochlorine pesticides on human health

Most organochlorine pesticides are known to be endocrine disruptors, neurotoxicants, and carcinogens. Their presence in the general population at the current background levels does not mean that they will result in direct adverse health effects, but they can cause disorders in the human body. As the human health effects of these chemicals are inconsistent in epidemiologic research, it is still debatable whether such exposure affects human health (Kaiser, 2000; Snedeker, 2001). This section focuses on the human health disorders associated with organochlorine pesticides. To investigate their potential health risks upon exposure, biomonitoring of organochlorine pesticides in human serum is essential. General toxicological and environmental data on hazardous substances are compiled in toxicological profiles published by the Agency for Toxic Substances and Disease Registry (ATSDR). On the basis of these data, the agency derives chemical-specific minimal risk levels (MRLs) that assist in evaluating public health risks associated with exposure.

5.1 Thyroid hormonal imbalance

Most organochlorine pesticides and organochlorines may cause thyroid hormonal inactivity. Thyroid hormone is produced by the thyroid gland, and its major forms are thyroxine (T_4) and triiodothyronine (T_3). An imbalance of thyroid hormeones can lead to various disorders. In 385 adult men, the serum concentrations of *p,p'*-DDE and HCB were found to be associated with thyroid hormone levels (Meeker et al., 2007). The former compound was associated with increased free thyroxine (T_4) and total triiodothyronine (T_3) levels, and inversely assosiated with thyroid-stimulating hormone (TSH). Hagmar et al. (2001) reported no associations between *p,p'*-DDE and thyroid hormones (Hagmar et al., 2001). Turyk et al. (2006) studied associations of total T_4 and TSH with organochlorines including *p,p'*-DDE using the National Health and Nutrition Examination Survey 1999–2000 data (Turyk et al., 2006). They found inverse associations of total T_4 with exposure to dioxin-like organochlorines, stronger associations in females, and a dose-dependent decrease in total T_4 with exposure to dioxin-like organochlorines at levels similar to those found in the general United States population. T_4 was positively associated with *p,p'*-DDE in all women but was negatively associated with this compound in men. Associations of thyroid-stimulating

hormone (TSH) with *p,p'*-DDE were insignificant and inconsistent (Turyk et al., 2007). A number of factors are related to the inconsistent human findings, including different detection methods as well as differences in age, gender, and other lifestyle factors.

5.2 Hormone-related cancers: breast and prostate cancers

Organochlorine pesticides may be associated with increased risk of hormone-related cancers including breast and prostate cancers. However, epidemiologic evidence is limited and inconsistent (Snedeker, 2001). Wolff et al. (1993) analyzed associations of serum *p,p'*-DDE concentrations with the risk of breast cancer (Wolff et al., 1993). During 1985–1991, cases (n = 58) and controls (n = 171) enrolled in the New York University Women's Health Study (NYUWHS), a prospective cohort study of hormones, diet, and cancer. The mean levels of *p,p'*-DDE were higher in patients with breast cancer than in control subjects. There were strong positive relationships between breast cancer risk and serum *p,p'*-DDE levels. On the other hand, the authors did not find associations of the risk of breast cancer with serum *p,p'*-DDE concentrations in a similar prospective investigation of the New York University Women's Health Study (NYUWHS) during the period 1987–1992 (Wolff et al., 2000). Cases (n = 148) and individually matched controls (n = 295) had similar serum levels of this compound, providing no evidence for an association of breast cancer risk with *p,p'*-DDE in blood. Recently, a case-control study was carreid out to investigate the association between breast cancer risk and organochlorines in Japanese women, which is the first large-scale study of an Asian population (Itoh et al., 2009). The serum samples were collected from Nagano during 2001–2005 and consisted of 403 breast cancer cases and matched controls. Organochlorine pesticides and PCBs were measured by using isotope dilution gas chromatography–high resolution mass spectrometry. The serum concentrations of organochlorine pesticides were not associated with increased risk of breast cancer in the Japanese population.

Xu et al. (2010) used the National Health and Nutrition Examination Survey 1999–2004 data to examine associations between serum concentrations of organochlorine pesticides and prostate and breast cancers (Xu et al., 2010). After adjustment for other covariates, the serum concentrations of β-HCH, *trans*-nonachlor, and dieldrin were significantly associated with prostate cancer prevalence. However, there was no positive association between these serum concentrations and the risk of prevalent breast cancer. The Japan Public Health Center (JPHC) prospective study consisted of 14,203 men aged 40–69 years who participated from 1990 to 2005 (Sawada et al., 2010). From the cohort, 201 prostate cancer cases and two matched controls were compared for the median concentrations of organochlorine pesticides and PCBs. The odds ratios (ORs) and 95% confidence intervals (CIs) for prostate cancer were estimated in relation to serum levels of *p,p'*-DDT, HCB, β-HCH, *trans*-nonachlor, oxychlordane, and mirex. No significant differences in the median levels of these pesticides were found between the cases and the controls. Further, no statistically significant association with total prostate cancer was seen with any organochlorine, suggesting the lack of an overall association between prostate cancer and organochlorine pesticides at the levels measured in the Japan Public Health Center population.

5.3 Childhood developmental disorders

As organochlorine pesticides are present in both cord blood and breast milk, fetuses and infants can be exposed to these chemicals during the prenatal and postnatal periods. Fetuses

and infants are more vulnerable to neurotoxic environmental chemicals even at very low-level exposure, which can affect brain development, decrease cognitive function, and result in development disorders in childhood. Lee et al. (2007a) investigated learning disability (LD) and attention deficit disorder (ADD) in children aged 4–15 years from the National Health and Nutrition Examination Survey 1999–2000 data (Lee et al., 2007a). Dioxins in children showed significant positive associations with learning disability (LD), but *p,p'*-DDE and *trans*-nonachlor were not associated with the prevalence of learning disability (LD). β-HCH was inversely associated with learning disability (LD) but not significantly. Sagiv et al. (2010) found higher risk for attention deficit hyperactivity disorder (ADHD) at higher levels of PCBs and *p,p'*-DDE in a longitudinal cohort study including 788 mother–infant pairs (Sagiv et al., 2010). The PCB and *p,p'*-DDE levels in cord serum were moderately associated with attention deficit hyperactivity disorder (ADHD) in children aged 7–11 years born in 1993–1998 in a PCB-contaminated area (ORs = 1.76). These results support the view that low-level prenatal organochlorine exposure is associated with attention deficit hyperactivity disorder (ADHD)-like behaviors in childhood. During 2001–2005, women of reproductive age in Mexico provided blood samples, and after birth, each child was checked for cognitive and psychomotor development. Among 244 mother–child pairs, *p,p'*-DDE levels in the first trimester of pregancy were significantly and negatively associated with the psychomotor development index (PDI) of the children (Torres-Sánchez et al., 2007). No significant association was found between *p,p'*-DDE levels and childhood neurodevelopment during the second or third trimester. Torres-Sánchez et al. (2009) also reported that the associations between prenatal exposure to *p,p'*-DDE in cord serum and neurodevelopment disappeared after 12 months of infant age (Torres-Sánchez et al., 2009).

5.4 Diabetes

The prevalence of diabetes has been increasing globally over the past few decades (King et al., 1998). Recent epidemiologic studies have shown that background exposure to persistent organic pollutants, especially organochlorine pesticides, is strongly associated with type 2 diabetes. Lee et al. (2006) demonstrated a very strong relationship between the levels of persistent organic pollutants in serum, particularly oxychlordane and *trans*-nonachlor, and the risk of type 2 diabetes in the general American population by extensive analysis of the National Health and Nutrition Examination Survey 1999–2002 data (Lee et al., 2006). This association was higher in obese people than among the non-obese. The associations between the serum concentrations of organochlorine pesticides and the prevalence of diabetes were examined in the Mexican-American population (Cox et al., 2007) and Korean population (Son et al., 2010). Exposure of *p,p'*-DDE was related to the incidence of diabetes in a cohort of Great Lakes sport fish consumers from 1994 to 2005 (Turyk et al., 2009). Lee et al. (2010) also investigated whether several persistent organic pollutants predict the risk of type 2 diabetes within the Coronary Artery Risk Development in Young Adults (CARDIA) cohort (Lee et al., 2010). Some persistent organic pollutants, such as *trans*-nonachlor and highly chlorinated PCBs, were associated with the incidence of type 2 diabetes over an 18-year period, especially in obese people. Persistent organic pollutants showed strong associations at relatively low exposures, resulting in inverted U-shaped dose–response curves instead of the traditional dose–response relationship with diabetes.

Country	Year[a]	n	β-HCH	HCB	Oxy	TN	DDE	DDT	reference
Korea	2006	40	49	16.7	4.8	6.5	224	18.6	Kang et al., 2008
Korea	2006	40	44	18.3	8.3	20.2	379	23.8	Son et al., 2010
Japan	2000	80	93.2	<LOD	9.0	20.9	221	<LOD	Tsukino et al., 2006b
Japan	-	18	50	13.5	7.3	21.9	218	11	Takasuga et al., 2006
Japan	1999	23	28[b]	7.8	60[c]	NA	230[d]	NA	Minh et al., 2005
Japan	1999	32	34[b]	13	26[c]	NA	220[d]	NA	Minh et al., 2005
Japan	1999	22	34[b]	9.8	28[c]	NA	200[d]	NA	Minh et al., 2005
Japan	1999	152	280	21.6	13.8	50	312	28	Masuda et al., 2005
Japan	02-03	32	26[b]	16.0	1.2	7.0	93	2.4	Fukuta et al., 2005
Japan	01-05	405	64	27	8.6	23	370	9.9	Itoh et al., 2009
China	2005	26	12[b]	39	NA	NA	600[d]	NA	Bi et al., 2007
China	2005	21	39[b]	31	NA	NA	2300[d]	NA	Bi et al., 2007
China	96-98	250	5065	62.7	NA	NA	7635	309	Lee et al., 2007b
New Zealand	96-97	60[f]	10.7	<LOD	<LOD	<LOD	919	<LOD	Bates et al., 2004
Sweden	96-97	205	51	65	13	23	497	NA	Glynn et al., 2003
Sweden	96-99	323	9	23	<LOD	5	88	<LOD	Glynn et al., 2007
UK	2003	154	12	11	NA	NA	100	2.9	Thomas et al., 2006
Spain	1998	682	NA	NA	NA	NA	118	<LOD	Zumbado et al., 2005
Spain	92-96	953	221	462	NA	NA	858	<LOD	Jakszyn et al., 2009
Belgium	1999	47[f]	6.0[e]	92.2	NA	NA	944.9	3.7	Koppen et al., 2002
Romania	2005	142	923	30		4.0	1975	339	Dirtu et al., 2006
Solvakia	2001	1009	48.6	690	NA	NA	2521	72.9	Petrik et al., 2006
Solvakia	2001	1038	44.0	639	NA	NA	1368	33.2	Petrik et al., 2006
USA	99-00	1702	<LOD	<LOD	<LOD	17.8	226	<LOD	CDC, 2005
USA	01-02	2307	<LOD	<LOD	11.1	17.9	250	<LOD	CDC, 2005
USA	03-04	1796	<LOD	14.9	10.3	14.8	203	<LOD	Patterson et al., 2009
USA	97-98	70	40	239	249	NA	1268	25	Rusiecki et al., 2008
USA	59-67	283	NA	NA	NA	NA	5200	1400	Bhatia et al., 2005
USA	63-67	399	NA	NA	NA	NA	5878	1611	James et al., 2002

[a]Year of collection; [b]sum of four hexachlorcyclohexane isomers; [c]sum of *o,p'*-DDT, *o,p'*-DDD, *o,p'*-DDE, *p,p'*-DDT, *p,p'*-DDD, and *p,p'*-DDE; [e]γ-HCH; [f]pooled serum
NA, not available; LOD, limit of detection; HCH, hexachlorcyclohexane; HCB, hexachlorobenzene; Oxy, oxychlordane; TN, *trans*-nonachlor; DDE, *p,p'*-dichlorodiphenyldichloroethylene; DDT, *p,p'*-dichlorodiphenyltrichloroethane

Table 2. The median organochlorine pesticide concentrations (ng/g lipid) in serum samples of different countries

6. Conclusion

Human biomonitoring data show that organochlorine pesticides are detectable in the body despite their ban a few decades ago. Nowadays, it is accepted that the general human population is exposed to background environmental levels of organochlorine pesticides. The exposure levels vary according to the region, population, and race, and have been decreasing over time. However, the current background concentrations of each these pesticides are still much higher than those of notorious organic pollutants such as PCDD/Fs, PCBs, and PBDEs. In addition, many epidemiologic studies have revealed that their current levels in the general human population are associated with several health disorders and may cause adverse effects on human health. In view of the number of factors related to the inconsistent human findings, including different detection and quantification methods, we recommend continuous biomonitoring study of organochlorine pesticides in human serum based on the analytical method using isotope dilution gas chromatography – high resolution mass spectrometry.

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10

Urea Pesticides

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1. Introduction

Urea herbicides form, together with phenoxy derivatives and triazines, the most important agricultural herbicide group. The urea-derivatives are typical pre-emergence herbicides applied usually as aqueous emulsions to the surface of soil. Almost all of the urea compounds with good herbicidal action are trisubstituted ureas, containing a free imino-hydrogen. According to the receptor theory, this hydrogen plays a role in the formation of the hydrogen bond being significant in the mode of action of ureas. Chemically, the urea type herbicides contain a urea bridge substituted by triazine, benzothiazole, sulfonyl, phenyl, alkyl or other moieties. Besides herbicidal activity, some analogous structures have other biological activity (Lányi&Dinya, 2005).

The general structure of a phenylurea herbicide (PU) is (substituted) phenyl–NH–C(O)–NR_2. The phenyl ring is often substituted with chlorine or bromine atoms, but methoxy, methyl, trifluoromethyl, or 2-propyl substitution is also possible. Most PU are N-dimethyl PU, but a combination of a methyl substituent and another group also occurs (Niessen, 2010). PU are used as selective and non-selective herbicides in substantial amounts, including the use as systemic herbicides to control broadleaf and grassy weeds in cereals and other crops, as total herbicides in urban areas, and as algicides in paints and coatings.

Sulphonylureas (SU) form a group of selective herbicides with R_1–NH–C(O)–NH–SO_2–R_2 as general structure. R_1 and R_2 generally are substituted heterocyclic rings such as 4,6-dimethylpyrimidin-2-yl and 2-(benzoic acid methyl ester) (Niessen, 2010). The mode of action of these herbicides consists of inhibiting acetolactate synthase (ALS) which is a key enzyme in the biosynthesis of branched amino acids (valine, leucine, and isoleucine). SU are low dose herbicides (10 – 40 g a.i. ha^{-1}) used to control broad leaved weeds in cereals exhibiting very low acute and chronic mammalian toxicities (Wang Y. S. et al., 2010).

Benzoylureas (BU), which were introduced in the early '70s, represent a class of insect growth regulators (IGRs) which act on the larval stages of most insects by inhibiting or blocking the synthesis of chitin, a vital and almost indestructible part of the insect exoskeleton during the molting stage; therefore, the failure to successfully cast off the old exoskeleton leads to the eventual death of the larvae. Diflubenzuron is the prototype of all benzoylurea chitin synthesis inhibitor insecticides (Shim et al., 2007).

The specificity of benzoylureas to species whose structural integrity depends upon chitin, their low acute toxicity to mammals along with their high biological activity, make them suitable for inclusion in integrated pest management programs for fruit and vegetables

(Shim et al., 2007). This kind of insecticide suffers a rapid degradation in both soil and water (Zhou et al., 2009). Nevertheless, residues can often reach populations through the food chain causing chronic exposure and long-term toxicity effects. Some studies show that several IGRs may affect nontarget arthropods, such as teflubenzuron and hexaflumuron at concentrations that are probably environmentally relevant (Campiche et al., 2006). Previous studies have shown that extremely low levels of metsulfuron-methyl, a SU herbicide, have phytotoxicity to sensitive crops in crop-rotation systems and have unintended side effects on non-target organisms (Wang H. Z. et al., 2010). Diuron, a PU herbicide, has been shown to cause a drop in photosynthesis in algal communities at concentration of 1.5 μg/L (Ricart et al., 2010).
The substituted urea herbicides are used for the control of many annual and perennial weeds, for bush control, and for weed control in irrigation and drainage ditches. Likewise, the benzoylurea insecticides are widely used on a large number of crops.
In this chapter, the more relevant contributions of the last 5 years to the current knowledge on several aspects regarding urea pesticides, such as degradation in soil and natural waters, occurrence of residues in food, legislation and analytical determination will be discussed.

2. Degradation studies

Several studies have investigated the degradation pathways of urea pesticides in aqueous solutions and soil. In soil, their persistence is mostly influenced by the rate of chemical and microbial degradation. Degradation is particularly dependent on the soil pH, moisture content and microbiological activity. The ultraviolet (UV) radiation in the sunlight is one of the most powerful forces for pesticide degradation. Studies on the photodegradation of pesticides in both homogenous and heterogeneous systems contribute to elucidate the transformation, mineralization and elimination of these xenobiotics in the different environmental compartments. In a review by Burrows et al. (2002) the photodegradation of pesticides is reviewed, with particular reference to the studies that describe the mechanisms of the processes involved, the nature of reactive intermediates and final products.
The more recent herbicide formulations are designed to offer advantages of the highest selectivity together with the lowest persistence in the environment: SU meet these requirements. But, unfortunately, lower persistence in the environment does not necessarily correspond to lower toxicity, since many herbicides undergo natural degradation reactions in the environment that do not lead to mineralization but to the formation of new species potentially more toxic and stable than the precursors (Bottaro et al., 2008).
The most important pathways of degradation of SU in soil are chemical hydrolysis and microbial degradation, while other dissipation processes such as volatilization and photolysis are relatively insignificant (Saha & Kulshrestha, 2008; Si et al., 2005; Wang Y. S. et al., 2010). SU typical field dissipation half-lives ($t_{1/2}$) are about 1-8 weeks in some cases, but within a few days in the case of some newer compounds. Chemical hydrolysis is pH and temperature dependent: in most cases the degradation is faster in acidic rather than in neutral or in weakly basic conditions, and at high temperature (Wang Y. S. et al., 2010).
Degradation of ethametsulfuron-methyl, a SU, in soils was pH-dependent; calculated $t_{1/2}$ values ranged from 13 to 67 days. Ethametsulfuron-methyl was more persistent in neutral or weakly basic than in acidic soil. Five soil metabolites were isolated and identified by LC-MS/MS analysis. Different authors have shown that soil pH is the most important factor in affecting both sorption behaviour and chemical degradation of metsulfuron-methyl in soil because of its ability to influence the ionization state of the herbicide (Wang H. Z. et al.,

2010). The mineralization rate was negatively correlated with soil pH, organic carbon contents, and clay contents, while it was positively correlated with soil microbial biomass carbon and silt contents. Regression analyses suggested that soil properties did not act separately but in an interactive manner in influencing the overall metsulfuron-methyl mineralization in soils.

The dissipation mechanisms of two SU herbicides, chlorsulfuron and imazosulfuron, were both chemical and biological. Half-life calculation followed the first-order kinetics. The $t_{1/2}$ of chlorsulfuron was 6.8–28.4 days and that of imazosulfuron was 6.4–14.6 days. Persistence is strongly influenced by the temperature and soil pH. Both compounds dissipate faster in a more acidic soil. The two SU changed the soil bacterial composition, and the change was larger with imazosulfuron at 50 mg/kg. The selectivity of survival for bacteria was stronger in more alkaline soil (Wang Y. S. et al., 2010).

In soil, the hydroxylation of the aromatic ring of chlorsulfuron has been reported in the presence of the fungus *Aspergillus niger*. Photolysis of imazosulfuron was reported in aqueous solution under UV light. Chemical cleavage was the main degradation pathway in aerobic conditions, whereas in anaerobic conditions, microbial degradation was the main degradative pathway to demethylate imazosulfuron (Wang Y. S. et al., 2010).

The hydrolysis rate of rimsulfuron was as high as the photolysis rate, and decreased on diminishing the pH values of the solution. Sorption and photolysis reactions of rimsulfuron on silica and clay minerals were also investigated and compared with a natural soil sample. The photochemical degradation of the herbicide was strongly affected by retention phenomena, showing that silica and clay minerals can retain and protect rimsulfuron from photodegradation much more than soil (Bufo et al., 2006). Degradation products of rimsulfuron can leach through sandy soils in relatively high concentrations and could potentially contaminate vulnerable aquatic environments (Rosenbom et al., 2010). Rimsulfuron is moderately persistent to non-persistent in aqueous solutions/soil suspensions under anaerobic/aerobic conditions, with $t_{1/2}$ of 6–40 d in soil. Most of the rimsulfuron and its degradation products are available for either leaching or formation of non-extractable residues (sorption, exclusion/trapping) since mineralisation is negligible.

The kinetics of hydrolytic degradation of sulfosulfuron was investigated to predict the fate of the herbicide in an aqueous environment. The study revealed that the hydrolytic degradation followed first-order kinetics. The degradation was dependent on pH and temperature. Hydrolysis rate was faster in acidic condition ($t_{1/2}$=9.24 d at pH 4.0) than alkaline environment ($t_{1/2}$=14.14 d at pH 9.2). Under abiotic conditions, the major degradation mechanism of the compound was the breaking of the sulfonylurea bridge yielding corresponding sulfonamide and aminopyrimidine (Saha & Kulshrestha, 2008).

The UV induced photodegradation of metsulfuron in water has been studied. The mechanism involved hydrolytic cleavage of the sulfonylurea bridge to form the corresponding phenyl sulfonyl carbamic acid and s-triazine, with the carbamic acid subsequently decarboxylating to form a phenyl sulfonamide and a cyclic derivative (Burrows et al., 2002).

BU adsorb readily in soil with little subsequent desorption and, even though its mobility in soil is very low, some BU may be present in surface water after application (Martinez et al., 2007).

Diflubenzuron is quickly degraded in the environment mainly by hydrolysis and photodegradation producing as major metabolites: 2,6-diflurobenzamide, 4-chlorophenylurea, 4-chloroacetanilide, 4-chloroaniline and N-methyl-4-chloroaniline, the last three of them classified as mutagens (Rodriguez et al., 1999).

In a review dealing with the degradation of phenylurea herbicides, Sorensen et al. (2003) reported that degradation proceeds mainly through a microbial way with the action of a wide variety of microbial strains.
Transport of pesticides from point of application via sub-surface drains can contribute significantly to contamination of surface waters. Many pesticides (particularly soil-acting herbicides) rely for their activity on a degree of mobility and persistence in soil, but these properties can confer vulnerability to leaching to sub-surface drains (Brown & van Beinum, 2009).
Due to the extensive use of urea pesticides for agricultural and non-agricultural purposes, their residues have been detected in wastewater effluents, surface water and raw drinking water sources, as well as food products, around the world, and have received particular attention because of their toxicity and possible carcinogenic properties. Among them, the highly persistent phenylurea herbicides can be found at concentrations reaching several μg/L in natural waters. Since their possible activity as carcinogens, the control of the levels of the residues of these compounds in the environment and in crops has an outstanding importance.

3. Legislation

Increasing public concern about health risks from pesticide residues in the diet has led to strict regulation of maximum residue levels (MRLs). Food Safety legislation is not harmonized through the world. However, well-known international bodies, the most representative of which is the *Codex Alimentarius* Commission established by Food and Agriculture Organization (FAO) and World Health Organization (WHO), create risk based food safety standards that are a reference in international trade and a model for countries to use in their legislation. Actually, the *Codex Alimentarius* (2009) set MRLs only for the following ureas: diazinon (0.01 to 5 mg/kg); diflubenzuron (0.01 to 5 mg/kg); novaluron (0.01 to 40 mg/kg) and teflubenzuron (0.05 to 1 mg/kg).
The European Union, as one of the world's largest food importers, exerts a major influence on food safety testing globally and has also strict legislation in this area (Hetherton et al., 2004). Legislation on food at the European Community level dates back to 1976 when Council Directive 76/895/EEC specified MRLs for pesticides (43 active substances) in and on selected fruits and vegetables, 7 of them were urea herbicides (monolinuron, metsulfuron-methyl, thifensulfuron-methyl, triasulfuron, azimsulfuron, chloraxuron and flupyrsulfuron-methyl). Linuron and monolinuron were also included in the so-called "black list" of the 76/464/EEC Council Directive on pollution caused by certain dangerous substances discharged into the aquatic environment of the Community. Later, in order to prevent the contamination of groundwater and drinking water, a priority list which considered pesticides used over 50,000 kg per year and their capacity for probable or transient leaching was published; chlorotoluron, diuron, isoproturon and methabenthiazuron were included in this list. The 80/779/EEC Directive on the Quality of Water Intended for Human Consumption stated a maximum admissible concentration of 0.1 μg/L for individual pesticide and 0.5 μg/L for the total pesticides, regardless of their toxicity. The regulation (EC) No. 396/2005 made an important step forward in its efforts to ensure food safety in the European Union, as a regulation revising and simplifying the rules pertaining to pesticide residues entered into force. The new rules set harmonised MRLs for pesticides. They ensure food safety for all consumers and allow traders and importers to do

business smoothly as confusion over dealing with 27 lists of national MRLs was eliminated. If a pesticide is not included in any of the above mentioned lists, the default MRL of 0.01 mg/kg applies (Art 18(1b) of Reg. (EC) No 396/2005). Recently, regulation (EC) No 901/2009 has been produced concerning a coordinated multiannual Community control programme for 2010 to 2012 to ensure compliance with MRLs and to assess the consumer exposure to pesticide residues in and on food of plant and animal origin. The Member States shall, during 2010 - 2012 analyse samples for the product/pesticide residue combinations, including the ureas: flufenoxuron, linuron, lufenuron, pencycuron and triflumuron.

4. Analytical methods

Various approaches are described in the literature for detailed analysis of urea pesticides in environmental, biological and food samples. Tables 1-3 summarize the more relevant studies, published in the last five years, concerning the analytical methodologies applied for PU (Table 1), SU (Table 2) and BU (Table 3) determination. Research has been carried out in ureas extraction, separation and specific detection. It is a tremendous challenge to develop sensitive and selective analytical methods that can quantitatively characterize trace levels of residues in the several types of samples. This challenge is most evident in the detection of ureas due to the low dose used, their water solubility and chemical instability. At present, there is still a lack of officially approved methods that would solve the difficulties associated with quantitative isolation of urea pesticides from the various matrices, clean-up of the extract without significant loss of the analyte, separation of all individual pesticides contained in the purified extract, detection of the separated components, unequivocal identification and quantification of the identified compounds.

Sample pretreatment processes are crucial steps to achieve clean-up and effective enrichment of the target analytes before analysis. For solid samples, traditionally, Soxhlet and manual/mechanical shaking have been used for ureas extraction (Buszewski et al., 2006; Cydzik et al., 2007; El Imache et al., 2009; Ghanem et al., 2008; Mou et al., 2008; Scheyer et al., 2005; Tamayo et al., 2005a, b; Tamayo & Martin-Esteban, 2005). On the other hand, for aqueous samples the classical methodology is liquid-liquid extraction (Moros et al., 2005). However, these techniques have inherent disadvantages, for example the large volumes of organic solvents required. They are also time-consuming and involve multistep processes that have always the risk of loss of some analytes. Supercritical-fluid extraction, matrix solid-phase dispersion, pressurized liquid extraction (Bichon et al., 2006), microwave-assisted extraction (Paiga et al., 2008, 2009a; Paiga et al., 2009b) and batch extraction enhanced by sonication (Boti et al., 2007a; Buszewski et al., 2006; De Rossi & Desiderio, 2005) have been developed as alternative techniques to replace classical extraction methods mainly for solid samples. All these methods reduce extraction time and the volumes of solvent required, but some have the disadvantages of high investment and maintenance costs of the instruments (i.e., supercritical-fluid extraction, pressurized liquid extraction, and microwave-assisted extraction). Supercritical-fluid extraction is less frequently used, probably due to a strong dependence of optimal parameters setting on sample composition and analytes, which is the cause of a rather low robustness of supercritical fluid extraction-based procedures. Matrix solid-phase dispersion is relevant for tissue analysis, such as beef fat, catfish muscle or oysters (Bichon et al., 2006). Matrices are blended with C18 or Florisil phases before analyte elution with an adequate solvent. The major drawback of this procedure is the manual preparation which complicates the routine application (Bichon et

al., 2006). Nowadays, microwave-assisted extraction and pressurised liquid extraction are applied successfully for urea residues control in soils (Paiga et al., 2008), vegetables (Paiga et al., 2009a; Paiga et al., 2009b) and in oysters (Bichon et al., 2006a). Low temperatures must be selected due to urea's thermolability. Studies have shown that once optimized, these new extraction techniques are comparably efficient, with similar standard deviations. However, the main drawback is the wide range of co-extracted compounds leading usually to more purification steps.

Solid phase extraction (SPE) is a well-established preconcentration technique that allows both extraction (for liquid samples) and concentration of traces of contaminants using low amounts of solvents. It represents the most often-applied method in environmental and food analysis (Tables 1-3). The popularity of SPE has increased in recent years as it is easily automated and a wide range of phases is available. Octadecylsilica is the largely preferred sorbent over other supports for all the three groups of ureas (Crespo-Corral et al., 2008; Piccirilli et al., 2008; Sa et al., 2007). Moreover new multi-functionalized and selective sorbents are exploited to improve enrichment and clean-up performances (Breton et al., 2006; Carabias-Martinez et al., 2005; Mansilha et al., 2010; Tamayo et al., 2005b; Tamayo & Martin-Esteban, 2005; Zhang et al., 2006).

Molecularly imprinted polymers (MIPs) are synthetic polymers possessing specific cavities designed for a target molecule. By a mechanism of molecular recognition, the MIPs are used as selective tools for the development of various analytical techniques such as SPE. MIPs possess many advantages, for instance, easy preparation, chemical stability and pre-determined selectivity. The enhancement of the selectivity provided by the MIP has been largely described in the literature (Pichon & Chapuis-Hugon, 2008; Pichon & Haupt, 2006). MIPs were developed for SU (Liu et al., 2007) and PU (Breton et al., 2006; Carabias-Martinez et al., 2005; Tamayo et al., 2005b; Tamayo & Martin-Esteban, 2005). They were compared to classical sorbents in order to demonstrate the possibility to obtain cleaner baseline when using the MIP than when using C18 silicas or hydrophobic polymers (Breton et al., 2006; Carabias-Martinez et al., 2005; Tamayo et al., 2005b; Tamayo & Martin-Esteban, 2005). There are more and more applications of MIPs directly to real samples without a preliminary treatment (Bettazzi et al., 2007; Breton et al., 2006; Pichon & Chapuis-Hugon, 2008; Pichon & Haupt, 2006). The selectivity was also demonstrated by spiking the sample with compounds belonging to the same range of polarity as the target analytes; the lack of retention of these compounds on the MIP demonstrates the selectivity of the extraction procedure on MIPs (Pichon & Chapuis-Hugon, 2008; Pichon & Haupt, 2006).

Room temperature ionic liquids (RTILs) containing relatively large asymmetric organic cations and inorganic or organic anions have recently been used as "green solvents" to replace traditional organic solvents for chemical reactions. The application of immobilized ILs in separation and clean-up procedures has recently raised much interest. (Fang et al., 2010) showed that cartridges with ionic liquid-functionalized silica sorbent allow a better simultaneous quantification of 12 SU than the reached with C18 sorbent.

Recently, solid-phase extraction with polystyrene divinylbenzene and multiwalled carbon nanotubes (MWCNTS) as the packed materials were successfully used for enhancing the detection sensitivity of PU (chlortoluron (Zhou et al., 2007); diuron and linuron (Ozhan et al., 2005)) and SU (nicosulfuron, thifensulfuron and metsulfuron-methyl (Zhou et al., 2006)). On the basis of their peculiar electronic, metallic and structural characteristics, they have also been exploited in other fields such as biosensors, field-effect transistors and so on (Zhou et al., 2006).

The need to reduce the overall sample preparation time and the quantities of organic solvents has led to the emergence of several new extraction approaches, including solid-phase microextraction (SPME) (Mughari et al., 2007b; Sagratini et al., 2007), liquid phase microextraction (Zhou et al., 2009) and dispersive liquid-liquid microextraction (Chou et al., 2009; Saraji & Tansazan, 2009). The SPME technique is a solvent-free extraction technique that was successfully coupled to GC and LC (Mughari et al., 2007b; Sagratini et al., 2007) in order to analyze PU in fruit juices and groundwater. In dispersive liquid-liquid microextraction, a water-immiscible organic extractant and a water-miscible dispersive solvent are two key factors to form fine droplets of the extractant, which disperse entirely in the aqueous solution, for extracting analytes (Chou et al., 2009). The cloudy sample solution is then subjected to centrifuge to obtain sedimented organic extractant containing target analytes. Saraji & Tansazan (2009) and Chou et al. (2009) used this technique to isolate and concentrate several PU herbicides from river water samples.

Several polymers have been developed which change their structure in response to surrounding conditions, such as the pH, electric field, and temperature. Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of these. There are considerable and reversible changes in the hydrophilic/hydrophobic properties of PNIPAAm-grafted surfaces in response to a change in temperature. Taking advantage of this characteristic, an LC column packed with PNIPAAm to selectively separate SU herbicides by controlling the external column temperature has been developed (Ayano et al., 2005).

Nowadays, the main analytical alternatives sufficiently sensitive for determining urea residues are gas chromatography (GC) and liquid chromatography (LC). GC is applied for the determination of many organic pollutants, but direct determination of ureas is difficult due to their low volatility and thermal instability (Crespo-Corral et al., 2008). Methods developed by GC usually involved a derivatization procedure with diazomethane or pentafluorobenzyl bromide (Scheyer et al., 2005). The derivative procedure made GC difficult to be a robust tool for monitoring ureas. However, (Crespo-Corral et al., 2008) showed the usefulness of the potassium tert-butoxide/dimethyl sulphoxide/ethyl iodide derivatization reaction to determine simultaneously PU, carbamate and phenoxy acid herbicide residues in natural water samples by GC-MS. They reached limits of detection for PU in the range of 0.12–0.52 ng/L which are ones of the lowest achieved (Table 1).

Methods based on LC coupled with different detectors are the most commonly preferred. Conventional UV, diode array or photodiode array detection have been extensively used in LC for the determination of PU, SU and BU in environmental samples (Zhou et al., 2006). MIPs were tested as stationary phases for PU separation (Tamayo et al., 2005b; Wang et al., 2005) before LC-UV detection.

Fluorescence detection (FLD) has been closely bound to the important development of LC instrumentation as it is generally more sensitive than classical UV absorption and less expensive that MS detection. It represents a very selective detector, overcoming matrix interferences (Mughari et al., 2007b). However, few compounds are fluorescent, although some of them possess the necessary degree of aromaticity and may be converted to fluorescent species by using derivatization methods. Several authors (Mou et al., 2008; Mughari et al., 2007a; Mughari et al., 2007b) studied the application of FLD combined with post-column photochemically induced fluorimetry derivatization to determine PU compounds in groundwater and rice and corn samples.

Amperometric detector has been also coupled with LC for the analysis of PU (Shapovalova et al., 2009).

Matrix	Phenylurea	Pretreatment	Extraction	Clean-up/ Elution	Detection	LOD	Ref.
Air samples	chlortoluron, diuron, isoproturon		Soxhlet with n-hexane/ CH_2Cl_2 (1:1, v/v)		GC-MS/MS after derivatisation with pentafluoro-benzyl-bromide	100-500 µg/L	Scheyer et al., 2005
Corn	fenuron, metoxuron, chlortoluron, isoproturon, metobromuron, linuron	manual shacking with ACN; supernatant was filtered through a 0.45 µm filter	several MIPs conditioned with toluene	MeOH	LC-PAD	not provided	Tamayo et al., 2005b
Corn, potato, pea and carrot	fenuron, metoxuron, chlortoluron, isoproturon, metobromuron, linuron	dried	manual shacking with ACN	MIPs elution with MeOH	LC-UV	not provided	Tamayo et al., 2005a
Corn and rice	fenuron, tebuthiuron, metoxuron, monuron, chlortoluron, fluometuron, isoproturon, diuron, monolinuron, metobromuron, buturon, siduron, linuron, chlorbromuron, neburon	finely ground	mechanical shaker with ACN	Florisil SPE conditioned by sequential washing with acetone/n-hexane (40:60, v/v) and n-hexane.	LC-FLD after UV decomposition and post-column derivatization	0.003-0.032 mg/kg	Mou et al., 2008
Courgette cucumber, lettuce and peppers	linuron, metobromuron, monolinuron	chopped	microwave-assisted extraction with ACN	filtration through a GF/C and 0.2 µm filters	LC-PAD	7.2-10.1 µg/kg	Paiga et al., 2009a
Drinking water	diuron, fluometuron, linuron, siduron, thidiazuron.		C18 disks, conditioned with MeOH water.	MeOH	LC-MS	0.010-0.026 µg/L	Li et al., 2006
Fresh and processed Tomato	linuron, metobromuron, monolinuron	chopped	microwave-assisted extraction with ACN	filtration through a GF/C and 0.2 µm filters	LC-PAD and LC-MS	2.0-7.1 µg/kg	Paiga et al., 2009b
Fresh and estuarine waters	chlortoluron, diuron, linuron, metobromuron, metoxuron, monolinuron, monuron, neburon,	pH adjusted to 7; filtered using GF/F glass microfibre filters (0.7 µm pore size)	HLB cartridge conditioned with MeOH and water	MeOH	LC-MS/MS	5-59 ng/L	Mazzella et al., 2009

Fruit juices (orange, strawberry, cherry and apple)	diuron, monuron, monolinuron	Centrifugation and filtration through 0.45 μm nylon membrane	SPME with 50 μm Carbowax/ templated resin, 60 μm (PDMS/DVB) and 85 μm polyacrylate		LC-MS and LC-MS/MS	0.005-0.01 mg/kg	Sagratini et al., 2007
Groundwater	monolinuron, diuron, linuron, neburon		SPME with a 60 μm (PDMS/DVB) fiber		LC-FLD after post-column photochemically induced fluorimetry derivatization with a Xenon lamp	0.019-0.031 μg/L	Mughari et al., 2007b
Groundwater	monuron, monolinuron, chlortoluron, diuron, neburon		C18 ethyl acetate followed by water	ethyl acetate	LC-FLD after post-column photochemically induced fluorimetry derivatization with a Xenon lamp	2.8-9.94 ng/L	Mughari et al., 2007a
Natural water samples	monolinuron, diuron, neburon	acidified to pH 2 with 0.1 mol/L HCl and filtered through 0.45 μm nylon membrane	C18 Sep-Pack cartridge conditioned with MeOH and water at pH 2.	of 5% ethanol in DMSO	GC-MS after potassium *tert*-butoxide/ dimethyl sulphoxide/ ethyl iodide derivatization	0.12–0.52 ng/L	Crespo-Corral et al., 2008
Natural Waters	diuron	filtered through a 0.45 μm syringe filter			one-shot screen-printed thylakoid membrane-based biosensor	1.3 μg/L	Bettazzi et al., 2007
Natural Waters	diuron		MIP based SPE conditioned with MeOH and water	Elution with MeOH/trifluoroacetic acid (98/2, v/v)	photo synthetic biosensor	0.35 μg/L	Breton et al., 2006
Natural waters and freshwater sediments	diuron, linuron	freeze-dried	Ultrasonic extraction with acetone, MeOH and DCM	Centrifugation and filtration through PTFE membranes (0.22 μm)	LC-DAD	0.6-0.7 μg/kg	Boti et al., 2007a
Natural waters	diuron, linuron		C18 conditioned with DCM: acetone (1:1, v/v)	DCM: acetone (1:1, v/v)	LC-DAD	1.4-5.7 ng/L	Boti et al., 2007b

Oysters	chlortoluron, diuron, isoproturon, linuron,		Pressurised liquid extraction	SPE CN/SiOH cartridge conditioned with cyclohexane SPE combined with LLE	LC–MS/MS	0.1-3.9 µg/kg	Bichon et al., 2006
Pesticide formulations	diuron		manual shaking with ACN		NIR spectrometry	0.08-0.18 mg/kg	Moros et al., 2005
Pure water	monuron, diuron, isoproturon, fenuron, chlortoluron, difenoxuron, metoxuron, neburon, buturon, fluometuron				LC-UV performed with a MIP column	not provided	Wang et al., 2005
River water	tebuthiuron, diuron, fluometuron, siduron, linuron, thidiazuron	filtered through a 0.45-µm nylon membrane filter	dispersive LLME with acetone, CS2 and toluene		LC-PAD	0.01–0.5 µg/L	Saraji & Tansazan, 2009
River water	fenuron, monuron, chlortoluron, isoproturon, diuron, metobromuron, linuron	Oasis HLB cartridges	several MIPs conditioned with ACN and toluene	ACN	LC-DAD	not provided	Carabias-Martinez et al., 2005
Sewage sludge	diuron		horizontal shaker; ethyl acetate–DCM 90:10 (v/v)	glass column with activated Florisil; elution with n-hexane–acetone (90:10 and 50:50 (v/v))	LC-MS/MS	0.4 µg/kg dw	Ghanem et al., 2008
Soil	chlortoluron, isoproturon, diuron, monolinuron, linuron, metobromuron, chlorbromuron, methabenzthiazuron.		mechanical shaker, fast speeding mixer, and sonicator with MeOH, DCM, ethanol, and acetone	activated silica gel; elution with MeOH/DCM (1:5, v/v)	LC-DAD	0.07 - 0.13 mg/kg	Buszewski et al., 2006
Soil	diuron, linuron	air dried	MeOH in an automated Soxhlet		LC-DAD	1 µg/kg	El Imache et al., 2009

Soils	linuron, metobromuron, monolinuron	air-drying and sieving to a grain size of 2 mm	microwave-assisted extraction with ACN	filtration through a GF/C and 0.2 μm filters	LC-PAD	0.65-2.4 μg/kg	Paiga et al., 2008
Surface waters	chlortoluron, diuron, isoproturon, linuron, metoxuron	pH 2	HLB cartridge conditioned with ultrapure water acidified at pH 2	ACN and DCM (1:1, v/v)	ultra-pressure LC-MS/MS	4-12 ng/L	Gervais et al., 2008
Surface waters	isoproturon	filtered through 0.45-μm cellulose acetate filters; pH adjusted to 7.5, stored at 4 ◦C.	sol–gel immuno sorbent	ACN	LC-MS/MS	5 ng/L	Zhang et al., 2006
Surface waters	chlortoluron, diuron, linuron, metobromuron, monolinuron	filtered through glass fiber filter (0.7 μm)	C18 cartridges activated with MeOH and water	ACN	LC-DAD	0.02-0.04 μg/L	Kotrikla et al., 2006
Surface waters	diuron, isoproturon	Filtration with 0.45 μm pore size cellulose nitrate membrane; pH adjusted to 4	on-line SPE		LC–MS/MS	0.5 ng/L	Stoob et al., 2005
Surface waters	diuron, linuron	filtered through 0.45 μm pore-size cellulosic membranes	C18 and styrene divinylbenzene cartridges (SDB)	DCM for C18 and ACN for SDB	dual-column LC-DAD	0.012-0.018 μg/L	Ozhan et al., 2005
Tap, underground and mineral waters	linuron	filtration	C18 silica gel disk conditioned with ACN and water	ACN	Flow-through photochemi-cally induced fluorescence optosensor	0.13-0.22 mg/L	Piccirilli et al., 2008
Tap water and beetroot juice.	linuron, metoxuron, dicuron	filtration	LLE with ACN		LC-amperometric detection	0.003-0.17 mg/L	Shapo-valova et al., 2009
Tap water, ground water, sewage water and snow water	chlortoluron	filtered through a 0.45 μm membrane	SPE multiwalled carbon nanotubes conditioned with MeOH and water	DCM	LC-UV	0.012 μg/L	Zhou et al., 2007

Urine, soil, water, pesticide formulation	fluometuron	soil was mixed with water and shaked	C18 conditioned with MEOH	ACN (10%)	photo-induced chemilumi-nescence in continuous-flow multi-commutation assembly	0.1 mg/L	Cydzik et al., 2007
Vegetables and vegetable processed food	diuron, isoproturon, linuron, metobromuron	chopped	MeOH or acetone in ultrasonic bath	carbograph cartridge eluted with DCM/MeOH (60:40, v/v)	Reversed phase capillary electro-chromato-graphy	not provided	De Rossi & Desiderio, 2005
Vegetable samples	isoproturon, metoxuron, fenuron, linuron, chlortoluron, metobromuron	dried	manual shacking during 10 min with ACN and centri-fugation		LC-UV performed with imprinted-stationary phases	not provided	Tamayo & Martin-Esteban, 2005
Water	fluometuron		C18 conditioned with MEOH	ACN (10%)	photo-induced chemilumine scence in continuous-flow multi-commutation assembly	0.1 mg/L	Sa et al., 2007
Water	linuron	pH 6	octadecyl silanized magnetite	ethanol		1.0 μg/L	Katsumata et al., 2007

Table 1. Methods for phenylurea determination.

HLB- hydrophilic–lipophilic balance; ACN-acetonitrile, MeOH- methanol; THF- tetrahydrofuran; DCM-dichloromethane; LLE- liquid liquid extraction; LLME- liquid liquid microectraction; MIPs- molecularly imprinted polymers; PDMS – poly(dimethylsiloxane); DVB – divinylbenzene

Chemiluminescence (CL) is becoming an attractive technique to be used as detection system in LC due to its high sensitivity, wide linear range and simple instrumentation. Despite these advantages, CL has been used less than fluorescence and absorbance for pesticide residue analysis, but has been successfully applied to the determination of some BU insecticides (Galera et al., 2008; Garcia et al., 2007)

Actually, LC combined with mass spectrometry (LC–MS) is widely applied to determine ureas in environmental and food analysis (Table 1-3) mainly due to the high selectivity and sensitivity of the MS-detector, as well as, the possibility of performing reliable identification and confirmation. As regards LC–MS interfacing, atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) are the most widely applied LC–MS ionization techniques (Losito et al., 2006).

Time-of-flight-mass spectrometry (TOF-MS) analysis generates increased resolution of signals on the m/z axis in comparison to other quadrupole mass spectrometers.

Matrix	Sulfonylurea	Pretreatment	Extraction	Clean-up/ Elution	Detection	LOD	Ref
Soil	Thifensulfuron-methyl, metsulfuron-methyl, chlorsulfuron, sulfometuron-methyl, rimsulfuron, ethametsulfuron, tribenuronmethyl, bensulfuron-methyl, prosulfuron, pyrazosulfuron, chlorimuron-ethyl and primisulfuron	60 mL of soil-extraction solution, pH adjusted to 4.8	Cart. with ionic liquid-functionalized silica equilibrated with 10mL of DCM–MeOH 95:5 (v/v) and water.	Water / Elution with 15mL of DCM- MeOH 95:5 (v/v).	LC-MS	0.08 – 1.0 µg/g	Fang et al., 2010
		60 mL of soil-extraction solution, pH adjusted to 4.5	Cart. C18 preconditioned with MeOH and water.	MeOH -water (2:8). / Elution with ACN	LC-MS	0.05 – 1.78 µg/kg	
Soil and orange juice	Thifensulfuron-methyl	-	50% ethanol solution	-	Polarography	36.3 µg/L	Inam et al., 2006
			-			159 µg/ L	
Soil	Cyclosulfamuron	-	-	-	Square wave stripping voltammetry	2.3 µg/L	Sarigul & Inam, 2009
Water						3.1 µg/L	
Soybean	Azimsulfuron and pyrazosulfuron-ethyl	-	ACN	AccuBOND C-18	LC-TOF-MS	1.3 - 9.7 µg/kg	Cui et al., 2007
Water	Thifensulfuron-methyl, metsulfuron-methyl, chlorsulfuron, sulfometuron-methyl, rimsulfuron, ethametsulfuron, tribenuronmethyl, bensulfuron-methyl, prosulfuron, pyrazosulfuron, chlorimuron-ethyl and primisulfuron	Filtered through a glass-fiber and pH adjusted to 4.8	Cart. with ionic liquid-functionalized silica equilibrated with DCM – MeOH 95:5 (v/v) and water.	Water/ Elution with DCM - MeOH 95:5 (v/v).	LC-MS	0.012 – 0.142 µg/L	Fang et al., 2010
		Filtered through a glass-fiber and pH adjusted to 4.5	Cart. C18 preconditioned with MeOH followed by water.	5 mL of MeOH –water (2:8). / Elution with 10mL of ACN.	LC-MS	0.013 – 0.175 µg/L	
Water	Amidosulfuron, bensulfuron-methyl, chlorsulfuron, iodosulfuron-methyl, metsulfuron-methyl, nicosulfuron, primisulfuron-methyl, prosulfuron, thifensulfuron-methyl, triasulfuron and triflusulfuron-methyl	-	-	-	LC-MS/MS	< 10 ng/L	Seitz et al., 2006
Water (drinking)	Metsulfuron-methyl	EDTA at 10 g/ L	MIP column washed with CH_3CN/H_2O (95:5, v/v) and then redistilled water.	ACN -water (10:90, v/v). /Elution with ACN -water (95:5, v/v).	LC-UV	6.0 ng/L	Liu et al., 2007

Water (drinking and paddy field)	Metsulfuron-methyl and bensulfuron methyl	-	Cart. C18 and HLB	-	LC-DAD	0.03 - 0.04 µg/L	Roehrs et al., 2009
Water (pure, tap and river)	Bensulfuron-methyl, flazasulfuron, pyrazosulfuron-ethyl, halosulfuron-methyl, Imazosulfuron	Ascorbic acid sodium salt 0.005%(w/v). River water was filtered through a glass-fiber	*N*-vinyl-pyrrolidone polymer resin (Oasis HLB Plus Extraction Cart.) equilibrated with MeOH and pure water.	Pure water / Elution with of MeOH.	UV using temperature-responsive chromato-graphy	1 - 4 µg/L	Ayano et al., 2005
Water (surfaces)	Amidosulfuron, azimsulfuron, nicosulfuron, rimsulfuron, thifensulfuron methyl, tribenuron methyl	-	Strata RP-18 cart. conditioned with ACN and water acidified (1.5%, v/v) for acetic acid.	Elution with 1.0mL of an acidified (1.5%, v/v acetic acid) water/ ACN mixture.	LC-UV	< 48.3 ng/L*	Polati et al., 2006
			Strata-X cart. conditioned with ACN and water acidified (2.0%, v/v) for acetic acid.		LC-MS^n	< 26.9 ng/L*	
Water (surface and drinking)	Chlorsulfuron	Filtration through a 0.45 µm membrane. pH adjust to 3.5	C18 disk conditioned with MeOH, ACN and Milli-Q water	Elution with 2 x 3 mL of DCM	LC-UV-DAD	0.035 µg/ L	Ozhan et al., 2005
Water (river)	Thifensulfuron, metsulfuron and chlorsulfuron	Filtration through a 0.45 µm membrane	On-line pre-concentration with a Supelguard cart. packed with 5µm, C18 silica-bonded phase.	-	LC-MS	0.012 - 0.026 µg/L	Losito et al., 2006
Water (well, tap, reservoir and seawater)	Nicosulfuron, thifensulfuron and metsulfuron-methyl	pH adjust to 3	Cart. with MWCNTS. Washing with ACN and ultrapure water.	Pure water. / Elution with ACN containing 1% of acetic acid.	LC-UV	0.0059 – 0.0112 µg/L	Zhou et al., 2006

Table 2. Methods for sulfonylurea determination.
ACN-acetonitrile, Cart- Cartridges; MeOH- methanol; DCM-dichloromethane; *LOQ

Furthermore, this enhanced resolving power benefits analyses involving complex matrices. Although LC-TOF-MS has not become a widely used technique for the determination of pesticides, it will probably become as one of the main techniques for the unequivocal identification of contaminants (Cui et al., 2007).

Recently, a wide range of immunoassays and sensors for environmental analytes such as pesticides (including ureas) are being investigated, using various detection systems such as amperometric, capacitative, conductimetric, potentiometric and fluorimetric (Bettazzi et al., 2007; Breton et al., 2006; Cydzik et al., 2007; Piccirilli et al., 2008; Sa et al., 2007). Despite offering a number of advantages, such as low cost, easy to use, often portable, disposable and rapid analyte detection, they are normally restricted to aqueous solutions or solutions

Matrix	Benzoylurea	Pretreatment	Extraction	Clean-up/ Elution	Detection	LOD	Ref
Apple, grape and wine	flufenoxuron (+6 other pesticides)		cyclohexane-DCM (9:1, v/v)	Isolute SPE silica cartridges and elution with THF	HPLC-UV	5 – 20 µg/kg	Likas & Tsiropoulos, 2009
Cucumber	diflubenzuron, flufenoxuron, hexaflumuron, lufenuron and triflumuron	chopped and homogenized	DCM (50 mL + 2×20 mL)	aminopropyl-bonded silica SPE cartridge elution with DCM	HPLC-CL	12 – 180 µg/kg	Garcia et al., 2007
Food (fruit, vegetable, cereals, and animal products)	chorfluazuron, diflubenzuron, flucycloxuron, flufenoxuron, fluometuron, hexaflumuron, lufenuron, teflubenzuron, triflumuron	chopped and homogenized	PLE (5 g of sample blended with diatomaceous earth in a 22 mL extraction cell; ethyl acetate as solvent at 80 ^{o}C and 1500 psi; evaporation and reconstitution in 0.5 mL of MeOH)		LC-MS/MS	0.7–3.4 µg/kg	Brutti et al., 2010
Peach juice	diflubenzuron, hexaflumuron teflubenzuron (+ 2 carbamates)	filtered through 0.45 µm cellulose acetate membrane filter	Floated organic drop microextraction (FDME) with 1-dodecanol; 15 mL sample; 40 ^{o}C; NaCl, 30 g/L; pH 4; 420 rpm; 25 min		HPLC-UV	5 – 10 µg/L	Zhou et al., 2009
Tomato	diflubenzuron, triflumuron	chopped and homogenized	QuEChERS	PSA	HPLC-CL	2 – 17 µg/L	Galera et al., 2008
Vegetables and water (river)	diflubenzuron, flufenoxuron, hexaflumuron, lufenuron and triflumuron	chopped and homogenized 400 mL H_2O+ 100 mL MeOH filtered through a 0.45 µm membrane	DCM (25 mL + 2×20 mL) for vegetable samples Plus C18 SPE cartridges (3 mL) containing 360 mg of the strongly hydrophobic silica-based phase for water samples	Elution with 5 mL of ACN and 5 mL of Cl_2CH_2	LC-MS	0.68 – 1.75 µg/kg (vege-tables) 2.6 – 7.5 ng/L (water)	Martinez et al., 2007

Table 3. Methods for benzoylurea determination.
ACN – acetonitrile; CL – chemiluminescence; DCM – dichloromethane; PLE – pressurized liquid extraction; PSA – primary secondary 2/2 amine; THF – tetrahydrofuran.

containing only small amounts of organic solvents. Although their great potential more research is needed since, in most cases, the LODs obtained are rather high for environmental analysis (Bettazzi et al., 2007; Breton et al., 2006; Piccirilli et al., 2008; Sa et al., 2007). On the other hand, electroanalytical methods offer useful applications in kinetic and equilibria studies. A differential pulse polarographic method for the determination of trace amounts of thifensulfuron-methyl in soil and orange juice was validated and the obtained LODs were 36.3 µg /L and 159 µg/L (9.37×10^{-8} and 4.1×10^{-7} mol/L), respectively (Inam et al., 2006).

The square-wave electrochemical mode offered favourable signal-to noise characteristics and was used by Sarigul & Inam (2009) for determination of cyclosulfamuron in tap water and soil achieving LODs of 3.1 μg/L and 2.3 μg/L, respectively.

5. Conclusion

Urea pesticides form, together with phenoxy derivatives and triazines, the most important agricultural pesticide group. Ureas undergo natural degradation reactions in the environment that may lead to mineralization and/or to the formation of new species potentially more toxic and stable than the precursors. Several studies have shown that degradation is mainly dependent on the soil pH, moisture content and microbiological activity. Nevertheless, residues can reach populations through the food chain causing chronic exposure and long-term toxicity effects.

Actually, sensitive and accurate methods are available to meet the needs for compliance of urea MRLs in environmental and food matrices. Methods based on LC are the most commonly preferred, and in particular those using mass spectrometric detection. Limits of detection and recoveries for ureas are compound, matrix and method dependent. The detection limits typically range from ng/L to mg/L.

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Liquid Chromatography in Studying Lipophylicity and Bioactivity of Pesticides

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1. Introduction

During the past decades the interest of new bioactive compounds for applications in medicine or agriculture has increased dramatically (Gocan & Cimpan, 2005; Kaliszan, 1987; Kaliszan, 1992; Krieger et al., 2001; Lipiński et al, 2001; Nasal et al., 2003; Sangster, 1997). Physicochemical properties of new bioactive compounds like pesticides, such as solubility, lipophylicity (hydrophobicity), stability and acid-base character, should be determined in the early stages of development. These properties affect absorption, distribution and transport of xenobiotics in biological systems. The hydrophobic effect is assumed to be one of the driving forces for the passive transport of xenobiotics through bio-membranes and to a certain degree responsible for interactions with receptor.

The expressions "lipophylicity" and "hydrophobicity" are frequently used as synonymous, but in scientific use their meaning should be distinguished. According to IUPAC suggestions (Pliška et al., 1996) hydrophobicity means the association of nonpolar species in aqueous environment arising from the tendency of water to exclude nonpolar molecules. Lipophylicity as a measure of relative affinity of a molecule for a lipophylic environment has a broader meaning than hydrophobicity because may be connected with polar interactions.

Lipophylicity as a property determining biological activity of substance was firstly recognized by Overton, Meyer and Baum (Kaliszan, 1987; Kaliszan, 1992). During the time hundreds of articles, among them some review papers, of lipophylic properties of different bioactive compounds in medicine, agriculture or environmental chemistry have appeared (Héberger, 2007; Kah & Brown, 2008; Komsta et al., 2010; Poole & Poole, 2003; Valkó, 2004; Wang et al., 1999; Sherma, 2003).

Lipophylicity is commonly measured by solute distribution in biphasic liquid system and universal lipophylicity scale is formed by the logarithms of partition coefficients (log P) in the case of neutral species or distribution ratio (log D) for ionisable compounds (Kah & Brown, 2008; Pliška et al., 1996). In early seventies octanol-water was proposed as reference system for lipophylicity measurements and to this day it remains as a standard for experimental and theoretical investigations. Due to experimental limitations connected with direct measurements of log P (log D) parameters by shake-flask method, chromatographic techniques are getting more and more popular in studying lipophilic properties of organic substances. Though partition parameters produce more universal scale of lipophylicity,

chromatographic approach is much more convenient, reproducible, fast and inexpensive. The shake-flask and liquid chromatography procedures are now standardized and officially recommended by the Organization of Economic Co-operation and Development (Guidelines for the Testing of Chemicals) (Nasal et al., 2003).

2. Theoretical background

The application of liquid chromatography in lipophylicity determination follows from the definition of retention factor k:

$$k = \frac{n_s}{n_m} = \frac{c_s}{c_m}\frac{V_s}{V_m} \tag{1}$$

where n is the average number of solute molecules while c is solute concentration at equilibrium, in the stationary and mobile phase, respectively; V_s and V_m are volumes of stationary and mobile phases. Introducing the phase ratio ϕ equal to V_s/V_m and taking into account the definition of partition coefficient K referring to Nernst's law:

$$K = \frac{c_1}{c_2} \tag{2}$$

where c is the concentration of neutral form of molecules in two immiscible solvents 1 and 2 at equilibrium, we can write:

$$\log k = \log K + \log \phi \tag{3}$$

Collander (Kaliszan, 1987) formulated the equation relating partition coefficients determined in different water-organic solvent systems:

$$\log P_a = m \log P_b + n \tag{4}$$

In equation 4 constants P_a and P_b are partition coefficients corresponding to two different systems: water-solvent a and water-solvent b. As it was already observed by Collander, the fit of equation 4 depended on the polarity differences between organic solvents a and b.
The combination of equations 3 and 4 leads to fundamental relationship in chromatographic determination of partition coefficient:

$$\log P_a = p \log k + q \tag{5}$$

where regression coefficients p and q should be evaluated for a set of structurally related standard substances.
Important advantage of chromatographic measurements is that partition coefficient can be evaluated independently of impurities and without quantitative analysis. Apart from that reversed-phase liquid chromatography is simple, rapid and relatively inexpensive, requires a much smaller amount of tested solute than shake-flask method and is free of some practical complications like formation of stable emulsions. In spite of all advantages there are important limitations of chromatographic measurements. Reversed-phase chromatographic system is only a partial and incomplete model for octanol-water partitioning. In contrary to liquid-liquid system in reversed-phase chromatography, apart

from interactions appearing in the bulk phase (solute-water, solute-organic solvent, water-organic solvent, water-water, organic solvent-organic solvent), different interactions in stationary phase occur: solute-stationary phase, water-stationary phase, and organic solvent-stationary phase. Moreover, specific interactions (e.g. ion-exchange) with residual silanol groups at the surface can appear as well as pore size effects such as size or ion exclusion (Kaliszan, 1987; Nasal et al., 2003). Next complications are connected with reproducibility of results on columns or chromatoplates delivered by various producers or even from different batches of the same manufacturer. The dissimilarities between chromatographic and shake-flask partition coefficients result also from problems with accurate measurements of the dead volume in column technique (Poppe, 1993) or thin-layer effects in planar chromatography (Nurok, 1989). Summarizing the above considerations it should be emphasized that chromatographic parameters depend not only on solute but also mobile and stationary phases properties and don't form universal scale of lipophylicity, which is limited for structurally diverse substances.

Kaliszan (Kaliszan, 1987) and Gocan and Cipman, 2005 (Gocan & Cipman, 2005) described in details first efforts and the history of applying liquid chromatography, thin-layer and column, for lipophylicity measurements. In the early studies stationary phases were impregnated with n-octanol and developed with mobile phases (aqueous or buffer solutions of organic modifiers) saturated with n-octanol. Such chromatographic systems resembled n-octanol-water (buffer) partitioning and many researchers obtained very good linear correlations between chromatographic parameters and experimental log P values. Due to technical inconvenience these methods are rarely used at present. Interesting results were described by Kaune et al. (Kaune et al., 1998) who investigated a group of commercial s-trazine herbicides and some of their degradation products using octadecyl column (LiChrospher 100, RP-18) coated with n-octanol and the buffer (pH 7.5) saturated with n-octanol as the mobile phase. Chromatographic partition coefficients were correlated to literature data (Figure 1) and, in comparison to gradient results obtained on LiChrospher

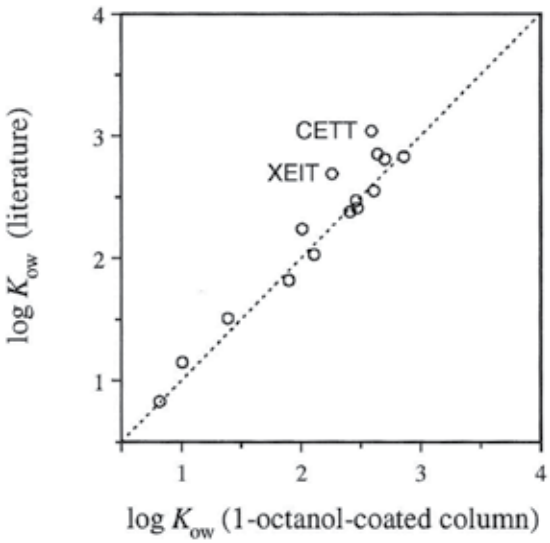

Fig. 1. Comparison between literature n-octanol-water partition coefficients and estimated by n-octanol-coated column method. (Kaune et al., 1998).

100 RP-18e with nonlinear water-methanol gradient, proved to be better lipophylicity indices characterized by an excellent reproducibility. Currently Sirius Analytical Instruments Ltd. offers the instrument called ProfilerLDA [http://www.sirius-analytical.com.] for determination of lipophylicity (log D) on HPLC columns dynamically coated with n-octanol and mobile phases buffered at desired pH value.
Spectacular increase of interest of applying chromatographic techniques in lipophylicity measurements was directly connected with introduction commercially available chemically bounded stationary phased commenced in the seventies of the last century. Reversed-phase stationary phases based on silica gel are stable, apolar and, similarly to biosystems, anisotropic (Kaliszan, 1987; Nasal et al., 2003).
For majority of substances it's extremely difficult to measure retention parameter for water (or buffer) as the mobile phase on apolar chemically bounded stationary phase. Due to general elution problem and long retention times, it's possible for moderately lipophylic solutes only. Moreover, water doesn't moisten most of RP layers and such experiments need forced flow, i.e., column or over-pressured-layer not thin-layer chromatography (Tyihák & Mincsovics, 2001).
In liquid chromatography the key lipophylicity parameter is retention factor log k or retention factor extrapolated to pure water log k_w. In HPLC the value of log k can be evaluated directly from chromatographic data, i.e. retention volume (V_R) or time (t_R) according to the following equations:

$$k = \frac{V_R - V_o}{V_o} \tag{6}$$

$$k = \frac{t_R - t_o}{t_o} \tag{7}$$

where V_o and t_o are the dead volume or the dead time, respectively.
In planar chromatography solute retention is described by retardation factor R_F equivalent to the ratio of the distance migrated by the solute zone to the distance moved by the solvent front and measured from the sample application position. In TLC practice the most frequently used is retention factor R_M defined as:

$$R_M = \log\frac{1 - R_F}{R_F} \tag{8}$$

The relationship existing between two factors, k and R_F:

$$k = \frac{1 - R_F}{R_F} \tag{9}$$

leads to the following equation:

$$R_M = \log k \tag{10}$$

Both factors, log k and R_M, are used alternatively, but in general R_M parameter is still in use in thin-layer chromatography.
Isocratic log k values are hardly ever applied as lipophylicity descriptors. These values strongly depend not only on stationary phase but also mobile phase organic modifier. The

functions describing the effect of organic modifier concentration in the effluent on retention parameters can cross each other for particular organic solvents. Nevertheless, some interesting results were described by Yamagami and Katashiba (Yamagami & Katashiba, 1996) who studied phenyl N-methyl and phenyl N.N-dimethyl carbamates using Capcell Pak C18 column (Shiseido, Japan) and buffered methanol as the mobile phase and obtained the best linear relationships (R^2=0.987-0.997) between log k and experimental log P parameters for 50% concentration of methanol in the effluent. De Kock and Lord (De Kock & Lord, 1987) received almost equally good results (R^2=0.95) for polychlorinated biphenyls applying 20% methanol in water on Resolve C18 (Waters) column. Ellgehausen et al. (Ellgehausen et al., 1981) correlated HPLC and TLC retention parameters of twenty commercial pesticides with partition coefficients log P. Xia et al. (Xia et al., 2005) obtained very interesting results on a new type of stationary phases, i.e. polystyrene-octadecene-encapsulated zirconia (C-18-PS-ZrO_2) in column technique. The authors examined retention of eighteen newly synthesized benzoylphenylureas on C-18-PS-ZrO_2 and commercial Kromasil C-18-SiO_2 columns using water-methanol as effluent. Four aqueous solutions containing 80%, 85%, 90% and 95% of methanol were applied. Very good linear correlations (R>0.97) between retention coefficients log k and structural descriptors (FHF, ECCR, HOMO, LUMO, DM, CMR and C log P) were obtained, especially for the new C-18-PS-ZrO_2 stationary phase.

Xu et al. (Xu et al., 1999) described the results obtained by HPLC technique with soil column and aqueous methanol, for eight commercial pesticides (fungicides, insecticides and one herbicide). They applied column chromatography for indirect evaluation of soil organic partition coefficients (K_{oc}). As chromatographic lipophylicity indices there were used retention factors obtained for particular mobile phase compositions (log k) as well as log k_w parameters linearly extrapolated to pure water. The authors observed very high linearity between retention factors and literature log K_{oc} parameters (R in the range 0.9784-0.791). Similar investigations were described by Bermúdez-Sadaña et al. (Bermúdez-Sadaña et al., 2006) who summarized results obtained on different columns: ODS, RP-CN or natural and artificial soils. Retention parameters of 48 substances, mainly pesticides, were used for indirect determination of soil-sorption coefficients K_{oc}. The authors considered the model proposed as a practical, alternative approach to estimating soil-sorption coefficients, though some deviations were observed for low retained compounds.

Pesticides mobility through soil structures can be investigated by thin-layer chromatography as well. Such experiments were performed by Ravanel et al. (Ravanel et al., 1999) on different types of soils and water or water-methanol mobile phases, and by Mohammad et al. (Mohammad et al., 2001) on silica, soil or mixed layers containing soil (silica, alumina and cellulose) with different mobile phases (aqueous ammonium or sodium salt solutions, with or without surfactant, aqueous surfactant or pure organic solvents).

The parameter most often applied as lipophylicity descriptor is retention factor extrapolated to pure water (or buffer) as the mobile phase, usually expressed as log k_w (or R_{M0} in TLC). The value of log k_w is calculated by extrapolation of experimental retention function, i.e. log k vs. φ, where φ is the concentration (volume fraction) of organic modifier in the effluent, towards pure water (φ=0). The results of calculations strongly depend on the range of extrapolation, i.e. the range of organic modifier concentration in the bulk phase, but also on mathematical formula applied for the purpose. The simplest description results from Soczewiński-Wachtmeister's equation (Kaliszan, 1987; Sangster, 1997):

$$\log k = \log k_w - s\varphi \tag{11}$$

where s, the slope of the experimental data after fitting to regression model, is related to specific hydrophobic surface are of the solute.
Within the intermediate range of organic modifier in the effluent an approximate linear relationship between log k and φ almost always is found. The majority of log k vs. φ plots are curved for mobile phases "rich" in water. Therefore quadratic equations are also proposed to describe the effect of mobile phase composition on log k values:

$$\log k = \log k_w - a_1\varphi - a_2\varphi^2 \tag{12}$$

where a_1 and a_2 are regression constants without physical significance (Gocan & Cipman, 2005).
Schoenmakers et al. (Kaliszan, 1987) proposed the following equation describing almost entire range of organic solvent concentration in the mobile phase:

$$\ln k = \ln k_w + A\varphi^2 + B\varphi + C\sqrt{\varphi} \tag{13}$$

Deviations from linearity of equation 13 are observed for higher than 90% concentration of organic modifier in water. According to results obtained by Pietrogrande et al. (Pietrogrande et al., 1985) retention factors calculated according to eq. 13 weakly correlate with log P values so they don't find application in practice.
The available evidence demonstrate that log k_w values calculated according to equations 11 or 12 differ significantly. Moreover extrapolated retention parameter depends on organic modifier that is in contrary with its physical significance. Some scientist recognize this parameter as an unacceptable lipophylicity descriptor, but majority considerate the extrapolation as a convenient method of standardization of chromatographic data (Gocan & Cipman, 2005; Kaliszan, 1987).
In our investigations we proposed new, alternative to extrapolation method of calculation of log k_w parameters [Janicka, 2009; Janicka et al., 2000). This numerical method, originating from thermodynamic description of the system solute-stationary phase-solution (Jaroniec, 1993), uses the following linear form of Ościk's equation:

$$G(x_{org}) = \frac{x_{org}(1-x_{org})}{\log k - x_{org}\log k_{org} - (1-x_{org})\log k_w} = ax_{org} + b \tag{14}$$

where x_{org} is a molar fraction of organic modifier in aqueous mobile phase and log $k_{org,}$ log k_w and log k are solute retention parameters corresponding to pure organic modifier, water and binary mixture as the mobile phase, respectively; a and b are regression factors. In the method the values of log $k_{org,}$ log k and x_{org} are known (experimentally evaluated) and log k_w parameter is determined by numerical fitting to equation 14 to obtain the linearity between the left term denoted as G and x_{org}. We tested numerical method for different groups of substances, using various mobile and stationary phases by thin-layer, over-pressured-layer and column reversed-phase chromatography techniques [Janicka, 2003; Janicka et. al., 2004; Janicka et al. 2004; Janicka et al., 2006; Janicka, 2007) and numerical log k_w parameters proved to be very good lipophylicity descriptors linear correlated to experimental and calculated log P values and moreover, in contrary to extrapolated, almost independent of the range of organic modifier concentration in the effluent.

In order to introduce the next chromatographic lipophylicity parameter one should return to equation 11. The value of parameter s, regression coefficient of this equation, is characteristic for chromatographed solute. Biagi et al. (Biagi et al., 1994) observed linear relationships between extrapolated log k_w values and s parameters for congeneric substances and, similarly to Waterbeemd et al. (Waterbeemd et al., 1996), designated the ratio equal to s/log k_w as lipophylicity descriptor, alternative to log k_w, for closely related substances. Moreover the linearity between these two parameters is a measure of congenerity of solutes tested.
Valkó and Slegel (Valkó & Slegel, 1993) introduced new hydrophobicity index φ_o defined as:

$$\varphi_o = \frac{\log k_w}{s} \tag{15}$$

According to equation 11 φ_o is a volume fraction of organic modifier in the mobile phase at which solute retention factor k is equal to 1, i.e. molar concentrations of solute molecules in stationary and mobile phases are equal to each other (equation 1) or a volume fraction required to get the substance retention time exactly twice of the dead time (equation 7). Important advantage of φ_o is that it's not only a parameter of adjustment but it has physical interpretation and, for majority of substances can be measured experimentally. According to Valkó and Slegel φ_o parameters evaluated for different organic modifiers correlate to each other as well as to experimental or calculated log P values.
Taking into account different lipophylicity parameters determined by isocratic reverse-phase liquid chromatography one can improve the basic correlation equation 5 by:

$$\log P = p_1 s + q_1 \log k_w + r_1 \tag{16}$$

or write another relationship:

$$\log P = d\varphi_o + e \tag{17}$$

3. Isocratic HPLC

Many researchers used isocratic reversed-phase liquid chromatography to measure lipophylic properties of bioactive compounds, also pesticides, both commercial and newly synthesized, using variety of mobile and stationary phases.
Braumann et al. (Braumann et al., 1983) compared log k_w parameters linear extrapolated from water-methanol mobile phase with log P values for a group of herbicides (phenylureas, s-triazines and phenoxycarbonic acid derivatives) and according to their suggestion chromatographic parameters may be a better model for the assessment of lipophylicity in biological system than log P values.
Hsieh and Dorsey (Hsieh & Dorsey, 1995) studied seven pesticides (among a group of PAHs and barbiturates) on homemade C-18 columns with water-methanol mobile phase. Extrapolated retention factors log k_w were compared with biotransfer factor for beef and milk and gave much better linearity (R^2=0.852-0.887) than n-octanol-water partition coefficients (R^2=0.501 and 0.584).
Darwish et al. (Darwish et al., 1993) and Cserháti and Forgács (Cserháti & Forgács, 1997) compared hydrophobicity indices (extrapolated log k_w and R_{M0}, and parameter s from equation 11) of twelve pesticides (herbicides and fungicides) evaluated by TLC and HPLC

on octadecylsilica stationary phase and water-methanol as the mobile phase. Principal Component Analysis (PCA) showed that chromatographic parameters from two techniques might be used alternatively. However, it was noticed that differences in biological activity of tested herbicides and fungicides couldn't be attributed to separate parameters (s and log k_w or s and R_{M0}). Similar results were obtained by Zhang et al. (Zhang et al., 2000) who studied 37 commercial pesticides (insecticides, herbicides fungicides and growth regulators) by HPLC and TLC on ODS type stationary phases and water-methanol mobile phases. Lipophylicity was described by retention parameters extrapolated to pure water (log k_w and R_{M0}) and specific hydrophobic surface area (s values). Linearity between log k_w (R_{M0}) and s parameters obtained by HPLC (R=0.9867) and TLC (R=0.9790) confirmed both indices as alternative lipophylicity descriptors, but PC analyses showed that particular groups of pesticides, different in chemical structure and bioactivity, couldn't be distinguished from each other considering only chromatographic parameters.

Cserháti and Forgács (Cserháti & Forgács, 1994) applied alumina support as stationary phases in HPLC and TLC techniques with n-hexane-dioxan as effluent for studying retention of 26 pesticides (commercial herbicides, fungicides, acaricides and insecticides). TLC and HPLC retention factors determined in the studies, though strongly intercorrelated, proved to have a negligible effect on the type of biological activity.

Pyka and Miszczyk (Pyka & Miszczyk, 2005) studied lipophylic properties of nine urea pesticides. Chromatographic lipophylicity indices (log k_w, R_{M0}, s and φ_o) were compared with partition coefficients (five different values: experimental and calculated from molecular structures) and Gutman and Randić topological indices. In experiments HPLC (Econosphere C-18 column) and TLC (RP-18W plates) techniques were used with water-methanol as the mobile phase. Chromatographic lipophylicities were intercorrelated and the results (R>0.99) indicated that studied pesticides could be regarded as compounds belonging to the same group. Very high linearity (R in the range 0.8450–0.9924) was observed between chromatographic parameters (log k_w, R_{M0}, s and φ_o) and partition coefficients, especially for φ_o calculated from HPLC. From all topological indices analysed in the studies only one, Gutman index M', correlated well with chromatographic parameters and experimental log P values (R>0.9).

Peculiar database are φ_o parameters collected by Valkó for different bioactive compounds (including thirty trade herbicides) and calculated from HPLC data (Brauman et al., 1983) obtained on C-18 column and acetonitrile as the mobile phase modifier.

Reversed-phase chromatography, both planar and column, is particularly attractive technique for studying new organic substances with potential or even defined bioactivity.

Perišić-Janjić et al. (Perišić-Janjić et al., 2001; 2003a; 2003b; 2005) applied planar chromatography to study lipophylic properties of newly synthesized s-triazine derivatives. These nitrogen-containing compounds are widely used as herbicides in agriculture and industry and some of them have fungicidal properties. In the TLC measurements there were used different stationary phases: silica gel impregnated with paraffin oil (Perišić-Janjić et al, 2003a), RP-18 (Perišić-Janjić et al, 2003b; Perišić-Janjić et al, 2005), RP-NH_2 (Perišić-Janjić et al, 2005) and aminoplast and cellulose (Perišić-Janjić et al, 2001). Aqueous solutions of methanol, acetone, acetonitrile, dioxane, 2-propanol, tetrahydrofuran or methanol-dilute acetic acid were compared as mobile phases (Perišić-Janjić et al, 2001). Linear extrapolated R_{M0} parameters were compared with software calculated log P values. In the studies the effect of mobile phase pH value on retention was used to calculate the approximate protonation constants pK_a of tested substances. The values were correlated with Hammett substituent constants σ.

In our studies homological s-triazines were investigated (Janicka et al., 2004) in TLC and OPLC technique with RP-8 and RP-18 stationary phases and water-acetone, water-acetonitrile, water-tetrahydrofuran and water-dioxane as mobile phases. We correlated linear extrapolated and numerical log k_w parameters with log P values calculated from molecular structures of solutes. The results obtained allowed to select numerical log k_w parameters calculated from OPLC technique in water-acetonitrile as the best lipophylicidy descriptors of s-trazines. The same group of homological s-triazines was analyzed in HPLC technique (Janicka et al., 2006). In this case we applied RP-18e (Purospher) column with methanol or acetonitrile as organic modifiers. Chromatographic lipophylicities, log k_w, calculated by linear and parabolic extrapolation and by numerical method were compared with different software calculated log P values. All log k_w parameters proved to be very good lipophylicity descriptors but the numerical seemed to be more universal (see Table 1).

		Alog *P*		IAlog *P*		clog *P*		log P_{KoWin}		xlog *P*		log $P_{Chem. Off.}$	
		r	*SD*	*r*	*SD*	*r*	*SD*	*r*	*SD*	*r*	*SD*	*r*	*SD*
log k_w	*	0.982	0.431	0.979	0.397	0.980	0.739	0.978	0.695	0.983	0.551	0.981	0.534
linear	**	0.943	0.766	0.939	0.676	0.935	1.311	0.943	1.109	0.934	1.083	0.946	0.893
log k_w	*	0.970	0.561	0.927	0.740	0.980	0.736	0.980	0.663	0.978	0.626	0.986	0.456
parabolic	**	0.949	0.729	0.933	0.706	0.935	1.317	0.946	1.081	0.933	1.095	0.949	0.870
log k_w	*	0.987	0.370	0.945	0.645	0.996	0.332	0.996	0.288	0.993	0.359	0.996	0.246
numerical	**	0.985	0.396	0.955	0.586	0.995	0.377	0.995	0.333	0.988	0.472	0.993	0.327

* – methanol as organic modifier; ** – acetonitrile as organic modifier

Table 1. Correlation matrix of log k_w vs. log *P* relationships. (Janicka et al., 2006).

Different scientists investigated new thiobenzanilides, substances with strong antibacterial or antimycotic activity. In the studies reversed-phase planar (Janicka et al., 2000; Matysiak et al., 1999; Janicka, 2006) and column (Niewiadomy et al., 1998; Jóźwiak, 2000; Jóźwiak et al., 2000) chromatography was applied. In TLC RP-8, RP-18 and RP-CN stationary phases and water-methanol or water-acetone as effluents were used, while in HPLC RP-18e (Eurosil Bioselect) or IAM stationary phases and buffered methanol (pH 4) or acetonitrile were applied as the mobile phases. As lipophylicity descriptors were analysed log k_w parameters, linearly extrapolated or numerical and/or φ_o values. Chromatographic lipophylicities were compared not only with calculated log P values but also with biological activities (described by minimal inhibitory concentration, MIC) against different dermatophytes, yeasts and moulds, giving very good results.

Djaković-Sekulić et al. (Djaković-Sekulić et al., 1987) evaluated lipophylic properties of potential pesticides, i.e. ten anilides (formanilide and 4-substituted acetanilides) using TLC technique with starch and cellulose and different mobile phases. Linear extrapolated R_{M0} parameters were correlated with calculated log P values giving excellent parabolic correlations. In the study very good linearity (R=0.839-0.911) for multivariable regression equation $R_{M0} = c_1 + c_2\pi + c_3\sigma + c_4 HB$, describing correlations between R_{M0} and molecular descriptors: Hansch parameters π, the Hammett electronic constant σ and the hydrogen-bond constant HB, was obtained.

The same authors studied newly synthesized anilides (pivalanilides, benzanilides and α-phenylacetanilides) using HPLC technique with LiChrosorb RP-18 column and water-methanol as the mobile phase (Djaković-Sekulić et al., 2001). Different chromatographic

lipophylicity indices (linear extrapolated log k_w, s and φ_o were correlated with calculated log P values as well as with molecular indices (the Hammett electronic constant σ and the hydrogen-bond constant HB) giving very good linear relationships (R=0.936-0.985). The authors claimed: "more rational and objective estimation and comparison of hydrophobicity is possible by multiple regression. Hydrophobic and steric parameters taken into account in this way contribute to the QSAR paradigm." Experimental log k_w values were subsequently correlated with topological indexes by Djaković-Sekulić et al. (Djaković-Sekulić et al., 2003).

Various scientists examined different groups of newly synthesized triazoles, well-known active compounds among others known as herbicides and fungicides. Perišić-Janjić et al. (Perišić-Janjić et al., 2000) studied ten 1,2,4-triazoles using HPLC and TLC techniques. In HPLC they applied RP-8 (Lichrosorb) and water-methanol and water-acetinitrile while in TLC silica gel impregnated with paraffin oil and water-methanol were used. Chromatographic lipophylicity indices (linear extrapolated log k_w and R_{M0} and φ_o) were correlated with calculated log P values giving very high linearity (R in the range 0.928-0.996). In the studies relationships between chromatographic lipophylices and antimicrobial activity against Salmonella enteritidis was analysed. Better results were obtained for R_{M0} and φ_o than log k_w, though deviation from linearity for some substances were observed. Sztanke et al. (Sztanke et al., 2008) studied a group of 14 derivatives of 1,2,4-triazoles - potential fungicides, applying HPLC technique on ODS stationary phase (Supelcosil LC-18) with buffered dioxane (pH 3.5). Unsatisfactory correlations between extrapolated log k_w parameters and calculated log P values (R=0.6707) observed in the studies were explained by the weakness of computer program [Pallas 3.1.1.2, CampuDrug, 2003] in the case of ortho, meta and para chloro isomers.

Matysiak et al. (Matysiak et al. 2002) determined lipophylicity of new 2-arylbenzothiazoles with potential antimycotic properties applying TLC technique on RP-8 and RP-18W stationary phases with different effluents. The author obtained linear correlations between chromatographic lipophylicities (extrapolated R_{M0} and s) and calculated log P values and Hansch parameters π. The same authors (Matysiak et al., 2004) investigated antimycotic compounds belonging to 2-(2,4-dihydroxyphenyl)benzothiazoles by HPLC on RP-18 (LiChrosorb) stationary phase and buffered methanol as an effluent (pH 4.0 and 7.4). Chromatographic descriptors log k_w, s and φ_o were compared with calculated log P values and afterwards, with fungistatic properties expressed by log 1/MIC.

Lipophylicities of nine newly synthesized N-hydroxyethylamides of aryloxyalkylene and piridine carboxylic acids were determined by Cipman et al. (Cipman et al., 1998) who applied TLC technique on RP-8 stationary phase and water-methanol effluent. Extrapolated R_{M0} parameters linearly correlated with calculated log P partition coefficients (R equal to 0.990 and 0.992) confirmed the significance of reversed-phase chromatography in determination of lipophylicity of congeneric compounds. Similar investigations were performed for propanoic acid amides by Djaković-Sekulić et al. (Djaković-Sekulić et al., 2002) who used silica gel impregnated by paraffin oil in TLC measurements. The authors additionally correlated extrapolated R_{M0} values with Hansch π parameters. Similar results were obtained for for n-phenylsuccinimide derivatives (Perišić-Janjić et al., 2002), benzimidazoles (Perišić-Janjić & Podunavac-Kuzmanović, 2008) or azaphenothiazines (Zięba & Prus, 2009) - potential herbicides, fungicides or insecticides. Perišić-Janjić et al. (Perišić-Janjić et al., 2007) used TLC technique and RP-18 stationary phase and variety of mobile phases (aqueous solutions of methanol, ethanol, 1-propanol, 2-propanol, acetonitrile,

acetone, tetrahydrofuran and dioxane) to determine lipophylic properties of eight 2-phenylacetamide derivatives and obtained good correlations between R_{M0} parameters and calculated log P values as well as with biological activity predictors.
Lipophylic properties of newly synthesized 2-(chlorophenoxy)acyl derivatives and n-aryltrichloroacetamides, potential herbicides, were examined by Janicka et al. (Janicka et al., 2004) by TLC technique on RP-18 stationary phase and buffered methanol as effluent (pH 5.3, 7.3 and 8.3). As lipophylicity descriptors log k_w parameters calculated by linear extrapolation and numerical method were applied. The best linear correlations (R^2 equal to 0.8057, 0.8084 or 0.9811) were observed between numerical log k_w and calculated log P parameters for mobile phases at pH 5.3 or 8.3.
Kostecka et al. (Kostecka et al., 2006) studied lipophylic properties of 2,4-dihydroxyphenylthioamide fungicides by TLC (RP-18/water-methanol) and HPLC (BDS C-18/water-methanol) techniques and their results confirmed log k_w, R_{M0} and s parameters as alternative lipophylicity descriptors for structurally similar compounds. The relationships between fungicidal activity and experimental lipophylicity indicated the possibility of reversed-phase liquid chromatography to choose compounds suitable for microbiological investigations.

4. Gradient RP HPLC

Important disadvantage of isocratic liquid chromatography application in lipophylicity studies is connected with the time of analysis. This technique usually requires preliminary measurements to establish optimal separation conditions; the range of organic modifier concentration should be wide enough to obtain accurate retention parameters; for more lipophylic substances and long retention times the measurements are much extended. Shortening chromatographic columns or variations of effluent flow rates can partially solve this problem. Another advantages offer planar techniques: the possibility of simultaneous measurements for many substances and, in the case of OPLC applying the forced-flow, really shortened analysis time. But the most attractive solution is gradient elution for the high throughput estimation of lipohylicity parameters.
For a linear solvent strength gradient in RPLC the retention time (t_g) is related to isocratic parameters by the following equation (Gocan & Cipman, 2005):

$$t_g = C + \left(\frac{t_M}{b} \right) \log k_o \tag{18}$$

where t_g is a gradient retention time, t_M is the column hold-up time, b is a gradient steepness parameter, log k_o is the isocratic retention parameter for the system at the start of the gradient, C is complex system constant.
Valkó et al. (Valkó et al., 1997; Du et al., 1998) using fast gradient liquid chromatography introduced chromatographic hydrophobicity index (CHI) as an independent measure of hydrophobicity which can be correlated with log P. CHI is an extension of the concept of lipophylicity parameter φ_o (equation 15) to gradient elution. CHI can be evaluated from the following formula:

$$CHI = At_g + B \tag{19}$$

Parameters A and B are characteristic for given gradient system and can be evaluated for a series of standards with experimentally determined gradient retention times t_g and isocratic values of φ_o. Equation 19 allows to calculate CHI parameter for any compound with measured gradient retention time t_g.

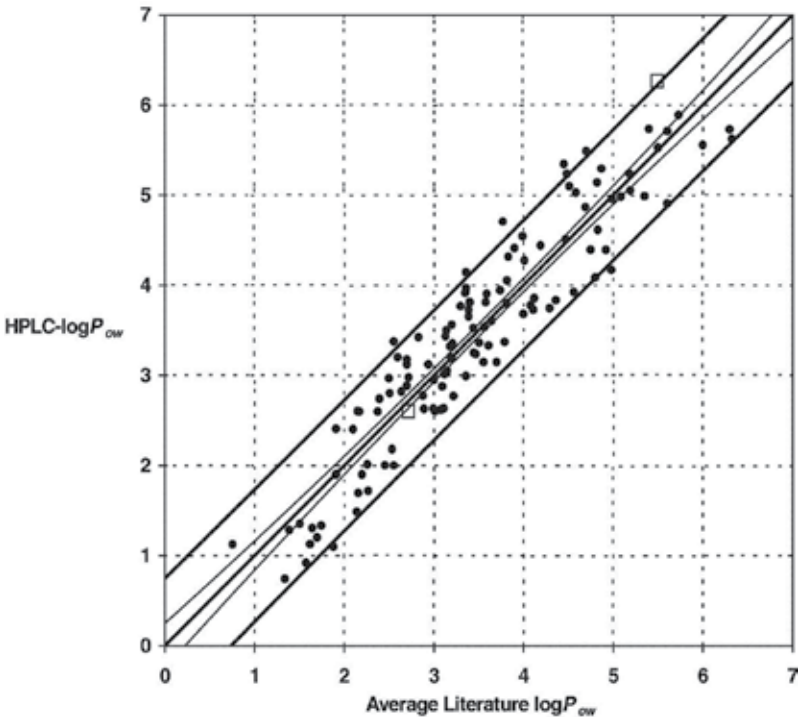

Fig. 2. Average literature log P_{ow} values compared to the HPLC log P_{ow} values. Ninety-percent confidence and prediction intervals displayed. Toluene and triphenylene used as the internal standards. (Donovan & Pescatore, 2002).

Kaune et al. (Kaune et al., 1998) used gadient HPLC studying s-triazine herbicides and some of their degradation products on RP-18e (LiChrospher) stationary phase with non-linear water-methanol gradient. Though they observed the linearity between retention factors and literature partition coefficient, especially for higher values, better results were obtained for isocratic HPLC with n-octanol coated column and buffered water saturated with n-octanol as the mobile phase. Verbruggen et al. (Verbruggen et al., 1999) also compared isocratic and gradient HPLC in measurements of lipophylicity of organic micropollutants (including pesticides). They used C-18 (Chrompack) column and water-methanol as the mobile phase. In isocratic elution methanol concentration in the effluent was in the range form 80% to 100% (with 5% step) while in linear gradient elution from 50% to 100%. The results showed linear correlations between chromatographic lipophylicity parameters and partition coefficients calculated from molecular structures of tested substances.
Forgács and Cserháti (Forgács & Cserháti, 1998) used gradient HPLC on home-made PEE_{sil} (polyethylene-coated silica) column and water-methanol as mobile phase to evaluate lipophylicity of twenty seven commercial urea pesticides. Linear extrapolated log k_w and s parameters were applied as lipophylicity descriptors and analysed by the use of PCA.

According to results obtained by Forgács and Cserháti, biological activity of pesticides tested cannot be predicted by their behaviour or lipophylicity parameters.
Donovan and Pescatore ((Donovan & Pescatore, 2002) proposed polymer stationary phase ODP (octadecyl-poly(vinyl alcohol) to determine lipophylicity for a group of 120 bioactive substances, among them 74 commercial fungicides, herbicides and insecticides, using linear gradient from 10 to 100% methanol in buffer. Chromatographic parameters (HPLC-log P_{ow} and retention time) were compared with literature log P_{ow} values (Figure 2) as well as with calculated log P_{ow} giving very good results. The authors established the method as a simple, quick, versatile and inexpensive due to: quick gradient and short column (20x4.0 mm). The additional advantage is the possibility of work at any given pH value in a wide range between 2 and 13.

5. Micellar liquid chromatography

Micellar liquid chromatography MLC is a mode of conventional RPLC using surfactant solution above critical micellization concentration (cmc) as the mobile phase (Medina-Hernándes & Sagrado, 1995; Garcia-Alvarez-Coque et al., 1997). The presence of micelles in the mobile phase provides a variety of interactions: solute association with the polar head of the surfactant, solute penetration into the micelle core, adsorption of surfactant monomers on the alkyl-bounded stationary phases as a result of hydrophobic interactions between surfactant tail and alkyl chain, and solute interactions with adsorbed surfactant and alkyl chains. The presence of organic modifier influences these interactions - it changes polarity of the bulk phase and cmc. Solvent which can solvate stationary phase reduces the amount of adsorbed surfactant. In such system the retention of solute is governed by three different equilibria: solute distribution between the micelle and the bulk phase, solute partition between the stationary phase and the bulk phase and the direct transfer of solute molecules between surfactant-modified surface and the micelle. The last equilibrium is significant in the case of highly non-polar solutes. MLC is very attractive analytical technique due to the low cost and nontoxicity of surfactant unique selectivity, compatibility of mobile phases with salts and water-insoluble compounds, short equilibrium times in gradient elution the possibility of direct application of biological samples.
Molecular interactions involving solute depend on its lipophylicity and micellar retention parameters and are the source of important information (Medina-Hernándes & Sagrado, 1995; Foley, 1990). The simplest theoretical retention model in MLC, used in lipophylicity studies, is based on the following relationship:

$$\frac{1}{k} = \frac{1}{k_m} + \frac{K_{AM}}{k_m}[M] \tag{20}$$

where [M] is a total concentration of surfactant in the mobile phase, K_{AM} is the constant describing solute-micelle binding, and k_m is solute retention parameter at zero micellar concentration, i.e. at surfactant monomer concentration equal to cmc. K_{AM} and k_m parameters can be evaluated form the slope and intercept of experimental 1/k vs. [M] relationships determined for three different surfactant concentrations in the mobile phase. In micellar chromatography k, k_m and K_{AM} parameters, or the logarithms of these values, are used as lipophylicity descriptors and are correlated with log P coefficients. The k_m parameter is considered to be analogical to k_w index in reversed-phase chromatography.

Various workers described the usefulness of micellar chromatography for the determination of lipophylicity of different substances (Medina-Hernándes & Sagrado, 1995; Garcia-Alvarez-Coque et al., 1997; Ruiz-Angel et al., 2004) and some of them observed linear relationships between micellar and partitioning parameters or chromatographic lipophylicity parameters while another reported the curvature of log k vs. log P plots.

Escuder-Gilabert et al. (Escuder-Gilabert et al., 2003) distinguished biopartitioning micellar chromatography BMC, a special case of MLC constituted by C-18 stationary phase and a polyoxyethylene (23) lauryl ether (Brij35) as a surfactant component of the effluent. BMC is helpful in describing the biological behaviour of different kinds of xenobiotics and a main micellar chromatography technique applied in the measurements of various pesticides. This technique was used to predict ecotoxicity of different organic pollutants (also pesticides) (Escuder-Gilabert et al., 2001). Retention parameters measured for buffered 0.06 M Brij35 (pH 7.4) were correlated to literature ecotoxicity parameters giving quadratic relationships and afterwards used in QSARs studies.

Bermudez-Saldaña et al. (Bermudez-Saldaña et al., 2005a) using correlated micellar retention parameters of 85 commercial pesticides with fish toxicity and obtained results allowed to classify studied pesticides as harmful, toxic and very toxic. The same authors (Bermudez-Saldaña et al., 2005b) studied another 85 pesticides from six chemical families, using buffered effluent (pH 7.0). Micellar parameters log k measured at 0.04 M Brij35 as the mobile phase, proved to be efficient factors in representing the bioconcentration process in fish. Ma et al. (Ma et al., 2006) applied BMC to evaluate micellar log k parameters of 79 heterogenous pesticides used subsequently in QSPR studies.

Biopartitioning micellar chromatography and micellar elektrokinetic chromatography MEKC techniques were compared in the measurements of lipophylic properties of phenoxy acid herbicides (Martin-Biosca et al., 2001). Evaluated retention parameters seemed to be capable of describing and predicting in vitro the toxicity of substances tested.

Modified MEKC, so-called microemulsion electrokinetic chromatography MEEKC, was used by Klotz et al. (Klotz et al., 2001) for the indirect measurements of log P values of over 80 different pesticide compounds. Retention parameters were compared with literature log P values giving very good linearity. Another modification of MEKC is vesicle electrokinetic chromatography VEKC applied by Klotz et al. (Klotz et al., 2002) for rapid estimation of lipophylic properties of different compounds - among them 43 pesticides. Very high linearity (R^2=0.968) between retention parameters and literature log P values showed this technique to be very effective in such investigations.

Until now planar techniques with micellar mobile phases are not frequently used for lipophylicity measurements (Shtykov & Sumina, 2002). In our investigations (Janicka & Pietras-Ożga, 2010) micellar TLC and OPLC techniques were compared in studying lipophylic properties of eight newly synthesized acetamides applying RP-18W stationary phase and water/Brij35/tetrahydrofuran as the mobile phase. As lipophylicity parameters were proposed micellar retention factors log k and log k_m, which were correlated with log P values calculated form molecular structures. Excellent correlations between log k_m and log P parameters (R=0.917-0.987) for OPLC results were observed and the results allowed evaluating micellar OPLC as suitable technique – very quick and trustworthy in prediction of lipophilic properties of new compounds. (see Table 3).

log P	TLC		OPLC	
	k_m	log k_m	k_m	log k_m
IAlog P	0.847	0.866	0.946	0.955
clog P	0.937	0.952	0.918	0.925
KowWin	0.938	0.961	0.908	0.917
Alog P_s	0.755	0.768	0.927	0.937
AClog P	0.772	0.789	0.930	0.939
AB/log P	0.950	0.961	0.957	0.957
milog P	0.790	0.813	0.938	0.953
Alog P	0.781	0.801	0.933	0.944
Mlog P	0.777	0.795	0.938	0.953
xlog P_2	0.792	0.814	0.938	0.953
xlog P_3	0.898	0.914	0.914	0.931
log $P_{average}$	0.873	0.893	0.975	0.987

Table 3. Correlation coefficients (R) obtained for k_m (log k_m) vs. log P relationships. (Janicka & Pietras-Ożga, 2010).

6. BioArena – the method of studying biological activity

Different parameters determined by reversed-phase liquid chromatography, both column and planar, are correlated with biological activity of commercial pesticides or newly synthesized compounds with potential biological significance. The results obtained by various workers confirm the importance of chromatography in studying lipophylicity or biological activity of pesticides and column and planar chromatography reveal their advantages and limitations. Apart from these applications planar chromatography is a unique technique enable to study the mechanism of action of different bioactive compounds. BioArena, a complex system of chromatographic and biological investigations, creates such possibilities.

BioArena was invented and developed by Hungarian scientists led by E. Tyihák (Tyihák et al., 2003; Tyihák et al., 2004b). This complex analytical system was preceded by extensive investigations on enzymatic methylation-demethylation processes and the role of formaldehyde HCHO in the biosphere. The studies demonstrated that formaldehyde is an endogenous component of all biological units and that there is a primary HCHO cycle in this system. Formaldehyde is an indispensable component of all cells and its reaction products play a fundamental role in different biological processes (Ahmad, 1995: Tyihák et al., 1998a; 1998c). The scientists introduced the term "formaldehydome" (Tyihák et al., 1998b; 2005; Tyihák et al., 2008b) which means: "the complete set of HCHO-cycle mediated and non-mediated HCHO pathways of a given biological unit (including the HCHO cycle itself) as the most important HCHO pathway" and being the part of the biological system." BioArena can aid understanding the mechanism of toxic action of different bioactive compounds - drugs or poisons. It's a combination of bioautography (Botz et al., 2001) and planar chromatography techniques (TLC, HPTLC or OPLC) making possible to examine the

mechanism of toxicity of substances tested directly on the plate, after chromatographic separation. The basic elements of BioArena are presented on Figure 3 (Tyihák et al., 2004b).
Bioautography, the first step of the system, is a very sensitive and selective method for visualization of bioactive compounds after chromatographic separation. Microorganisms can be easy applied to the plate, by immersion or spraying, and the inhibition or promotion of growth in the spots can be visualized by dying living cells. As the next stage post-chromatographic investigations are performed: in the adsorbent bed are possible interactions between microbes and co-factor molecules added to the culture medium (Tyihák et al., 2003; 2004b). Co-factors added to the cell culture influence formaldehyde level: capture compounds (e.g. L-arginine or glutathione) decrease HCHO level while promoters (e.g. Cu^{2+} ions) enhance the antibacterial activity of HCHO (Tyhák et al., 2004a). BioArena makes possible not only the arrangement of all processes on the layer, but also visual and spectroscopic evaluation of the results and even extending the monitoring time for several days (Tyhák et al., 2004b; Tyhák et al., 2005).
BioArena was used to investigate the mechanism of biological action of different substances such as trans-resveratrol and chamomile extracts (Tyhák et al., 2008a), aflatoxins (Móricz et a., 2007; 2008a; 2008b), paclitaxel (Tyhák et al., 2008b), ascorbigens (Kátay et al., 2009).

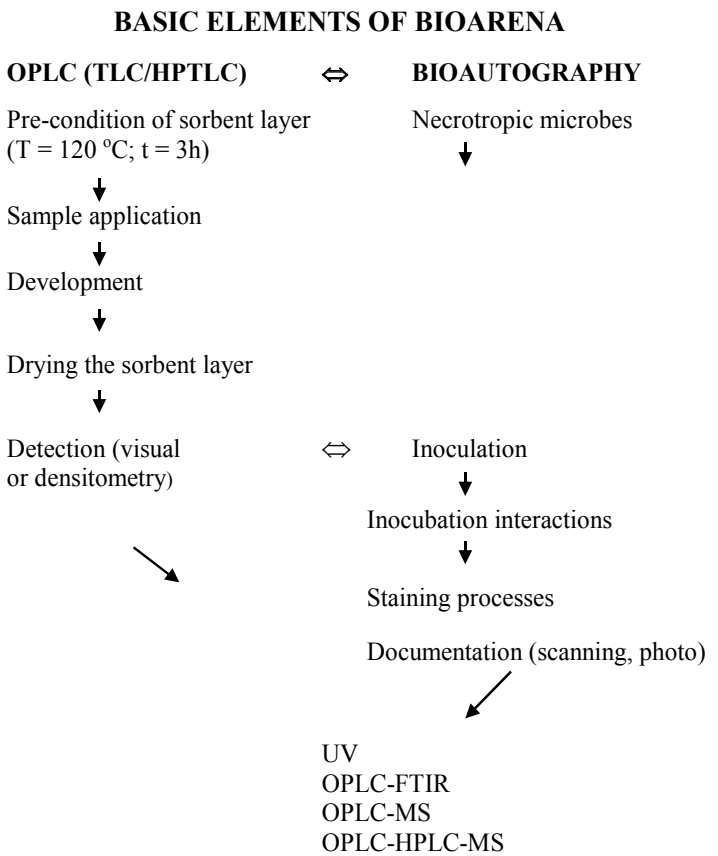

Fig. 3. Combination of off-line OPLC with bioautography and interactions. (Tyhák et al., 2004b).

In our investigations BioArena system was applied for studying the mechanism of action of 13 newly synthesized potential herbicides - acetamides and chlorophenoxy derivatives (Janicka et al., 2008). At the end of chromatographic separation the plates with substances tested were dried and immersed in the bacterial suspensions. Bacterial biotest was conducted on Pseudomonas savastanoi pv. Phaseolicola race 6, which causes halo blight on beans. After bioauthography detection five substances demonstrating toxicity against Pseudomonas, were chosen for further investigations. Plates with these substances were immersed into bacterial suspensions enriched in L-arginine or reduced glutathione (formaldehyde captures) or Cu^{2+} ions (formaldehydr promoter). After incubation dry plates were visualized by immersing for 5 minutes in aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Triton-100. Living bacterial cells cause the plate violet color while bright spots (killed bacteria) confirm toxicity of given compound against Pseudomonas.

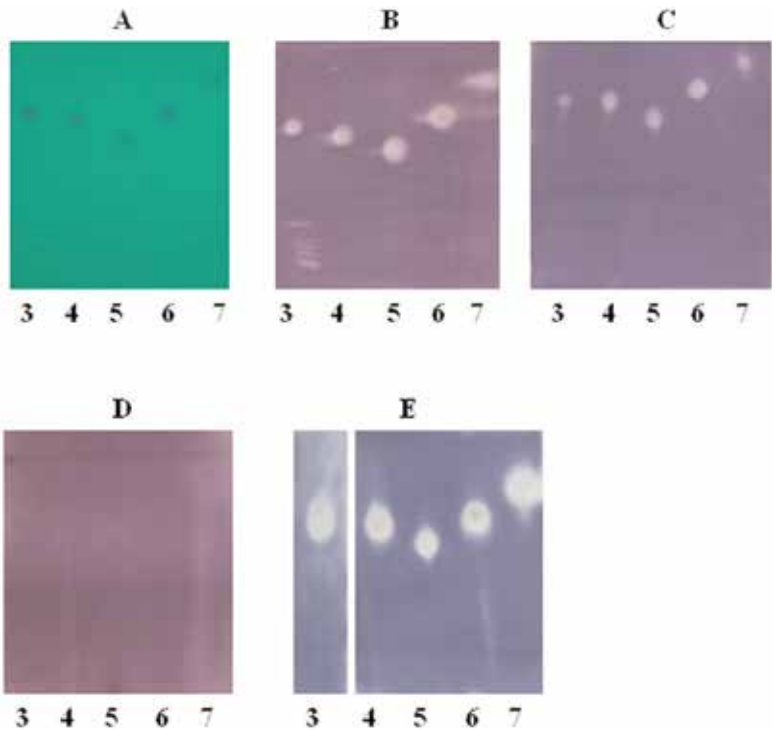

Fig. 4. Solutes 3–7 developed with heptane–1,2-dichloroethane before (A) and after (B) biological detection, and the effects of L-arginine (C),glutathione (D), and Cu2+ ions (E) on the antibacterial activity of the compounds. (Janicka et al., 2008).

The photos presented on Figure 4 and densitograms on Figure 5 show that L-arginine reduced while glutathione completely inhibited antibacterial activity of the substances. The effect of Cu^{2+} ions is also unambiguous: copper ions dramatically increase toxicity. These phenomena are visible only in the spots, not on the whole plate, and must be connected with compounds tested. The investigations confirm the mechanism of toxic action as connected with HCHO cycle and formaldehydome system. In the study biochemical informations were correlated with chromatographic lipophylicity parameters log k_w calculated by numerical method from TLC measurements.

The BioArena system is convenient, inexpensive and easy to do experimental method for studying different bioactive compounds, especially newly synthesized, and is a real alternative to animal experimentation.

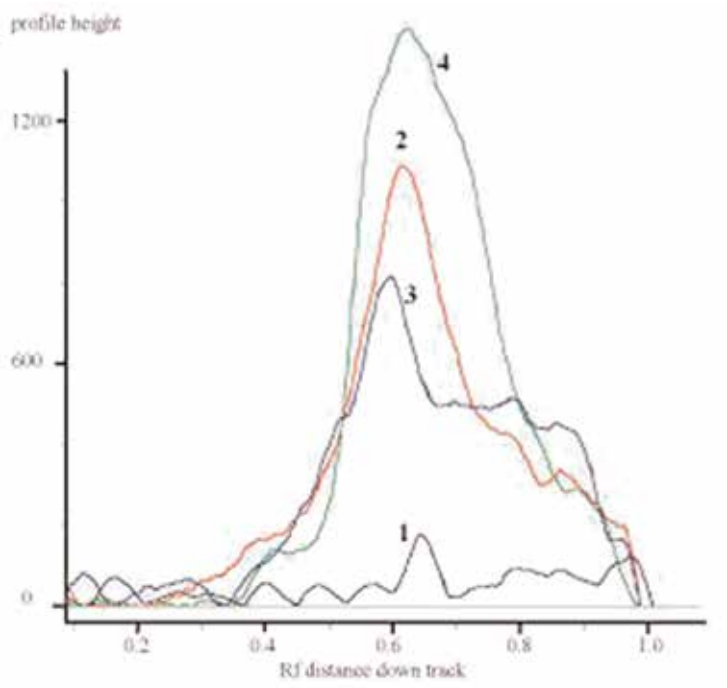

Fig. 5. Densitograms obtained from solute no. 1 before (trace 1) and after (trace 2) biological detection, and the effects of L-arginine (trace 3) and Cu2+ ions (trace 4) on the antibacterial activity of the compound. (Janicka et al., 2008).

7. Conclusions

Liquid chromatography, at the first place very effective separation technique, is more and more often accepted in different outside separation applications. Reversed-phase liquid chromatography is very useful and reliable in studying lipophylic properties of different bioactive compounds like pesticides. The advantages of chromatographic evaluation of lipophylic properties of such substances are beyond dispute. In Table 3 there are summarized applications of different RP LC techniques, i.e. column with isocratic and gradient elution and planar chromatography, as well as techniques applying micellar mobile phases, from last two decades, related to pesticides – commercial and newly synthesized. In the case of new substances being at the stage of investigations and with potential significance in industry or agriculture, chromatographic techniques seems to be especially attractive. They are the source of interesting and valuable informations about solute lipophylicity, structural properties, partition or even biological activity.

Though chromatographic lipophylicity indices are recognized as standard parameters characterizing new bioactive substance, they are not universal. In chromatographic measurements various stationary and mobile phases are used and systematical studies could produce a large chromatographic lipohylicity database similar to log P values.

So far micellar techniques, very attractive in lipophylicity studies due to simplicity and short analysis time, are not very popular in studying pesticides.

Special attention should be paid to BioArena, the system combining separation with biological investigations. BioArena was with great success used to investigate different active substances but so far only occasionally for pesticides.

compounds investigated	**chromatographic system**	**Ref.**
isocratic reversed-phase column chromatography RP HPLC		
herbicides	RP-18/water-methanol	Braumann et al., 1983
pesticides	RP-18/water-methanol	Hsieh and Dorsey, 1995
herbicides, fungicides insecticides,	RP-18/water-methanol	Cserháti & Forgács, 1997
herbicides, fungicides, growth regulators	RP-18/water-methanol	Zhang et al., 2000
herbicides, fungicides, acaricides, insecticides	alumina/n-hexane-dioxan	Cserháti & Forgács, 1994
urea pesticides	RP-18/water-methanol	Pyka & Miszczyk , 2005
herbicides	RP-18/water-acetonitrile	Braumann et al.,1983
pesticides	RP-18/water-methanol	Verbruggen et al., 1999

compounds investigated	**chromatographic system**	**Ref.**
herbicides, fungicides, insecticides	soil/water-methanol	Xu et al. [31]
pesticides	ODS, RP-CN, soils/different effluents	Bermúdez-Sadaña et al. 2006
phenylureas	C-18-PS-ZrO_2/water-methanol RP-18/water-methanol	Xia et al., 2005
carbamates	RP-18/water-methanol (1:1, v/v)	Yamagami & Katashiba, 1996
biphenyls	RP-18/water-methanol (4:1, v/v)	De Kock & Lord, 1987
s-triazines	RP-18/water-methanol RP-18/water-acetonitrile	Janicka et al., 2006
thiobenzanilides	different systems	Niewiadomy et al., 1998 Jóźwiak, 2000 Jóźwiak et al., 2006
acetanilides	RP-18/water-methanol	Djaković-Sekulić et al., 2001
triazoles	RP-18/water-methanol	Perišić-Janjić et al., 2000
triazoles	RP-18/buffered dioxane (pH 3.5)	Sztanke et al., 2008
thiazoles	RP-18/buffered methanol (pH 4.0 and 7.4).	Matysiak et al., 2004
thioamides	RP-18/water-methanol	Kostecka et al., 2005
s-triazines	n-octanol coated column/ buffer saturated with n-octanol	Kaune et al., 1998
gradient reversed-phase column chromatography		
s-triazines	RP-18e/water-methanol	Kaune et al., 1998
pesticides	RP-18/water-methanol	Verbruggen et al., 1999
pesticides	PEE_{sil}/water-methanol	Forgács & Cserháti, 1998
fungicides, herbicides, insecticides	ODP/buffered methanol	Donovan & Pescatore, 2002

compounds investigated	**chromatographic system**	**Ref.**
reversed-phase planar chromatography		
herbicides, fungicides insecticides, herbicides	RP-18/water-methanol	Darwish et al., 1993
fungicides, growth regulators	RP-18/water-methanol	Zhang et al., 2000
herbicides, fungicides, acaricides, insecticides	alumina/n-hexane-dioxan	Cserháti & Forgács, 1994
urea pesticides pesticides	RP-18W/water-methanol soil/water-methanol	Pyka & Miszczyk, 2005 Ravanel et al., 1999
pesticides	silica, soil, mixed layers/different effluents	Mohammad et al., 2001
s-triazines	different systems	Perišić-Janjić et al., 2001; 2003a; 2003b; 2005
s-triazines	RP-8 and RP-18/different effluent	Janicka et al., 2004
thiobenzanilides	different systems	Janicka et al., 2000; 2006 Matysiak et al., 1999
acetanilides	starch, cellulose/different effluents	Djaković-Sekulić et al., 1987
triazoles	silica gel impregnated with paraffin oil/water-methanol	Perišić-Janjić et al. 2000
thiazoles	RP-8 and RP-18W/different effluents	Matysiak et al., 2002
acetamides	RP-8/water-methanol	Cipman et al., 1998
acetamides	silica gel impregnated by paraffin oil/different effluents	Djaković-Sekulić et al., 2002
acetamides	RP-18/different effluents	Perišić-Janjić et al., 2007
acetamides	RP-18/buffered methanol (pH 5.3, 7.3 and 8.3)	Janicka et al., 2004
thioamides	RP-18/water-methanol	Kostecka et al., 2005

compounds investigated	**chromatographic system**	**Ref.**
liquid chromatography with micellar mobile phases		
pesticides	BMC: RP18/buffered Brij35 (pH 7.4)	Escuder-Gilabert et al., 2001
pesticides	BMC: RP18/buffered Brij35 (pH 7.0)	Bermudez-Saldaña et al., 2005a
pesticides	BMC: RP-18/water-Brij35	Ma et al., 2006
herbicides	BMC: RP-18/ water-Brij35	Y. Martín-Biosca et al., 2001
acetamides	TLC MLC: RP-18W/ water-Brij35-tetrahydrofuran	Janicka & Pietras-Ożga, 2010
pesticides	MEEKC	Klotz et al., 2001
pesticides	VEKC	Klotz et al., 2002

Table 3. Application of different liquid chromatography techniques in lipophylicity measurements of different commercial and newly synthesized pesticides

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Pesticide Immunoassay

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1. Introduction

Pesticides are used globally for enhancing crop yields. However, their excessive use/misuse, especially in the developing countries, results in widespread food and environmental contamination. The presence of pesticide residues in food and environment has posed a serious threat to human health and caused a great concern. In order to keep human from being affected, analytical and monitoring system of pesticide residues in food and environment must be developed. Conventional methods employed to detect/analyze the pesticide residue are chromatographic techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC), which are time consuming and require sophisticated equipment only available in well-equipped laboratories. In addition, the conventional methods usually require a lot of complex pre-treatment of samples. Therefore, convenient and rapid pesticide detection system is urgently needed. Immunoassay (IA) technology is such an analysis system with simple, rapid and cost-effective characteristics and widely used in pesticides detection. Thus, the topic of "pesticide immunoassay" will be introduced in this chapter.

An "immunoassay" is a quantitative or qualitative method of analysis for a substance which relies on an antibody (Ab), or mixture of antibodies, as the analytical reagent. Antibodies are a class of proteins with the unique ability to bind with high specificity to one or a very limited group of molecules. A molecule that binds to an antibody is called an antigen (Ag). In addition to binding specificity, another important feature of immunoassays is its ability to produce a measurable signal in response to a specific binding. Most immunoassays today depend on the use of an analytical reagent that is associated with a detectable label, such as radioactive elements, enzymes and so on.

Immunoassay has a rather long history and has become a widely accepted technique, particularly in the clinical area. Beginning in the middle 1950's, Berson and Yalow were investigating the disease, diabetes. To test the hypothesis that diabetic individuals eliminated insulin too rapidly, they injected radio-labelled insulin into normal and diabetic subjects, and found that the radio-labelled insulin was actually cleared more slowly in diabetics. Then, they begin to experiment with combinations of labelled and unlabeled insulin, and found the phenomenon of competition between labelled and unlabeled insulin. Using the same principles, they described the first radioimmunoassay and this work was considered as the beginning of modern immunoassay (Herzog, 1997).

The use of enzymes for labels in immunochemical reactions dates back to the mid-sixties when enzyme-labelled antibodies were applied to the identification and localization of

antigens in histological preparations. The use of enzyme labels in an immunoassay was first published in 1971 by a Dutch group ((Herzog, 1997). Although a large variety of other labels (such as fluorescent, phosphorescent, and chemiluminescent dyes) have been applied in immunoassay over the past decades, the enzyme immunoassay (EIA) was still the most popular one.

The application of immunoassays to pesticide residue analysis dates back to 1971, when Ercegovich *et al.* (1971) described their early work on the detection of pesticides by immunochemical means and indicated the potential usefulness of this method for routine analyses. Following Ercegovich's work, more and more researchers paid their attention to pesticide immunoassay, and this was confirmed by the related publications (Figure 1). At the same time, the American Chemical Society (ACS) had its first symposium on pesticide immunoassay in 1989 during its national meeting. In addition, the Association of Official Analytical Chemists (AOAC) held its first workshop on immunoassay in 1990, and offered an immunoassay workshop at its national meeting again in 1991(Herzog, 1997).

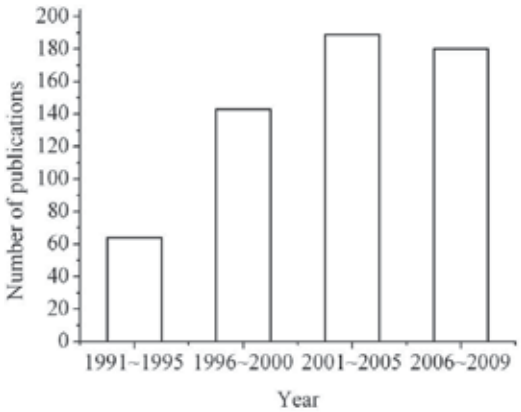

Fig. 1. Number of publications in the topic of "pesticide immunoassay" from 1991 to 2009 (data from "web of knowledge").

2. Principle of immunoassay

As stated, an immunoassay is a quantitative or qualitative technique that depends on the reaction between the molecule of interest, "the antigen", and a complementary molecule, "the antibody". An antigen is a molecule that can be recognized by the immune system (immunogenicity) and bound specifically to an antibody (reactogenicity). Molecules with both immunogenicity and reactogenicity are called as "complete antigens"; while molecules that only possess reactogenicity are defined as "incomplete antigens" or "haptens". Pesticide molecules are typical haptens that can induce the immune system to produce antibody only when attached to a large carrier such as a protein. Antibody is the key regent of immunoassay, and can be produced by animal immunization, hybridoma technique or recombinant antibody technique. As to the specific procedure of immunoassay, many kinds of immunoassay format have been developed. However, the competitive immunoassay was the most frequently used one in pesticide immunoassay. In this section, the principle of immunoassay for pesticides detection will be introduced, including the immunogen preparation, antibody production and relevant immunoassay formats.

2.1 Immunogen preparation

The first step of pesticide immunoassay system development was to design and prepare a rational immunogen for antibody production. The typical procedure to prepare immunogen in pesticide immunoassay system development was to link the pesticide molecules to a carrier protein by a coupling spacer (linker) (Figure 2). The site of coupling to the carrier, the length of coupling spacers, the selection of optimized carriers, the coupling procedure as well as the number of haptens bound to one carrier molecule can be of major importance for the sensitivity and selectivity of the resulting antibody (Dankwardt, 2001).

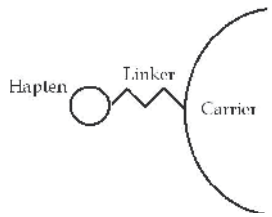

Fig. 2. Sketch map of the immunogen for pesticide specific antibody production.

The desired haptens should be one that hapten-carrier conjugates can induce specific immune response and produce high quality hapten-specific antibodies. While designing/selcting haptens, the most important thing is to ensure the final chemical structure and stereochemistry to be identical or similar with the original haptens (analytical targets). If haptens contain active groups such as -COOH, -NH_2 and -OH, they can be directly coupled with the carrier proteins. Otherwise, derivatives of the haptens should be prepared to introduce reactive groups into the structure. In addition, the haptens themselves should possess complicated chemical structures. Generally, most of these desired haptens are characterized by the following aspects: (1) amino group or carboxyl group or both; (2) aromatic compounds; (3) high branch; (4) heteroatom rings (Tong *et al.*, 2007).

Another very important factor is the length of the spacer. If it is too long, the haptens can overlap along the spacer and change their stereo-structures. If it is too short, the carrier protein can cover the hapten and can not produce specific antibody. In addition, the spacer should be non-polar, or, they can change the distribution of the electric density of the hapten (Dankwardt, 2001).

The carrier protein is not only to simply increase the molecular weight of the hapten-carrier conjugate; it can also affect the quality and quantity of immune responses. Bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), and human serum albumin (HSA) are usually used as carriers for the preparation of immunogen. Among these proteins, BSA is the popular one because its physical and chemical stability is higher, no expensive, easily available, more lysine residues and more amino groups. In addition, BSA can also present excellent solubility under various pH value and ionic strength and react with the targeted haptens in organic solvents such as pyridine and N, N-dimethylformamide (DMF) (Tong *et al.*, 2007). Generally, the carrier should be heterogenous with the experimental animals, since it is easier to induce strong immune response and to produce high-titer and hapten-specific antibody.

Linking the desired haptens to the carriers is the critical step in immunogen preparation. If the haptens possess active groups such as –COOH, -OH, and -NH_2, as described above, they can directly react with the carrier protein, otherwise, structural modifications are required. Based on the chemical and stereo-structure of haptens, various synthetic approaches were

employed: (1) Carboxyl-containing haptens can be coupled with the carrier proteins using N-hydroxysuccinimide active ester/carbon-diimine or Woodward reagent protocol. (2) Amino-containing haptens can be linked to carriers by employing glutaraldehyde, diisocyanate, halo-nitrobenzene, hiophosgenation, diimine ester, or diazotization protocol. (3) Hydroxyl-containing haptens can be directly connected to the carrier proteins through succinic anhydride or azobenzoic acid protocol. (4) Carbonyl-containing haptens (ketone or aldehyde) are usually connectd to carriers using amino-ox-acetic acid protocol. (5) Homogeneous or heterogeneous difunction reagents can be used to synthesize the immunogen for mercapto-containing haptens (Tong *et al.*, 2007). The general principles of some frequently-used immunogen syntheitc method were outlined in Figure 3.

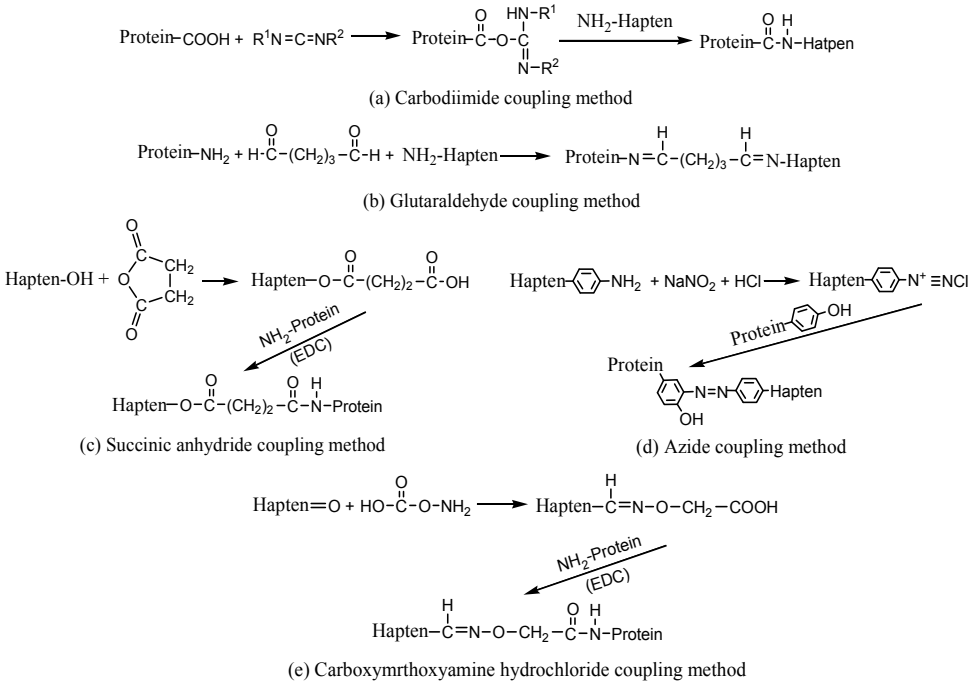

Fig. 3. General principles of some frequently-used immunogen syntheitc method (modified after "Tong *et al.*, 2007").

After the artificial hapten-protein conjugate is prepared, a purification step is usually proceeded, since the unreacted hapten molecules, salts and other impurity affect the quality of antibody. Usually, dialysis and chromatography are employed. Comparing the two techniques, dialysis takes long time (usually 2 days or more). However, it can obtain well purified conjugate and the process is simple, which is suitable in various laboratories. Chromatography such as ion-exchange gel chromatography, gel chromatography needs sophisticated equipments and the process is complicated. Anyway, how to select the best purification technique and the specific process is dependent on the substrates. After purification, it is necessary to carry out an identification procedure with two purposes to confirm whether the desired haptens have been successfully connected on the carriers and how many haptens have been bound to one carrier molecule. The most popular technique employed to identify artificial antigen is UV spectrometry.

As to the issue concerns the optimal number of haptens bound to the carrier protein (i.e. optimal epitope density), highly substituted carriers usually lead to better results. For bovine serum albumin (BSA) molar ratios of 10 : 1 to 20 : 1 (hapten : carrier) are desirable; for larger molecules such as hemocyanin, ratios of 800 : 1 to 1000 : 1 should be obtained. However, very high ratios may reduce immunogenicity because of either the changes in tertiary structure of the protein caused by masking of the essential free amino groups or the removal of critical determinant sites on the carrier by haptenic blocking (Dankwardt, 2001).

2.2 Antibody production

2.2.1 Antibody structure

Immunoassay is based upon the specific reaction between an antibody and its corresponding antigen. Antibodies are serum glycoprotein of the immunoglobulin class (Ig) produced by the immune system against foreign material such as pathogens or xenobiotics, and bind the target substance with high selectivity and affinity. Although there are five distinct classes of antibody in most higher mammals (IgA, IgD, IgE, IgG, IgM), IgG accounts up approximately 80% of the total Ig in human serum. In reality, most immunoassay systems rely upon IgG as the major antibody.

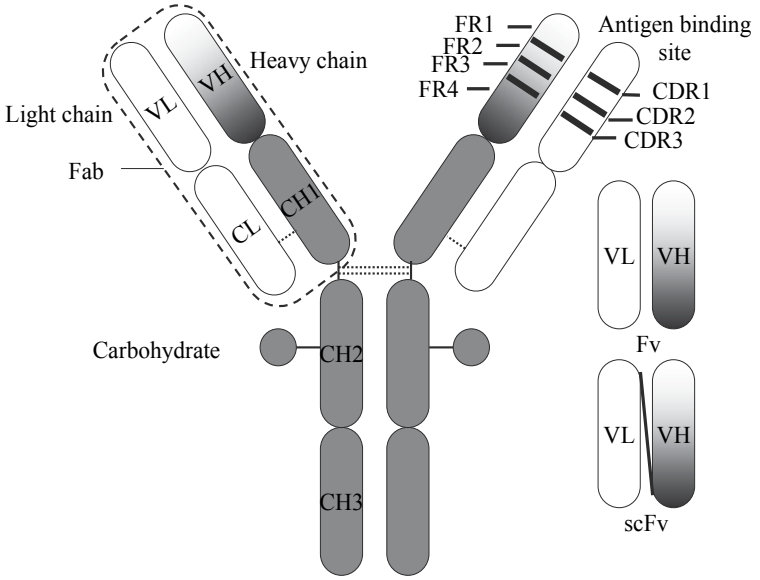

Fig. 4. Structure of antibody and its fragments (modified after "Dankwardt, 2001").

The basic structure of an Ab molecule is shown in Figure 4. It consists of two identical heavy (H) chains and two identical light (L) chains stabilized and linked by inter- and intra-chain disulfide bonds. The H- and L-chains are organized into variable and constant regions. The antigen binding site (combining site) is formed by the association of parts of the variable regions of the H- and L-chains, located at the amino terminal end. The variable regions of both chains are organized into three hypervariable or complementary determining regions (CDRs) separated by four framework regions. The greatest amino acid sequence variation occurs within the CDRs whereas the framework regions are more conserved. It is assumed that the association of the CDR regions forms the combining site. The lower part of the

molecule contains the last heavy chain domains (crystallizable fragment, Fc) which is responsible for some important biological effector functions such as complement fixation and is not necessary for antigen binding. The whole of the Ig molecule or antibody fragments, F(ab)2 and Fab (antibody fragments containing the antigen binding site(s)) can be used in immunoassay. Moreover, recent research results exhibited that the recombinant antibody fragment, scFv (single-chain Fv fragment) can form intact antigen binding site and can also used for immunoassay.

2.2.2 Polyclonal antibody production

Antibody production is conveniently carried out in warm blood animals, e.g. rabbits, sheep, mice or chickens. In this procedure, polyclonal antibodies (pAbs) are obtained from the serum and comprise a mixture of different antibody populations.

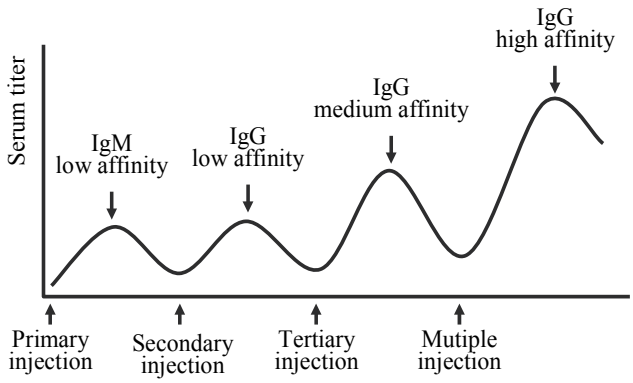

Fig. 5. Kinetics of a typical immune response (modified after "Hefle *et al.*, 2006").

Rabbits are the species almost exclusively used for pesticide pAbs production, principally because they are of a size capable of producing adequate amounts of antibodies, have a relatively long life span, and are relatively easy to handle. Usually, adjuvant is used to improve the immune response to the immunogen. After emulsifying the immunogen by adjuvant, this combination can be injected subcutaneously on the back in at least 4 sites per rabbit with a maximum of 0.25ml in each site and with the total not to exceed 1ml. The immunization schedule has a decisive influence on the result, and must be rationally designed. Specific recommendations for the interval between primary and booster immunizations are usually not cited. In general, a booster can be considered after the antibody titre has plateaued or begun to decline and 2 to 3 weeks is recommended between each booster injections. In most cases, the endpoint of pAb production should be judged when the antibody titre has reached an acceptable level. This should usually occur after 3 to 5 boosters (Figure 5). Then, the blood of the immunized rabbit can be collected, and serum can be separated for immunoassay development. (Leenaars *et al.* 1999)

2.2.3 Monoclonal antibody production

Monoclonal antibodies (mAbs) consist of a single monospecific antibody population. These antibodies are produced in cell culture by a single hybridoma cell derived from the fusion of B-lymphocytes with myeloma cells. The hybridoma cells can then be propagated almost indefinitely in culture and will continue to produce the antibody of the lymphocyte parent.

Since an individual lymphocyte produces only a single antibody type, all of the antibody molecules produced by a hybridoma cell line derived from a single hybrid cell are identical and have the same binding properties. Therefore, the hybridoma technology guarantees the unlimited production of mAbs with constant characteristics.

Figure 6 outlines the protocol of mAbs production. In general, mice are used as B-lymphocyte doner for mAbs production. Mice are immunized every 2-3 weeks till a sufficient antibody titer is reached in serum, then immunized mice are euthanized and the spleen removed to use as a source of cells for fusion with myeloma cells. Next, single spleen cells from the immunized mouse are fused with the previously prepared myeloma cells. Fusing antibody-producing spleen cells, which have a limited life span, with cells derived from an immortal tumor of lymphocytes (myeloma) results in a hybridoma that is capable of unlimited growth. After that, hybridoma cells are followed by selection for antigen binding. Then the selected cell line can be used for mAbs production under in vivo or in vitro procedures or combinations thereof. (Marx *et al.*, 1997)

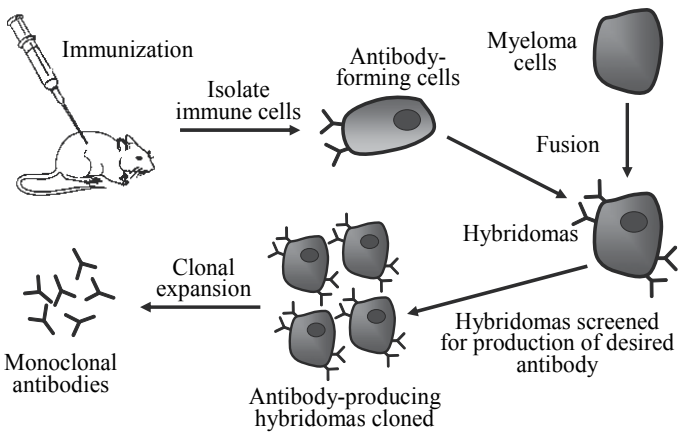

Fig. 6. The protocol of mAbs production (modified after "Michnick & Sidhu, 2008").

2.2.4 Recombinant antibody production

Recently, a third possibility (recombinant antibody (rAb) techniques) for creating antibody has emerged. Here, antibody genes are cloned, introduced and expressed in inexpensive and relatively simple host systems. Although several non-mammalian host systems (yeast, plant and insect cells) have been used to produce rAbs, the most common one is *Escherichia coli*.

Phage display technology is one of the most widely used approachs for recombinant antibody fragment production. The primary task for making antibody by phage display is to design and construct a phage displayed antibody library. Figure 7 outlines the general protocol of phage displayed antibody library construction. Briefly, antibody fragment gene are constructed by relevant gene operation technology, and then cloning it into a special vector for gene expression in *E. coli* host. Based on the difference of gene source, antibody library can be classified into naive, immune and synthetic library. Based on the difference of expressed antibody fragment, it can be classified into scFv library, Fab library and so on (Hoogenboom *et al.*, 1998). For more information about antibody library construction, relevant handbooks or monographs, such as "Antibody Phage Display: Methods and Protocols (Aitken, 2009)", can be refered.

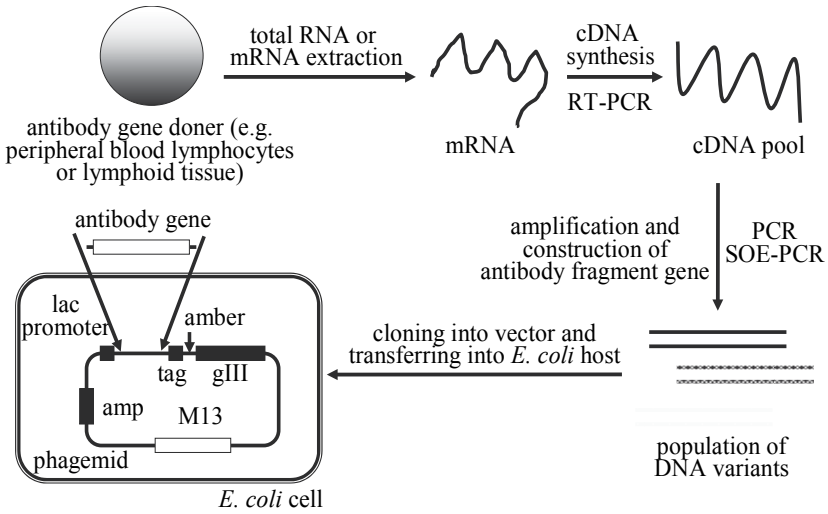

Fig. 7. General protocol of phage displayed antibody library construction.

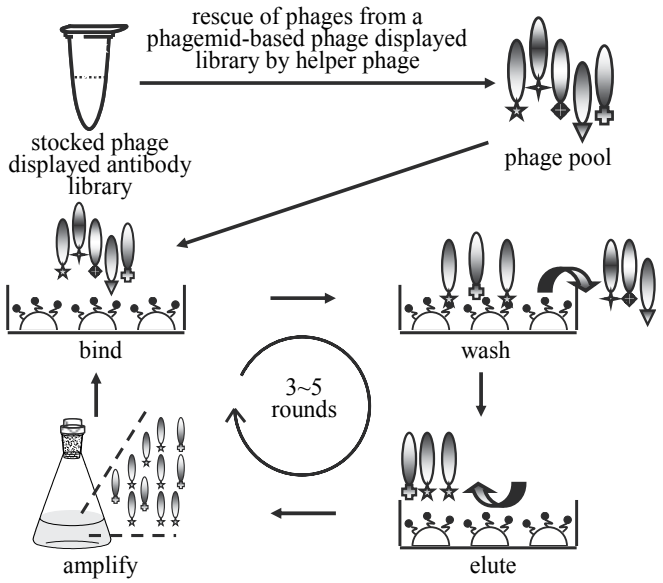

Fig. 8. Isolation of specific antibody fragment from phage-displayed library by biopanning. (modified after "Sheedy, *et al.*, 2007")

After the antibody library is constructed, specific antibody fragment can be isolated from it by biopanning technology. The general procedure of biopanning is outlined in figure 8. Briefly, antibody phages are firstly rescued from the previously constructed phage displayed antibody libraray by helper phage, and then three to five rounds of "bind-wash-elute-amplify" procedure are repeated. This process can be operated in solid phase or solution phase, in the former the target are immobilized by adsorption or covalent effect, while in the latter, biotinylated target are used for binding then target-antibody complexes are isolated from solution by immobilized streptavidin. In general, a panning optimization

experiment is needed for isolating desired antibody fragment (Sheedy, *et al.*, 2007). After this biopanning process, the target specific antibody fragment can be screened by moloclone immunoassay, and those fragments can be used for immunoassay under fusion expression or soluble expression status.

2.3 Immunoassay formats

2.3.1 Immunoassay labels

Depending on the label, immunoassays are classified into different groups. Radioisotopes are used in RIAs, enzymes in enzyme-linked immunosorbent assays (ELISAs or EIAs), fluorophores in FIAs or PFIAs and chemiluminescent compounds in CLIAs. There are some additional types of immunoassays, but are not commonly used in pesticide analysis. A more detailed description of these immunoassays can be found in relevant reference.

EIAs are most commonly used in pesticide analysis as they avoid the necessity of working with radioactive material and low detection limits can be reached. Simple and cheap photometers which give an extremely rapid measurement capability and long-lasting stability of the colored product make EIAs superior to fluorimetry or luminometry, even though with these methods lower detection limits may be reached. Enzymes used as labels in EIAs including alkaline phosphatase, β-Galactosidase and peroxidase, of which the peroxidase derived from Horseradish (HRP) is the most commonly used one.

2.3.2 Traditional pesticide immunoassay formats

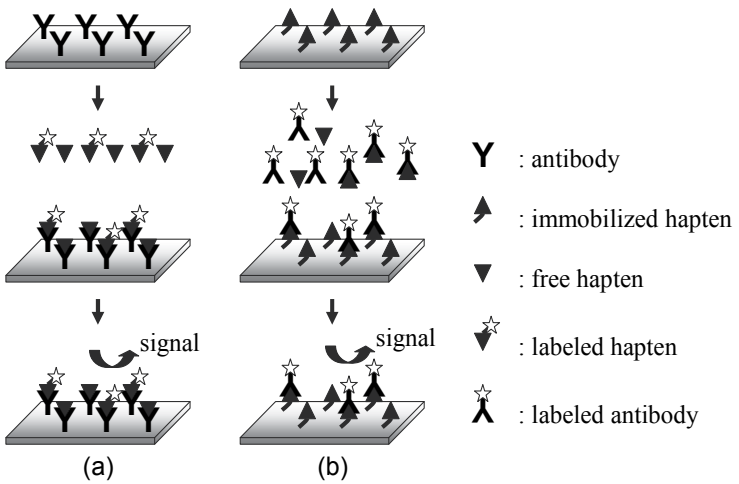

Fig. 9. Competitive immunoassay formats.

Traditionally, detection of low-molecular-mass analytes (haptens) such as pesticides in solution must employ competitive immunoassay formats. There are two different competitive formats available for pesticide immunoassay, (1) with immobilized antibody (Figure 9a) and (2) with immobilized coating conjugate (Figure 9b) (Dankwardt, 2001). In variant (1), analyte and labeled analyte (tracer) compete for the free antibody binding sites. After removal of unbound reactants the bound tracer yields a signal. The variant (2) employs an immobilized hapten-carrier conjugate on the solid phase to which analyte and antibody are added. Antibody binds to the free analyte or to the immobilized hapten in certain ration of the

reactants concentration. If a labeled antibody is used, the amount of antibody bound to the solid phase can be directly determined after a washing step. Alternatively, a secondary labeled antibody may be used to detect the bound antibody. In these competitive immunoassay formats, the signal is inversely proportional to the amount of free analyte in the sample.
In enzyme labled competitive immunoassay, separation of unbound reagent from bound reagent is needed, i.e. heterogeneous assay is performed. While for other labels, homogeneous assay system can be employed. An example for a homogeneous assay system is the polarization fluoroimmunoassay (PFIA) (Eremin & Samsonova, 1994). PFIA measures the increased polarization of fluorescence when a fluorophore-labeled hapten (tracer) is bound by a specific antibody and the decreased signal when free analyte competes with the tracer for binding. While these assays are easy to carry out and very suitable for automation, they usually show a poorer repeatability than enzyme labled immunoassays.

2.3.3 Novel immunoassay formats with pesticide detection potential

Although the traditional competitive immunoassay format have widely applied in pesticide detection, some difficulties also exist for specific target. For example, sensitivity and specificity of immunoassay to pesticide are inferior to macromolecule analytes due to the weakness of immunogenicity. Hence, novel formats have been developed by researchers to overcome those difficulties.

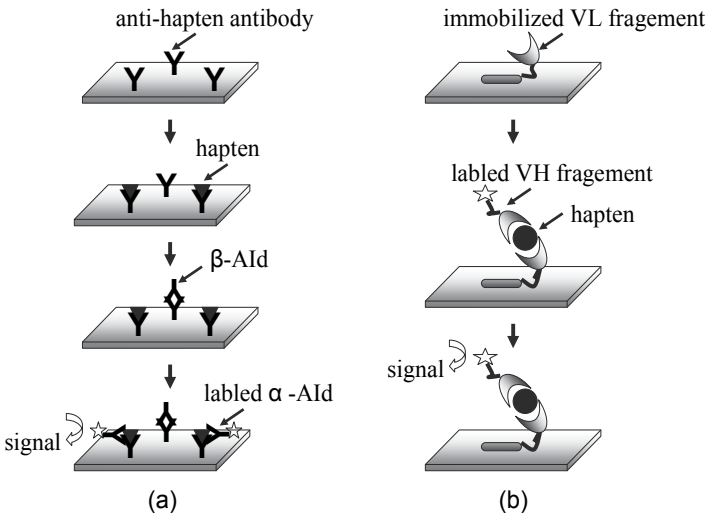

Fig. 10. Novel immunoassay formats with pesticide detection potential.

The theoretical study of Jackson and Ekins (1986) has demonstrated that noncompetitive immunoassays are superior to competitive immunoassays in terms of sensitivity, precision, kinetics and working range of analyte. Sandwich ELISA is a widely used noncompetitive immunoassay to determine antigen concentration. However, it has a fundamental limit that the antigen to be measured must be large enough to have at least two epitopes to be captured. Hence, it can not be used to measure low-molecular weight compounds, such as pesticides. In order to overcome these drawbacks, novel immunoassay approachs, "idiometric assay" (Figure 10a) and "open sandwich immunoassay" (Figure 10b) were previously proposed. The "idiometric assay" utilizes α-type and β-type anti-idiotype

antibody (AId) which have the ability to recognizes different regions of the anti-hapten antibody. The α-AId recognizes an epitope close to the binding site and is not affected by the presence or absence of the analyte but is sterically hindered from binding to the anti-hapten antibody in the presence of the β-AId. The β-AId recognizes an epitope at the unoccupied binding site, which is masked in the presence of the analyte (Barnard & Kohen, 1990). Meanwhile the "open sandwich immunoassay"is based on the phenomenon of stabilization of an antibody variable region by the bound antigen (Ueda *et al.*, 1996). For more imformation about these two immunoassay formats, relevant paper can be refered. Although they are not widely applied in pesticide detection yet, these two novel formats could be the trend in pesticide immunoassay.

3. Application of immunoassay in pesticides detection

Ercegovich (1971) has described his early work on the detection of pesticides by immunochemical means and indicated the potential usefulness of this method for routine analyses in 1971. Since then, more and more immunoassay papers were published annually. Most of those works were targeted to a single specific pesticide, while broad-specificity immunoassay of pesticides could be the tend in the future. In addition, as the great potential of noncompetitive immunoassay format, researchs in this area have been carriing out as well. In this section, literature review of these areas will be provided.

3.1 Single target immunoassay of pesticides

Binding specificity is the key feature of all immunoassays. In initial period, the works of pesticide immunoassay were targeted to a single specific pesticide, i.e. single target immunoassay. These works have been summarized elsewhere, such as Hennion & Barcelo (1998) and Dankwardt (2001). Most of them were just developed in laboratories and only a few of them have been commercially available yet. For example, a commercial immunoassay kit was developed and applied by Watanabe *et al.* (2007 a) to direct determination of insecticide imidacloprid in fruit juices.

Depending on the conjugate used for immunization and the class of chemicals under investigation, cross-reactivities of the antibdoy with haptens similar to the analyte are frequently observed. For single target immunoassay, cross-reactivities can produce false positive values. This problem could be solved, however, by sophisticated sample preparation technology or (and) antibody purification technology. For example, an antibody for alachlor was found to react very strongly to the sulfonic acid metabolite using an alachlor screening kit, but Aga *et al.* (1994) solved this problem by using solid-phase extraction (SPE) prior to immunoassy and sequential elution of the two compounds with different organic solvents.

Pesticides are widely used in fruit, vegetable and grain production, hence pesticide residues on/in fruit (fruit juice), vegetable and grain are the most important things to cause food safety problem, which deserve special attention. A variety of pesticides have been determined in those food samples by immunoassay. For example, residues of Carbaryl (Abad & Montoya, 1995), Azinphos-methyl (Mercader & Montoya, 1997), Carbofuran (Abad *et al.*, 1997) and Imidacloprid (Watanabe et al., 2007) in fruit juice, and Procymidone (Fernandez-Alab *et al.*, 1995), Fenitrothion (Cho *et al.*, 2004), Isofenphos (Lee *et al.*, 2006) and Iprodione (Watanabe & Miyake, 2007 b) in vegetables have been detected by immunochemical analysis. In general, direct detection can be applied to liquid food sample, while for solid food sample, the target

pesticides must be extracted with an organic solvent, such as acetone, ether, petroleum ether, methanol, acetonitrile or hexane. Matrix effects in food samples frequently occur owing to colored extracts or to the content of lipids, proteins or polyphenols that may be coextracted during sample preparation. Hence, the liquid food samples or extracts of solid food sample are usually further diluted with buffer prior to the immunoassay for eliminating matrix effect. For example, in the work of Abad *et al*, (1995) a monoclonal antibody based immunoassay was applied to direct determination of carbaryl in apple and grape juices. The influence of matrix dilution was investigated using different dilutions of the samples. And their result indicated that, the samples should be diluted at least 1 : 5–1 : 10 for proper analysis and the most accurate and precise results were obtained with a dilution of 1:100. In some cases a further cleanup step is needed, in which the analyte of interest is separated from the matrix. Usually, this can be carried out by SPE columns. For example, an ELISA commercial kit was used to quantitate residues of Procymidone at very low levels (< 20 pg/kg) in pepper samples by Amadeo *et al*. (1995). In this work, samples were extracted with ethyl acetate-sodium sulphate and an aliquot is evaporated to dryness and reconstituted in 10 ml of light petroleum. Then, sample clean up is accomplished by aspirating 2 ml of the light petroleum extract through a silica gel solid phase disposable cartridge. Following aspiration, the sample was eluted with 2 ml of ethyl ether-petroleum ether (1 : l). The eluted fraction was evaporated and dissolved with sonication in 2 ml of water before immunoassay analysis.

Pesticide residue in soil and water environment is another serious problem. Immunoassay has been used intensively for the determination of pesticides in soil and water sample. For soil sample, matrix effects exist as per the food sample, hence similar sample preparation processes could be suitable. For example, in the work of Johnson *et al*. (2001), supercritical fluid extraction (SFE) was coupled with a commercial semi-quantitative enzyme-linked immunosorbent assay for detection of polychlorinated biphenyls in an oily soil matrix. While for water sample, direct detection can be applied as per the liquid food sample, but sample dilution is usually not required.

The third application of pesticide immunoassay is biomonitoring, which involoves the measurement of a parent chemical and/or metabolites or a product of its reaction with cellular components in selected tissues, body fluids such as blood, milk, urine or sweat, or expired breath of an exposed individual. Most immunoassays for pesticides are sensitive enough for biomonitoring, and many analytes could be determined without any sample preparation except for dilution with water or buffer. As in the work of Chuang *et al*. (2005), for quantitative measurement of 2,4-dichlorophenoxyacetic acid (2,4-D) in human urine by immunoassay, samples were diluted (1 : 5) with phosphate-buffered saline containing 0.05% Tween and 0.02% sodium azide. Result indicated that, recovery rates of 2,4-D in spiked urine samples were over 70%.

Usually, after immunoassay system development, a validation of the results obtained by immunoassay should be carried out. To a limited extent this can be done by immunoassay itself. Dilution of the samples as well as spiking of the authentic sample with known amounts of the contaminant can be used to check whether the matrix interferes with the immunoassay. But for more strict, an immunoassay should be validated by a different established method like high-performance liquid chromatography (HPLC), gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS). Many groups have used this approach and have usually obtained correlation coefficients of >0.9. Often a slight overestimation of the immunoassay in comparison with HPLC or GC is observed owing to cross-reactivities of the antibody or matrix effects. (Dankwardt, 2001)

3.2 Broad-specificity immunoassay of pesticides

As stated, immunoassays have been developed for effectively measuring individual pesticides; however, it is now more desirable to use the technique to identify more than one target simultaneously. In another word, there is a need now to increase sample throughput and to screen samples for more than one analyte per text (Spinks, 2000). To achieve this it is possible to raise mumberous antibodies that recognise individual targets and incorporate all the antiboies into a single test. However, a more economical alternative is to raise a single antibody that is able to determine several analytes in one test, which is called a broad-specifcity antibody.

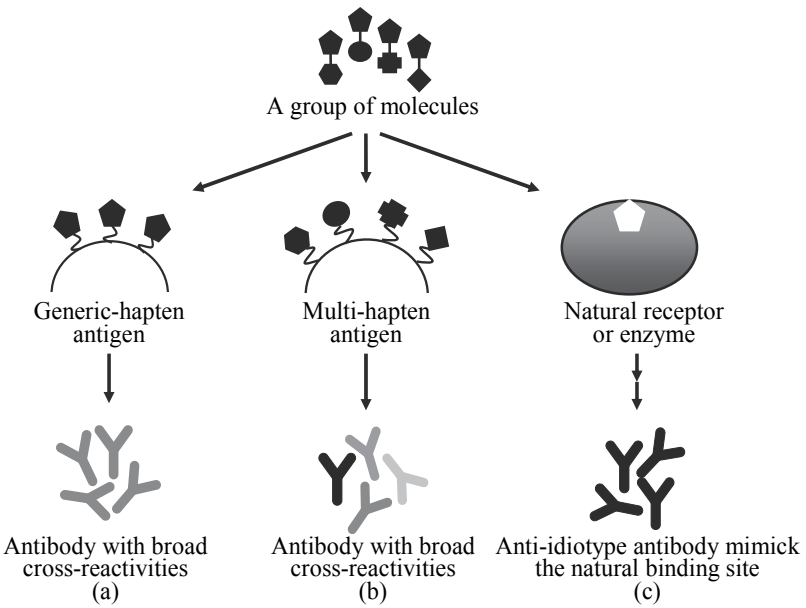

Fig. 11. Strategies for broad-specificity antibody production.

A valid strategy for broad-specificity antibody production is to synthesize generic hapten based on the similar structure of a group molecules, and then prepare an antibody with broad cross reactivities (Figue 11 (a)). Examples of broad specifity antibody production by this strategy are summarized in table 1. As in the work of our group (Liang *et al.*, 2008 a, b), three generic haptens of O,O-dimethyl organophosphorus pesticides with different spacer-arms were synthesized for broad-specifcity antibody production, then a general and broad-specificity enzyme-linked immunosorbent assay was developed for malathion, dimethoate, phenthoate,phosmet, methidathion, fenitrothion, methyl parathion and fenthion. The haptens were conjugated to bovineserum albumin (BSA) for immunogens and to ovalbumin (OVA) for coating antigens. Rabbits were immunized with the immunogens and six polyclonal antisera were produced and screened against each of the coating antigens using competitive indirect enzyme-linkedimmunosorbent assay for selecting the proper antiserum. The antibody-antigen combination with the most selectivity for malathion was further optimized and tested for tolerance to cosolvent, pH and ionic strength changes. The IC_{50} values, under optimum conditions, were estimated to be 30.1 μg L^{-1} for malathion, 28.9 μg L^{-1} for dimethoate, 88.3 μg L^{-1} for phenthoate,159.7 μg L^{-1} for phosmet, 191.7 μg L^{-1} for methidathion, 324.0 μg L^{-1} for fenitrothion, 483.9 μg L^{-1} for methyl parathion, and 788.9 μg L^{-1} for fenthion. Recoveries of

malathion, dimethoate, phenthoate, phosmet and methidathion from fortified Chinese cabbage samples ranged from 77.1% to 104.7%. This assay can be used in monitoring studies for the multi-residue determination of O,O-dimethyl organophosphorus pesticides.

Target Pesticides	Antibody	References
organophosphorus pesticides	mAb	Wang *et al.*, (2010)
O, O-dimethyl organophosphorus pesticides	pAb	Liang *et al.*, (2008 a, b)
O, O-dimethyl organophosphorus pesticides	mAb	Liu *et al.*, (2009)
O,O-diethyl organophosphorus pesticides	pAb	Xu *et al.*, (2009 a)
O,O-diethyl organophosphorus pesticides	mAb	Jang *et al.*, (2002)
pyrethroid insecticides	pAb	Kuang *et al.*, (2009)
type I pyrethroid insecticides	pAb	Watanabe *et al.*, (2001); Zhang *et al.*,(2010)
type II pyrethroid insecticides	pAb	Lee *et al.*, (1998 a, b); Mak *et al.*, (2005); Zhang *et al.*, (2010)
benzoylphenylurea insecticides	pAb	Wang *et al.*, (1998)
triazine	mAb	Bertoncini *et al.*, (2003).

Table 1. Examples of broad-specifcity antibody production by "generic hapten" strategy.

Another approach for broad-specificity antibody production is preparing a multi-hapten antigen for antibody produciton (Figue 11 (b)). For example, a multi-determinant artificial antigen was prepared by haptens of four pesticides (chlorpyrifos, triazophos, carbofuran and parathion methyl) conjugating to the carrier protein BSA in turn. Then, male New Zealand white rabbits were immunized with this multi-determinant immunogen to produce the polyclonal antibodies, which can react with the four pesticides. Characterization studies of the polyclonal antibodies showed that it has high affinity and specificity to the four relative pesticides. At last, an indirect competitive enzyme-linked immunosorbant assay was developed for multi-residue determination. The IC_{50} value for the four pesticideswas 0.290, 0.065, 0.582 and 2.824 μg ml^{-1}, with the detection limit (IC_{10}) of 0.022, 0.005, 0.015 and 0.115 μg ml^{-1} for carbofuran, triazophos, chlorpyrifos and parathion methyl, respectively (Wang *et al.*, 2007).

The third strategy for broad-specificity antibody production is relied on the concept of β-type anti-idiotype antibody (Figue 11 (c)). In nature, there are some receptors or enzyme molecules can bind groups of structurally related compounds. If these naturally occurring binding can be mimicked by β-type anti-idiotype antibody, a broad-specificity antibody will be obtained. For example, cutinase can bind to kinds of carbamates and organophosphate pesticides, based on this point, a broad-specificity immunoassay of organophosphate pesticides was developed by Ward *et al*. (1999). After preparation of a monoclonal Ab against cutinase, this antibody was used as antigen, an anti-idiotype antibody (monoclonal antibody) which mimicks the active site of cutinase was acquired. Results indicated that this anti-idiotype antibody was able to bind the organophosphate pesticides, chlorfenvinphos, ethyl paraoxon, tetrachlorfenvinphos and demeton-s-methyl.

3.3 Noncompetitive immunoassay of pesticides

As stated above, it was widely accepted that noncompetitive immunoassays are superior to the competitive counterparts. Example of noncompetitive immunoassay of pesticides was reported by Anfossi *et al.* (2004). This work highlights the characteristics of a general method of performing noncompetitive immunoassays for low-molecular-mass analytes, which was developed and applied to 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) determination in aqueous samples. The method is based on the separation of the analyte-bound antibody from the excess of the free antibody by a chromatographic step, followed by the dissociation of the complex and the capture of the previously bound antibody on a solid phase. The measured signal is linearly correlated to the concentration of the complex and, consequently, to the analyte concentration. The 3σ limit of detection (LOD, 8 ng L^{-1}) obtained by the above method enabled decidedly improve the sensitivity of the corresponding enzyme-linked immunosorbant assay and of all reported immunoassays for DDT. In addition, by applying this new format, even if a very selective antibody was used, a broad selectivity could be observed, which allowed DDT + DDD+ DDE to be determined instead of only p,p'-DDT as in the assay performed with the same antibody. Further, real water samples were validated in a recovery test. Very good recovery rates were obtained, confirming the validity of the proposed method to accurately determine the total DDT content in water.

From the example above mentioned, it is known that, development of noncompetitive immunoassay for small molecules such as pesticide is a complex task. It was due to small molecules present single epitope (monovalent antigens), and hence non-competive sandwich immunoassay could not be applied in small molecules as per the large molecules such as protein. Idiometric assay (Figure 8a) and open sandwich immunoassay (Figure 8b) are novel noncompetitive immunoassay formats with small molecules detection potential. The former format has sucessfully applied in the detection of small molecules such as estradiol (Barnard & Kohen, 1990; Barnard *et al.*, 1991; Mares *et al.*, 1995), progesterone (Barnard *et al.*, 1995), UDCA 7-NAG (a bile acid metabolite) (Kobayashi *et al.*, 2000; Kobayashi *et al.*, 2003 a), 11-deoxycortisol (Kobayashi *et al.* 2003 b) and cortisol (Niwa *et al.*, 2009). And the latter has successfully applied in the detection of gibberellin (Lee *et al.*, 2008), benzaldehyde (Shirasu *et al.*, 2009), zearalenone (Suzuki *et al.*, 2007) and 11-Deoxycortisol (Ihara *et al.*, 2009). Although they are not widely applied in pesticide detection yet, thess two novel formats could be the trend in pesticide immunoassay. In our recently unpublished work, a noncompetitive immunoassay based on anti-idiotype antibodies was developed for O, O-dimethyl organophosphorus pesticides detection. Positive and negative primary antibodies were affinity purified and used for biopanning of anti-idiotype antibody (AId) from phage displayed antibody library. After three round of panning, three α-AId and nine β-AId clones were achieved. At last, based on clones D11 and B9 (identified as β-AId and α-AId respectively), a broad-specificity noncompetitive immunoassay of O, O-dimethyl organophosphorus pesticides was developed.

4. Conclusion and future outlook

Immunoassay technique provides a simple, powerful and inexpensive method for pesticide analysis, and has become a popular research field. Coupled with the development of relevant theory and technology, some more convenient immunoassay techniques have developed and successfully applied in pesticide detection.

Dipstick immunoassay is a typical example of convenient immunoassay format. Its main advantage is easy to use without sample prepatation, just by dipping the strips into the water. The dipstick techniques involve first the competitive immuno-reaction and then the colour development. Test strips are usually prepared by applying a coating of primary antibody and then incubating with the specific antibody. Once the strip is prepared, the immunoassay dipstick is dipped into the solution and the enzyme tracer, and then incubated a certain time. The enzyme substrate is added and incubated if necessary. The dipstick is then removed from the solution and quantitative measurements can be obtained photometrically (Henion & Barcelo, 1998). Another strip format has been described, based on gold nanoparticles (GNPs), and is described below. Such a system was described for the determination of dichlorodiphenyltrichloroethane (DDT): an organochlorine pesticide. GNPs with definite size were synthesized and conjugated to anti-DDT antibodies (IgY), which served as the detecting reagent. DDA-BSA conjugate (antigen) was immobilized on to nitro cellulose (NC) membrane containing strip. GNPs conjugated anti-DDT antibodies were treated with different concentrations of free DDT ranging from 0.7 ng ml^{-1} to 1000 ng ml^{-1} to form an immunocomplex. This immunocomplex solution was further reacted with DDA-BSA conjugate immobilized NC membrane containing strips by dipping the strip in the immunocomplex solution. The free GNPs conjugated anti-DDT antibodies present in the immunocomplex solution were targeted for competitive binding with immobilized DDA-BSA on NC membrane containing strip. Depending on the concentration of free DDT in the sample the binding of GNPs conjugated anti-DDT antibodies to the immobilized DDA-BSA varied and was detected by the development of red color (due to gold nanoparticles) in the detection zone of NC membrane containing strips. The intensity of color development was inversely proportional to the DDT concentration with maximum intensity at zero DDT concentration. The lowest detection limit of DDTwas determined to be 27 ng ml^{-1} with the optimized conditions (Lisa *et al.*, 2009).

Immunosensor is the most sophisticated immunoassay format to date. It is designed so that the formation of the immune complex on the transducer surface is directly determined by measuring the physical changes (electrical or optical) induced by the binding reaction. Either the antibody or the antigen is immobilized on the transducer carrier surface forms a sensing device and allows reacting with the complementary antigen or antibody to form an immune complex. This formation alters the physical properties of the surface. Depending upon the transducer technology employed, immunosensors are broadly classified into optical, piezoelectric (PZ), electrochemical and micromechanical. Immunosensors have great potential to become costeffective, sensitive devices for on-site monitoring of pesticide pollutants. Recent developments in immunosensors for pesticides detection have been summarized by Suri *et al.* (2009).

However, the progress of development of new immunoassays and related immuno-technologies is still limited by the availability of antibodies with the desired affinities and specificities for given applications. Efforts are still to be made for developing antiboies for both common and new pesticides. As stated, the first and critical step for developing immunoassay system for pesticide detection is to design and prepare a rational hapten molecule. The importance of careful hapten design during immunoassay development can never be underestimated. Traditionally, however, design of hapten molecules in immunoassay development is primarily based on trial and error. For example, in order to generate a desired antibody with high affinity and specificity, a series of immunogen were designed and used to immunize animals, and then immunoassay methods were used to

screen for the best antibody. These procedures are time-consuming and very laborious, and sometimes the obtained antibodies lack the required features to develop a useful immunoassay. Recently, the computer-assisted molecular modelling (CAMM) provides a useful tool for hapten design. CAMM can provide insights into molecular structure and biological activity that are difficult or otherwise impossible to obtain, and it has been successfully applied in rational hapten design. For example, Julicher *et al.* (1995) applied CAMM to investigate the structure of the hapten and evaluated the influence of spacer arm on hapten conformation and electronic nature as compared with the analyte. A suitable molecule based on CAMM results was selected for immunization, and two polyclonal antibodies were raised; one was selective to 2,4,6-trinitrotoluene, and the other was able to recognize important structurally related compounds at low concentrations (2-1000 μg L^{-1}). More details about application of CAMM to rational hapten design have been reviewed by Xu *et al.* (2009 b). It is believed that, CAMM will be a useful and potential tool in helping develop immunoassays with better sensitivity and specificity in the future.

Continued academic and industrial research offers additional less traditional methodologies for production of binding species (molecularly imprinted polymers, aptamers) against pesticides. Molecularly imprinted polymers (MIPs) are synthetic polymeric materials with specific recognition sites complementary in shape, size and functional groups to the template molecule, involving an interaction mechanism based on molecular recognition. These recognition sites mimic the binding sites of biological entities such as antibodies and enzymes, and the molecularly imprinted polymers have been widely recognised as synthetic and robust alternatives to antiboies ("plastibodies"). While, aptamers are single stranded DNA or RNA ligands which can be selected for different targets starting from a huge library of molecules containing randomly created sequences. The selected aptamers can bind to their targets with high affinity and even discriminate between closely related targets, hence it can also be considered as a valid alternative to antibodies.

The first successful emulation of a biological immunoassay by using molecularly imprinted polymers (pseudo-immunoassay) was early reported by Mosbach's group in 1993 (Vlatakis *et al.,* 1993). Up to now, molecularly imprinted polymers based immunoassays have been successfully applied to the detection of pesticdes, such as 2,4-D (Lu *et al.*, 2007; Vandevelde et al., 2007), Atrazine (Prasad *et al.*, 2007), Parathion (Li *et al.*, 2005) and so on. While, aptamers that recognize small molecules, such as amino acids, antibiotics, organic dyes and so on, have been successfully obtained (Famulok, 1999). In addation, aptamer based biosensor system has been developed and appiled to the detection of cocaine, adenosine, and so on (Lee *et al.*, 2010). It is believed that, these alternatives of antibodies will be critical materials for rapid detection of pesticides in the future.

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13

Organophosphorous Compounds-Toxicity and Detection Approach

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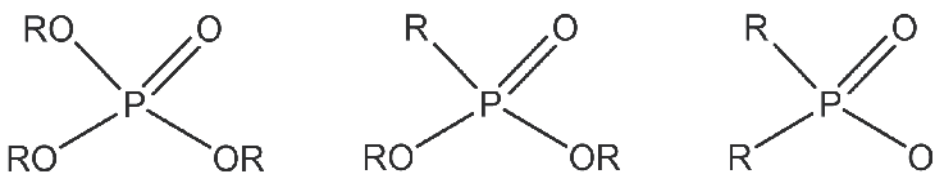
1. Introduction

The first organophosphorus (OP) compound tetraethyl pyrophosphate (TEPP) was synthesized by de Clermont in France. Later, von Hofmann synthesized methyl-phosphoryl dichloride (Fest & Schmidt, 1982). OP compounds are widely used in the agriculture industry around the world as pesticides and insecticides. Phosphorous compounds play a central role in the living organism; it is pertinent to mention photosynthesis, metabolism, and involvement in coenzyme systems etc. It can have a variety of oxidation states 3 and 5, generally OP compounds based on their derivatives of phosphorous. Organophosphate triesters, phosphonates, phosphonofluoridates and phosphonothioates comprise a broad class of chemical neurotoxins (Fig 1). The hydrolysis of OP compounds follows several patterns, depending upon the type of ester, the solvent, the pH range or upon catalytically active additives.



(a) (b) (c)

Fig. 1. Structures of (a) Phosphate, (b) Phosphonates and (c) Phosphinates

However, the high toxicity of the OP compounds had not been recognized until the 1930s, when Lange and Krűger described effects, which they noticed during synthesis of some OP with the P-F bond (Holmstedt, 1963). German Chemists subsequently became interested in synthesizing insecticides. G. Schrader, in 1936, synthesized highly toxic OP insecticide ethyl-N,N-Dimethylphosphoramidocyanidate (tabun) and isopropyl methylphosphonofluoridate (sarin) in 1937 (Robinson & Leitenberg, 1971). Schrader synthesized the toxic OP compounds in search of better insecticides. Nerve agents are also OP compounds such as sarin (GB), tabun (GA), soman (GD) and VX are categorized as chemical warfare (CW) agents. During World War II, the Germans possessed large quantities of tabun and sarin although they

were not used in that conflict. Nerve agents are divided into two main groups: the G-agents and V-agents. The G-agents are nonpersistent (sarin, soman, & tabun) and cause casualties primarily by inhalation. Sarin is highly volatile compared to tabun and soman. The V-agents are persistent (VX) they can therefore cause casualties by both inhalation and absorption through the skin.

In 1944, G.Schrader synthesized the parathion series of OP compounds. The first member of parathion series is O,O-diethyl O-4-nitrophenyl phosphate (paraoxon). These compounds have excellent insecticidal properties, but on the other hand they are highly toxic to mammals. Schrader therefore sought to synthesize esters with as low toxicity as possible to ensure maximum safety for all users. Therefore, he changed the ethyl group to methyl esters i.e. parathion methyl (O,O-dimethyl O-4-nitrophenyl phosphorothioate (Fig.2).

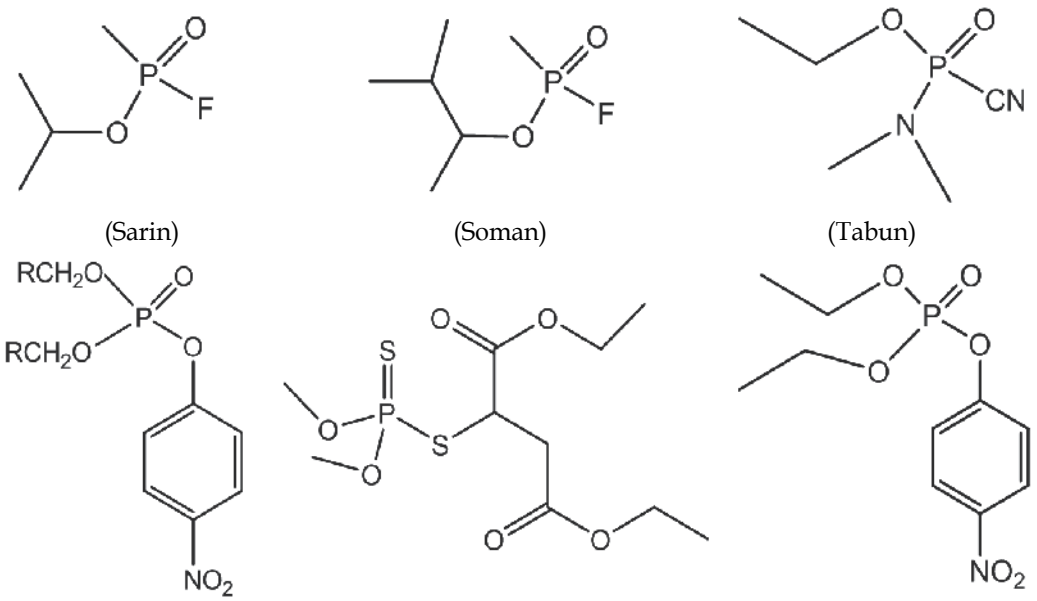


Fig. 2. Structures of OP compounds- Nerve agents and Pesticides

OP pesticides and insecticides are extensively used by farmers all over the World (Gilliom et al., 1999). The general chemical structure of these types of deadly OP compounds consist of a tetrasubstituted phosphorous (V) center, an oxygen or sulfur atom double bonded to the phosphorous, a leaving group, and two substituents that vary widely depending on the subclass. Due to their widespread presence, great environmental concerns have recently arisen around this type of pollution (Fig 3). These effective broad-spectrum compounds used against insect and arthropod, pests, are highly toxic to humans by different routes of exposures, such as dermal absorption, ingestion or inhalation. 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.

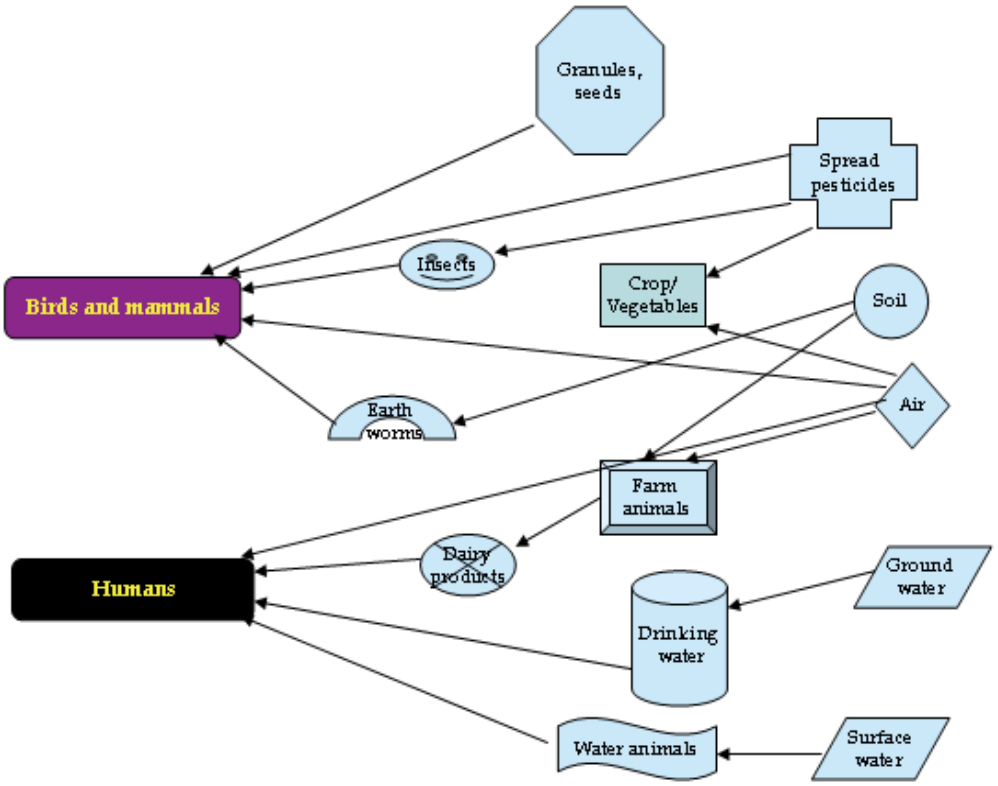


Fig. 3. Possible routes of environmental exposure of OP Pesticides/nerve agents to humans and wildlife

2. Mode of action of OP compounds on acetylcholineesterase enzyme

The toxicity or mode of action of OP compounds can be attributed to the inhibition of the enzyme acetylcholinesterase (AChE). AChE is a globular protein and its three-dimensional structure is known. Its physiological substrate is acetylcholine. The active site of AChE consists of two subsites, anionic and esteratic sites. The anionic site is represented by a glutamate ion. The esteratic site has serine moiety and histidine as well as tyrosine residues (Schumacher et al., 1986). This enzyme is essential for the central nervous system, and being present in both humans and insects. The normal function of AChE is the hydrolysis of acetylcholine neurotransmitter in the synaptic membrane to prevent its accumulation, and as a result forming acetylated enzyme and releasing choline. The high percentage of released choline is transported back into the nerve ending for reconversion to acetylcholine and storage (Fig. 4). This degradation process results in a lowered level of acetylcholine, and ultimately the termination of nerve impulses.

OP compounds covalently block the active site of serine residue of AChE by undergoing nucleophilic attack to produce a serine-phosphoester adduct. This irrevrsible inactivation leads to an excess accumulation of acetylcholines in the peripheral and central nervous system causing cholinergic manifestations. At high doses, there is depression of the respiratory centre in the brain, followed by peripheral neuromuscular blocked causing respiratory paralysis and

death (Baigar, 2004; Somani, 1992; Vijayaraghavan et al., 2010). The pharmacologic effects and toxicity of these OP compounds are dependent on their stability, rate of absorption by various routes, distribution ability to cross the blood-brain barrier, rate of reaction with AChE.

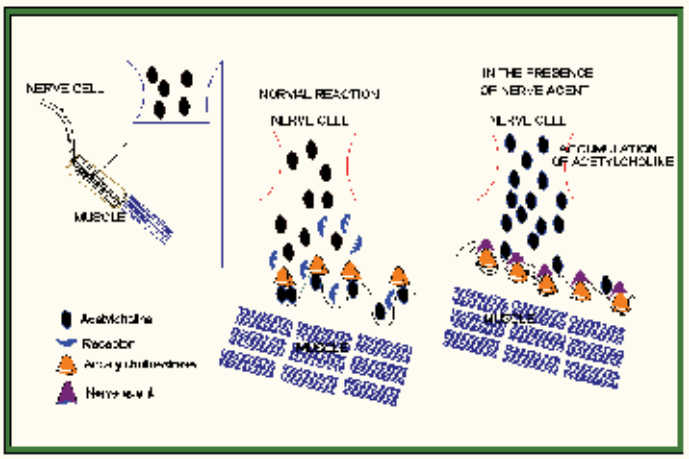


Fig. 4. Action of OP compounds on acetylcholinestrase

3. Toxicity of OP compounds and treatment

The nerve agents (also known as nerve gases) are organophosphorus compounds (OP). All OP compounds do not qualify as war gases due to their differential toxicity. Some of the OP compounds are less toxic to humans and are used as insecticides. Agents that fall in the nerve agent category are tabun, sarin, soman and VX. The absorption of these agents into the system is through inhalation, and if the skin is also exposed, they can be absorbed appreciably.

The effects of nerve agents are the result of the action on the muscurinic and nicotinic receptors within the central nervous system. They include constriction of the pupil (meiosis), increased production of saliva, running nose, increased perspiration, urination, defecation, bronchosecretion, bronchoconstriction, decreased heart rate and blood pressure, muscular twitches and cramps, cardiac arrhythmias, tremors and convulsions. The most critical effects are paralysis of the respiratory muscles and inhibition of the respiratory center. Ultimately death is due to respiratory paralysis. If the concentration of the nerve agent is high, death is immediate (Baigar, 2004; Munro et al., 1994; Somani, 1992; Vijayaraghavan et al., 2010)).

LD_{50} is the dose that may kill 50% of the population exposed. LCt_{50} is the product of concentration of a vapour or an aerosol and the time for which one is exposed, that may kill 50 % of the population. Toxicological parameters of nerve agents and OP pesticides are shown in Table 1.

4. Treatment of OP compounds poisoning

The treatment of nerve agent poisoning requires to be done under the supervision of medical personnel (Marrs et al. 2006; Thiermann et al.2007) The treatment schedule can be classified as:

i. Termination of further intoxication
ii. Artificial respiration or oxygen therapy, and
iii. Antidote therapy

5. Termination of further Intoxication

Like any other poison, the first and the foremost step is removal of the subject from the contaminated environment and removal of the toxicant from the skin.

NerveAgents/ Insecticides/	LD_{50} (bare skin) mg	LD_{50} (oral) mg	LCT_{50}(inhalation) $mg.min.m^{-3}$
Tabun	200-1000	25-50	100-200
Sarin	100-500	5-20	50-100
soman	50-300	5-20	25-50
VX	5-15	3-10	5-15
Dichlorvas	>7000	300-6000	500-1000
Malathion	>25000	400-40000	-
Parathion	1470	70	-
Methidathion	> 100000	1400	-
Fenthion	>23000	>15000	-
Mevinpos	>300	>250	-

Table 1. Toxicity data of nerve agents and insecticides for a 70 Kg man (Median lethal dose).

6. Artificial respiration or oxygen therapy

Artificial respiration is very important since it assists the patient in breathing, and should be initiated as early as possible either manually or by mechanical respirators. Artificial respiration must continue until natural breathing of the patient is restored.

7. Antidote therapy

The principle of antidote therapy is based on the effects of the nerve agents as shown in the Table 2.

Effects	Treatment	Drugs
Excess of acetylcholine	Antagonists of acetylcholine	Cholinolytics
Cholinesterase Inhibition	Reactivation of cholinesterase	Oximes
Convulsions	Anticonvulsants	Diazepam

Table 2. Treatment of OP compounds exposure.

8. Cholinolytics

These drugs are very important to block the excess action of acetylcholine. They are competitive inhibitors of muscarinic receptors. As atropine has been studied extensively in this group, it is invariably used. Atropine should be administered immediately and

repeatedly starting with an initial dose of 2 mg intravenously, till it is adequate (atropinisation), as indicated by dryness of mucosa of nose and mouth, and an increase in heart rate. The administration of atropine has to be continued for several days or weeks, depending on the severity of intoxication (2-4 mg per week for moderate exposure). The dosage of atropine should not hinder the performance of a non-intoxicated individual. Side effects of 2 mg atropine in a normal individual are increased heart rate, drying of secretions, mydriasis (dilatation of pupil) and paralysis. Most of the effects are reversible. Inhibition of sweating in a non-nerve agent poisoned individual is hazardous and is temperature dependent.

9. Oximes

Oximes are used as cholinesterase reactivators, thereby restoring the inhibited AChE. The oximes in common use are pralidoximechloride (2-PAM) and obidoxime (toxogonin). But, these oximes are not effective for soman poisoning. For this H-series oximes are preferred e.g. HI-6.

The oximes should be administered in combination with atropine. The dose of pralidoxime chloride is 15 - 25 $mg.kg^{-1}$ by slow intravenous injection. Autoinjectors like Combopen type contain 600 mg of pralidoxime chloride in 2 ml solution. Commercially available vials containing 1 g of pralidoxime can be dissolved in 3 ml of sterile water or saline and 2 ml administered intramuscularly. The usual dose of obidoxime is 300 mg. Since these oximes are quickly excreted, a second or third dose may be needed at regular intervals.

10. Diazepam

Nerve agent poisoning leading to serve convulsions and may cause brain damage in severely exposed patients. Diazepam is used as an adjunct to reduce the convulsions. The usual dose of diazepam is 5 - 10 mg.

11. Self treatment

It is important that the antidotes should be administered very quickly in the field itself in the form of first aid. This is done by the use of autoinjectors. These autoinjectors contain a cholinolytic (atropine) and an oxime (2-PAM or obidoxime). The autoinjectors are simple to use and are for intramuscular injection only. Reusable autoinjectors are also available (Autoject Injectors) for atropine sulphate and pralidoxime chloride in which the drug cartridges can be replaced. This is ideal for mass causality management.

12. Prophylaxis against nerve agent poisoning

There are no accepted prophylactic antidotes for nerve agent poisoning, i.e., drugs administered before exposure to the agent. Physostigmine or pyridostigmine, a reversible cholinesterase inhibitor, has been tried with some success in the prophylaxis of nerve agent poisoning. Pyridostigmine bromide has been introduced as a pretreatment drug. The dose is 30 mg, three times a day. Though it may give some protection against nerve agent poisoning it has side effects.

13. Detection of OP compounds

In recent years the determination of OP compounds and nerve agents have became important because of the widespread use of OPs as pesticides and clear threat to people from the potential use of nerve agents by terrorists. Several organizations regulate the maximum levels of permissible pesticide residues in drinking water and in food for human and animal consumption. Among them, notable are Food and Agricultural Organization (FAO) of the United Nations, the World Health Organization (WHO), the European Union (EU), the US Environmental Protection Agency (EPA) and the US National Institute for Occupational Safety and Health (NIOSH). The Organization for the Prohibition of Chemical Weapons (OPCW) regulates the use of CW agents, through the implementation of the provisions of Chemical Warfare Convention (CWC).

Therefore, there is need to develop fast, sensitive, and field-deployable screening technology for quick response. The most common ways for detecting OP pesticides are chromatographic methods coupled with different detectors and spectrometry (Gundel & Angerer, 2000; Hernandez et al., 2005). This method is sensitive and reliable but can not carried out in field, it is expensive and time consuming too. In addition to this, variety of approaches have been investigated for sensors, including enzymatic assays (Russell et al., 2003), molecular imprinting coupled with luminescence (Jenkins et al., 1997; Rudzinski et al., 2002), colorimetric methods (Wallace et al., 2005), surface acoustic waves (Nieuwenhuizen & Harteveld, 1997), fluorescent organic molecules (Yamaguchi et al., 2005; Zhang & Swager, 2003), interferometry (Sohn et al., 2000) and enzyme biosensors based on inhibition of cholinesterase activity (Evtugyn et al., 1996; Trojanowicz, 2002).

14. Enzyme inhibition based biosensors

Enzyme-based biosensors have emerged during past few years and based on the principle of inhibition of AChE and electrochemical or optical based detection. Analytical devices based on the determination of inhibition of AChE have been widely used for the detection of OP compounds (Pavlov et al., 2005; Schulze et al. 2003; Tran-Minh et al. 1990). The screen-printed biosensors were used for the determination of methamidophos pesticides. The inhibition of AChE is measured by direct or indirect measurement of its activity. In the case of the direct method, the assay is based on the spectrophotometric or electrochemical measurement of thiocholine produced from the following reaction:

AChE

$$\text{acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{acetic acid} + \text{thiocholine} \quad (1)$$

The rate of inhibition (%) is calculated before and after incubation with OP compounds as $100 \times (I_o\text{-}I_i) / I_o$ where I_o is current before inhibition and I_i is current after inhibition (Amine et al. 2006).

In the development of biosensors immobilization of enzymes is the critical step in maintaining enzyme activity, stability and shelf life of electrode. Various techniques are used such as physical entrapment, microencapsulation, covalent binding, adsorption and cross-linking. AChE was encapsulated in sol-gel film on a glass cap that could be fixed on an optical fiber (Doong & Tsai, 2001). Sol–gel technology provides an attractive way for the immobilization of biological entities including full cell, enzyme, protein and antibody or antigen due to the inert low temperature process (Pandey et al. 2000). Recently, the

nanoparticles and carbon nanotube (CNT) have received considerable attention to increase the sensitivity of the biosensor due to then high conductivity, catalytic and electrical properties (Pavlov et al., 2005). AChE was immobilized on silica sol -gel assembling gold (Au) nanoparticles for the inhibition study with OP compounds. Due to the large quantities of hydroxyl groups in the sol-gel composite provide a biocompatible environment for AChE enzyme. Immobilized AChE catalyze the hydrolysis of acetylthiocholine chloride and produce thiocholine which is again oxidized to produce signal (Fig.5). The Au nanoparticles catalyze the electro-oxidation of thiocholine. After incubation with OP compounds, peak current decreases and it shows the inhibition of immobilized enzyme and the inhibition is directly proportional to the concentration of OP compounds. In another approach reaction of thiocholine with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) generates fluorescent product which can also be monitored for detection purpose based on inhibition approach (Parvari et al. 1983).

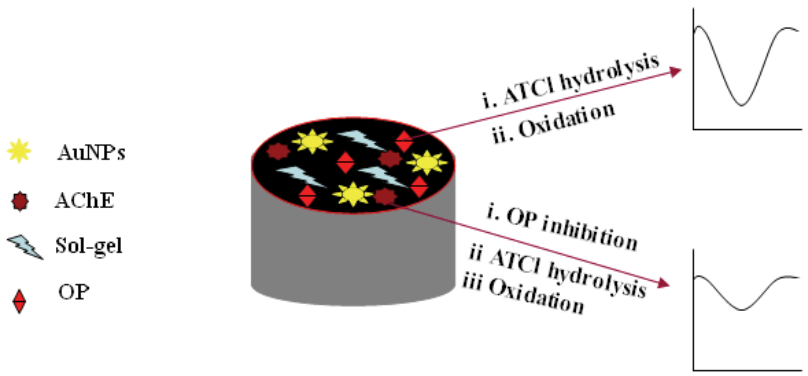


Fig. 5. Acetylcholine esterase biosensor principle for the detection of OP compounds based on AuNPs immobilized in silica sol-gel process

The detection of OP compounds have also been developed on the basis of two-enzyme approach. Acetylcholinesterase (AChE) and choline oxidase (ChOx) are used to recognize acetylcholine and choline. In this reaction, choline is acted upon by ChOx and resulted hydrogen peroxide (H_2O_2) is determined. Finally, oxidation of H_2O_2 is monitored amperometrically.

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{Choline} + \text{Acetic acid}$$

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{ChOx}} \text{Betaine} + 2H_2O_2 \quad (2)$$

Various sensor based on bienzymatic approach have been developed for the detection of OP compounds (Ciucu et al., 2003, Ferapontova et al., 2001,Lin et al., 2004, Ferapontova et al., 2001, Kok et al., 2002, Upadhyay et al. 2009). The ferrophthalocyanine (FePC) modified carbon paste electrodes are used for the construction of bienzymatic amperometric biosensor which is operated at low potential (0.35 V) for the detection of OP pesticides (Ciucu et al. 2003). They reported detection limit up to 10^{-10} M of paraoxon and carbofuran. A disposable CNT based amperometric biosensor developed for the detection of these compounds (Lin et al., 2001). Here, also the biosensor is comprised of co-immobilization of CNT with AChE/ChOx enzymes on a screen printed electrode. In this method multiwall

CNT (MWCNT) showed a significant catalytic effect for the reduction and oxidation of H_2O_2, Leading to the development of an effective biosensor for the assay of chlorpyrifos, fenitrothion and methyl parathion with detection limit up to 0.05 µM. These improved characteristics are attributed to the catalytic effect to H_2O_2 and the large surface area of MWCNT material. The application of bimetallic nanoparticles (Bi-MNPs) is also reported (Fig. 6) for the sensitive detection of OP pesticides and nerve agents (Upadhyay et al., 2009). A novel sensitive amperometric biosensor based on electrodeposition of gold-platinum bimetallic nanoparticles onto 3-aminopropyltriethoxy silane modified glassy carbon electrode for the detection of paraoxon ethyl, aldicarb, and sarin has been developed. The AChE and ChOx are coimmobilized on the Au-PtNPs modified electrode by cross-linking through glutaraldehyde. The key idea for using Au-PtNPs modified glassy carbon electrode is to improve the electrocatalytic activity of H_2O_2 on the modified electrode. Inhibition of enzyme depends upon the preincubation time of enzyme with inhibitors. It is observed that the inhibition level of enzyme increases or remaining enzyme activity decreases with increasing incubation time. The detection limit and linear working range of biosensors were reported at 30-40% inhibition level 150-200 nM, 40-50 nM and 40-60 µM for paraoxon ethyl, sarin and aldicarb respectively. It can be reached below this range but to avoid the interference, 30-40% enzyme inhibition level was considered as optimum. This result showed that the biosensor has good analytical characteristics for these inhibitors due to electrochemical catalytic efficacy of Au-Pt NPs.

Biosensor based on flow injection amperometric detection of OP compounds/nerve agents has been developed. AChE is immobilized on the negatively charged CNT surface by alternatively assembling a cationic poly(diallyldimethylammonium chloride) (PDDA) layer and followed by self assembly of the negatively charged AChE layer. Under optimum conditions, the biosensor is used to measure as low as 0.4 pM paraoxon with a 6-min incubation time (Liu & Lin, 2006). In some other method AChE is immobilized on the pH sensitive redox polymer (polyaniline), which is coated on the vertically aligned thiol terminated ss-DNA-SWCNT on gold electrode for the detection of methyl parathion and chlorpyrifos. The key step of this biosensor is AChE-acetylcholine enzymatic reaction which causes small changes of local pH in the vicinity of an electrode surface. The pesticides are determined through inhibition of enzyme reaction (Viswanathan et al., 2009).

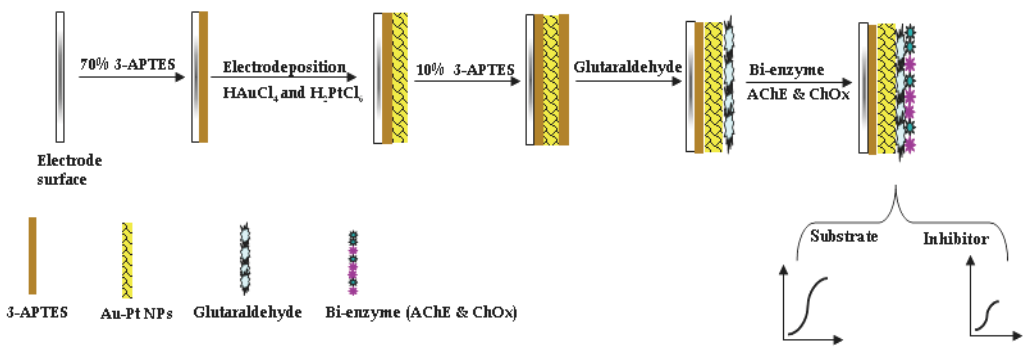


Fig. 6. Preparation of the bienzyme biosensors on Au-Pt NP modified electrode.

The reactivation of AChE enzyme after the inhibition by OP compounds has been investigated by using pyridine-2-aldoxime methyliodide (2-PAM) and 4-formylpyridinum

bromide dioxime (TMB-4) (Amine et al., 2006, & Upadhyay et al., 2009). It has been found that the enzyme activity retains nearly 60% of initial activity with TMB-4, where as in the case of 2-PAM, the enzyme activity retention dropped to less than 50% of initial activity. Thus, it is recommended that the pesticide inhibited enzyme should be reactivated within 10 min to achieve the maximum reactivation of enzyme. In case of nerve agents owing to aging of inhibited AChE, a fraction of enzyme will irreversibly inhibited. In case of bienzymatic approach the relative proportion of AChE/ChOx will change. Therefore, it is not easy to get 100% recovery of enzyme after inhibition.

15. Fluorescence based detection of pesticides and Organo-phosphorous compounds

Fluorescence-based sensors (biosensors/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 of the cases it shows almost instantaneous response. Fluorescence methods are capable of measuring concentrations of analytes 10^6 times smaller than absorbance techniques (Martinez et al. 2003). A variety of analytical techniques have been developed which exploit changes in fluorescence properties of a molecule in different environments, whether those changes are quenching (Chen et al. 2000), and surface modified fluorescence (Kummerlen et al. 1993; Lichlyter et al. 2003). Molecular beacons provide an example of the use of surface modified fluorescence for the detection of DNA with sensitivity down to the mid nanomolar level (Bonnet and Libchaber 1999). The requirements for a successful sensor are: (1) high specificity in binding between recognition molecule and target, and (2) ability to easily manipulate the distance between nanoparticle and fluorophore in response to the target molecule concentration.

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. The principle behind sensor operation is based on nanoparticle- associated optical biosensors for the direct detection of organophosphate chemical warfare agents and pesticides is shown in Fig 7a and Fig 7b. As shown in Fig. 7a gold nanoparticle is covalently bound to an enzyme molecule. A fluorophore decoy, being a weak competitive inhibitor of organophosphorus hydrolase (OPH) with a similar chemical structure to the substrate (analyte of interest), is introduced to the solution and is bound to the OPH active site. If the gold particle attached via amino- or sulfhydryl groups to the OPH is at the certain distance from the decoy (size ranging from 10 to 40 nm), enhancement of fluorescence will be observed. If the nanoparticle is at a distance of greater than about 40 nm from the fluorophore, then fluorescence will be unaffected by the presence of the gold, leading to a reduction in fluorescence signal. Once the decoy is bound to the OPH active site, then it is possible test for the presence of the analyte of interest (which is a substrate of OPH). If the substrate is present, then the analyte will displace the decoy because of its much higher affinity for the OPH active site, and the fluorescence signal of the sample will change. As seen in Fig. 7b, for the case of an enhancement-based sensor, the analyte (indicated by S), will displace the decoy bound to the enzyme active site. As the decoy moves away from the gold nanoparticle, its fluorescence intensity will change. The change in fluorescence intensity is related to the concentration of analyte present in the solution.

Rogers et al. used a pH-sensitive fluorescent dye, consisting of AChE linked to the pH-sensitive compound fluorescein isothiocyanate (FITC). 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 compounds (Rogers et al. 1991). A number of biosensors have been developed based on fluorescence polarization immunoassays (FPIA) (Kolosova et al. 2003; Kolosova et al. 2004; Lee et al. 2005; Tang et al. 2008). A rapid, fiber-optic biosensor assay for the direct detection of organophosphates was developed to provide continual remote monitoring and spectral fluorescent notification. In this study, the bio-recognition element, organophosphate hydrolase (OPH), was conjugated with both biotin and a fluorescence marker i.e. carboxynaphthofluorescein (CNF). Avidin was attached to the polystyrene waveguide surface of a fluorescent detector, and the OPH–CNF–biotin biosensor conjugate was bound to the avidin. The recognition element (OPH) and reporter (CNF) molecules were designed to entertain OP samples with concentrations of neurotoxin as low as 0.05 μM. Quantitative detection could be determined from 1 to 800 μM for paraoxon and from 2 to 400 μM for DFP (Viveros et al. 2006).

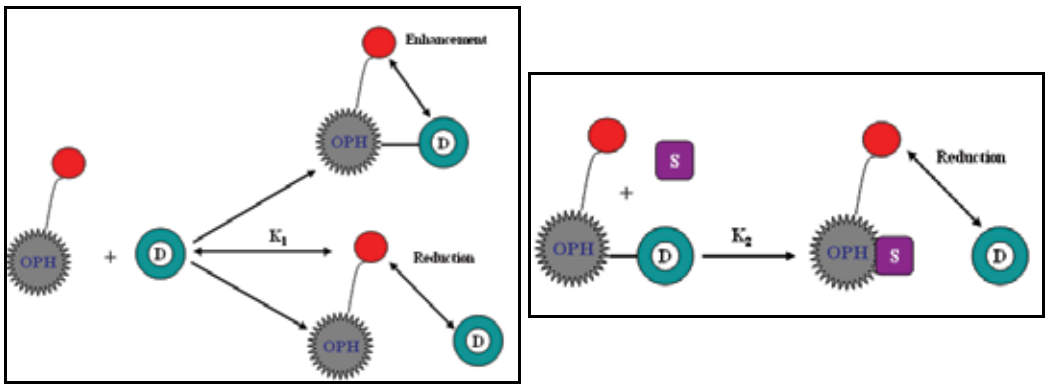


Fig. 7. (a) Decoy-enzyme interaction for enhancement in the absence of substrate. Decoy binds to enzyme-nanogold conjugate (organophosphorus hydrolase-OPH), leading to a surface enhanced fluorescence of the decoy; (b) Analyte (S) displacement of decoy (D) from OPH-gold complex (OPH), leading to decrease fluorescence signal from the decoy.

Gold nanoparticle based surface enhanced fluorescence (NSEF) spectroscopy for rapid and sensitive screening of organophosphorus agents (OPA) was reported. In this technique, the fluorescent from Eu^{3+} ions that are bound within the electromagnetic field of gold nanoparticles exhibit a strong enhancement. In the presence of OPA, Eu^{3+} ions are released from the gold nanoparticle surface and thus a very distinct fluorescence signal change was observed with the high sensitivity of 1 μM (Samuel et al. 2008). Dale et al presented a small molecule sensor that provides an optical response to the presence of an organophosphorus (OP)-containing nerve agent mimic. Exposure to an OP nerve agent mimic triggers phosphorylation of the primary alcohol followed rapidly by an intramolecular substitution reaction as the amine displaces the created phosphate. The quaternized ammonium salt produced by this cyclization reaction no longer possesses a lone pair of electrons, and fluorescence readout is observed as the nonradiative PET quenching pathway of the fluorophore is shut down (Dale et al., 2006). Anandakathir et al. reported the synthesis of

stilbene-based fluorophore, 3,4-dihydroxy-4'-aminostilbene (DHAS) for the detection of chemical warfare agents such as organophosphorus nerve gases. The interaction of DHAS with nerve agent simulant, diethyl chlorophosphate (DCP) was investigated in solution and vapor phase by fluorescence spectroscopy (Anandakathir et al. 2009).

16. Immunological determination of pesticides and Organo-phosphorous compounds

Bioanalytical assays based on enzymatic or immunochemical principles have been proposed as promising alternatives, as they are highly sensitive, selective, specific, rapid and reliable. A selective enzyme-linked immunosorbent assay (ELISA) for the insecticide chlorpyrifos was developed using the sera of highest specificity. This shows an I_{50} of 160 ppb with a detection limit of 10 ppb (Cho et al., 2002). Sensitive, simple and rapid enzyme linked immunosorbent assay (ELISA) methods have been reported for the determination of four organophosphorus pesticides diazinon, fenthion, malathion and chlorpyrifos in extra virgin olive oil. The limits of detection for the pesticides in olive oil are from 46 ng ml^{-1} for diazinon to 10 ng ml^{-1} for fenthion (Garcia et al., 2006). Teller et et al developed a combined piezoelectric/amperometric sensor based on the modular assembly of different recognition elements. Acetylcholinesterase was chemically modified by benzoylecgonine-1,8-diamino-3,4-dioxaoctane (BZE-DADOO), thus providing an additional recognition element for anti-cocaine antibodies or butyrylcholinesterase, respectively. It was possible to determine cocaine in dynamic range of 10^{-7} to 10^{-9} mol/L using polyclonal antibody. At the same time the in-situ inhibition of the adsorbed BZE-AChE by the organophosphate chlorpyrifos-oxon could be monitored by amperometric activity measurement (Teller et al., 2008). Liang et al developed immunoassay method for the O,O-dimethyl organophosphorus pesticides, including malathion, dimethoate, phenthoate, phosmet, methidathion, fenitrothion, methyl parathion and fenthion. Three haptens with different spacer-arms were synthesized. The haptens were conjugated to bovine serum albumin (BSA) for immunogens and to ovalbumin (OVA) for coating antigens. The IC_{50} values, under optimum conditions, were estimated to be 30.1 μg/L for malathion, 28.9 μg/L for dimethoate, 88.3 μg/L for phenthoate, 159.7 μg/L for phosmet, 191.7 μg/L for methidathion, 324.0 μg/L for fenitrothion, 483.9 μg/L for methyl parathion, and 788.9 μg/L for fenthion (Liang et al., 2008). A nanoparticle-based electrochemical immunosensor has been reported for the detection of phosphorylated acetylcholinesterase (AChE), which is a potential biomarker of exposure to organophosphate (OP) pesticides and chemical warfare nerve agents. Zirconia nanoparticles (ZrO(2) NPs) were used as selective sorbents to capture the phosphorylated AChE adduct, and quantum dots (ZnS@CdS, QDs) were used as tags to label monoclonal anti-AChE antibody to quantify the immunorecognition events. The voltammetric response of the immunosensor is highly linear over the range of 10 pM to 4 nM phosphorylated AChE, and the limit of detection is estimated to be 8.0 pM. The immunosensor also successfully detected phosphorylated AChE in human plasma (Liu et al., 2008).

17. Microfluidics based detection

Rapid detection of OP compounds/agents is required to take a quick decision or efficient decontamination for a particular site. Miniaturization of analytical devices is attracting considerable interest due to the potential for greatly enhancing the speed of analytical

separations or characterizations. Over the last decade, micrototal analysis system (μTAS) or Lab-on-a-chip for various purposes have been developed that aim for the rapid high throughput analysis of molecules, such as DNA and proteins, point-of-care testing and microchip for the fast screening of OP compounds (Figeys & Pinto, 2000; Vilkner at al., 2004; Wang et al., 2002). Microfluidic system is a potential platform for biochemical/chemical analysis with numerous advantages including low sample/reagent consumption, high sample throughput and total analysis on the same platform (Vilkner et al., 2004).
An integrated microfabricated device that performs automated enzymatic assays was developed. Active and precise microfluidic control of reagent transport throughout the interconnected channel network was achieved using electrokinetic-induced motion. They controlled the reagent dilution and mixing by regulating the applied potential at the terminus of each channel, using voltages derived from an equivalent circuit model of the microchip. Assay of enzyme (β-galactoside) was monitored by using β-D-galactopyrranoside resorfuin a substrate that is hydrolyzed to resorfuin, which is a fluorescence product (Hadd et al., 1997). The separation and sensitive electrochemical detection of OP compounds have been developed by using on-chip micellar electrokinetic chromatographic (MEKC) techniques (Wang et al., 2001). In this study they microfabricated capillary electrophoresis glass chips with planar thick film amperometric detectors for the separation and detection of toxic OP compound. The integrated microsystem offers rapid (~ 2.5 min.) simultaneous measurements of micromolar levels of OP compounds. The detection of regenerated sarin in human blood samples has been developed in a lab-on-a-chip device. This device should allow early detection of sarin exposure in human being. The device is based on continuous-flow microfluidics with sequential stages for lysis of whole blood, regeneration of free nerve agent from its complexes with blood cholinesterase, protein precipitation, filtration, enzyme-assisted reaction and optical detection (Tan et al., 2008). The reactor for nerve gas regeneration is designed as a micromixer based on chaotic advection with herring-bone structures. The reaction chamber is located on the other side of the main device layer, with herring-bone patterns to improve the transport of reagents to the glass surface with immobilized enzyme. Detection of sarin in whole blood spiked with a low level sarin concentration, 200nM is achieved. They also reported the kinetics of inhibition reaction to estimate the required flow rate and inhibition time. In the present system the pumping and valving processes were carried out outside the lab-on-a-chip. The device is suitable for other applications in occupational hygiene in agriculture. Microfluidics system for OP compound detection holds great promise for a timely warning and alarm in the emergency case and its also can be carried any where for on site detection. Microfluidics provide better platform with combination of nanoparticles to enhance the sensitivity and selectivity on the chip.

18. Protective measures against OP compounds/nerve agents

The threat of OP compounds or commonly used pesticides and chemical manufacturing by products act as anticholinesterases, posing an occupational low-dose exposure hazard to workers in a variety of professions as well as public (Aas, 2003). Protective equipment which is used by an individual to achieve physical protection is termed as individual protection equipment (IPE). These include gas masks and protective clothing such as trousers, jacket, over boots and gloves. These physical protections create an artificial barrier between the OP

compounds and the subject (human being), and they have provision for breathable air. The barrier has to provide protection against liquids, aerosols or gases and should possess the essential characteristics of air tightness and non-permeability to gases. The basic materials are used in these barrier include spherical carbon coated fabric, activated charcoal, polyurethane foam and for aerosol High efficiency Particulate Aerosol (HEPA) filter media is composed of glass fibers of diameter 1.0 to 10 microns. The aerosol particles from the contaminated air captured over the surface of the filter medium by van-der wal's force.This type of protection comes under physical protection. Medical countermeasures have been discussed earlier in the section of toxicity of OP compounds. Three drugs atropine, pralidoxime chloride and diazepam are used to treat nerve agent exposure. Atropine is very important to block the excess action of acetylcholine. It is a competitive inhibitor of muscarinic receptors. Atropine should be administered immediately and should be repeated starting with an initial dose of 2 mg intramuscularly or intravenously.

19. Conclusions and future perspectives

We have described a brief summary about OP compounds/nerve agents and mode of action toxicity of these compounds and various detection approach. Despite the considerable research activity towards the development of detection system for monitoring the OP compounds in the lab and on site it is not easy to discriminate various OP compounds in the same sample. Current research activity involves numerous efforts for improving the analytical performances of the biosensing systems in order to be able to monitor a wide range of pollutants in environmental and food samples. The use of nanoparticles/CNT leads to a greatly improved electrochemical detection due to its electrocatalytic activity. For more sensitive detection fluorescence method is also beneficial. However, with the increasing threat of terrorism, and large scale use of OP pesticides the roles of detectors are also increasing in civil emergency responses. In these instances, the detectors are used to monitor the presence of these compounds in the atmosphere, provide an indication of their levels in order to determine the necessary level of protection. Due to the structural similarity of OP compounds, it is also of paramount importance that the designed sensors must be fabricated such that they are highly selective towards specific OP compounds. The Microfluidics and nanotechnology offer a promising technology for the miniaturized detectors which can be used for onsite and easy to operate. By using this sensitivity, real time detection, response time, and selectivity can be improved. It requires very low sample volume and other reagents and the interference can also be minimized. The engineered variants of enzymes could be another approach in biosensor design for the discrimination and detection of various enzyme-inhibiting compounds when used in combination with chemometric data analysis using artificial neural network. New opportunities are considered with the application of novel enzymes or enzyme sources as well as biocomponents with necessary enzyme activity. Combined with traditional biosensors and test kits this biosensing can be applied as alarm monitors of environmental pollution. In this combination nanoparticles will play a very important and effective role to increase the sensitivity and selectivity. When we introduce nanoparticle in the combined system all the physical parameters will change and it creates a new phenomenon. The development of antidote is also necessary for medical countermeasures. For this new drug development research is promising to counter the effect of OP compounds once it is exposed to any person.

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14

New Materials in Electrochemical Sensors for Pesticides Monitoring

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1. Introduction

Pesticides are substances or mixtures thereof, natural or synthetic, formulated to prevent or control any species of plants or pests. The term pesticide includes substances intended for use as plant -growth regulators, defoliants or desiccants.
Once a pesticide is introduced into the environment, either through an application, removal or a spill, it is influenced by processes, such as adsorption, transfer, breakdown and degradation, which determine their persistence, movement and its final fate.
In recent years the growing awareness of the risk associated with intensive use of pesticides has led to a more critical attitude towards the use of agrochemicals. At the same time, many national environmental agencies have been involved in the development of regulations to eliminate or severely restrict the use and production of a number of pesticides.
However in the third millennium, pesticides will continue to play an important role in plant diseases and pest management. Even in developed countries, where farmers are shifting from subsistence agriculture to modern agriculture, pesticide use may increase at a high rate. Therefore, their use should be optimized, bearing in mind the safety to producers and consumers as well as the environmental impact.
Monitoring and exposure data are critical to accurately determine the impact of pesticides on human health and environment. The analytical methods, faster and more cost-effective, can facilitate the collection of data concerning particular target pesticides that may impact on human health and the environment. Advances in miniaturization and microfabrication technology have led to the development of sensitive and selective electrochemical devices for field-based and *in situ* environmental monitoring (Pellicer et al., 2010). Electrochemical sensors can provide fast, reliable and cost-effective measurement and monitoring methods. Hanrahan et al. (2004) examine the role electrochemical sensors play in environmental monitoring, focusing on recent technological advances in terms of microfabrication, analytical improvements and remote communication capabilities, including also microfluidic integration and submersible devices for remote, continuous monitoring.
The development of *in situ* electrochemical devices requires proper attention to major issues including reversibility, long-term stability, specificity and changes in natural conditions that may affect the response of interest.

This review covers publications related to electrochemical sensors that appeared in print between 1999 and 2010. The focus of the chapter is centered on the development and research in electrochemical sensors based on chemical or biological recognition process and the advantages that the nanomaterials provide on the fabrication and electrochemical sensing systems development for pesticides monitoring.

2. Molecularly imprinted polymer strategy applied to the development of biomimetic sensors

Chemical sensors and biosensors are very powerful tools in modern analytical sciences. The new demands of environmental analysis, have driven the development of more selective and sensitive sensing systems. An essential component of any detection system is a recognition platform, which is able to bind selectively to a target analyte in the presence of competing analytes. Both synthetic and biologically derived recognition platforms have been successfully incorporated into molecular sensors. Synthetic recognition systems include synthetic molecular receptors (Schrader & Hamilton, 2005) and functionalized polymers (Senaratne et al., 2005). Biological recognition systems include immobilized antibodies, enzymes, DNA, and aptamers.

Biological recognition platforms generally show higher levels of affinity and selectivity than their synthetic counterparts and may be more easily targeted to specific analytes. In this respect, the molecular imprinting strategy, represents an important field of application for the generation of sensing systems as substitutes for biological antibodies and receptors (Ye & Mosbach, 2008). Probably, due to the fact that the selectivity against desired target molecules is considerably improved when these type of devices are employed. This technique lies in synthesizing selective recognition sites in a polymeric matrix with a molecular template during the polymer formation. The removal of the template molecule from the polymer matrix reveals binding cavities that are complementary in size and shape to the template molecule and are lined with appropriately positioned recognition groups.

The choice of the functional monomer to generate a proper MIP is very important because it is the component involved in forming chemical bonds with the template (Lanza & Sellergren, 1999). In order to optimize the selection of the monomer for a target analyte, several studies have suggested that the synthesis takes place by combinatorial approaches (Takeuchi et al., 1999; Yilmaz et al., 1999). Other authors proposed a method which included computational screening of a virtual library of functional monomers against a target molecule, followed by selection of those ones able to form the strongest complex with the template (Haupt, 2001; Subrahmanyam et al., 2001).

Molecularly imprinted polymers (MIPs) are highly cross-linked polymers that share the advantages of both biological and synthetic recognition platforms. MIPs have excellent mechanical, chemical and thermal stability (Svenson & Nichols, 2001). However, they also possess the ability to be tailored like biological systems with specificity for a molecule of interest such as pesticides (Fuchiwaki et al., 2007; Shoji et al., 2003; Yamazaki et al., 2001).

The inherent specificity of molecularly imprinted materials and their ability to adapt to different formats, have provided many opportunities for integration into sensing applications (Haupt & Mosbach, 2000; Piletsky et al., 2001).

The development of highly sensitive transducers capable of monitoring the binding process, the development of polymers capable of interacting with the template in terms of required affinity and specificity and the integration of MIP with the transducer, are the critical issues

in the design of sensors based on MIPs (Blanco-Lopez et al., 2004a; Suryanarayanan et al., 2010).

Electrochemical methods have been widely used for the detection of rebinding almost at the same extent as the optical and mass-based ones, as discussed by Piletsky & Turner (2002) and Blanco-Lopez et al. (2004b). This is in part due to the easy integration of MIPs in electrochemical sensors.

The MIPs are primarily recognition platforms that do not have any innate signalling properties. Thus, an important challenge in developing MIP-based chemical sensors is the interface between the MIP and the signal transduction device. The coatings of electrodes with molecularly imprinted polymers (MIPs) are an important tool to achieve this goal (Malitesta et al., 1999).

Despite being a well established analytical technique, the use of electrochemical methods incorporating MIPs provides the researcher the challenge of finding a suitable format for the integration of an imprinted film or particles on the electrode. Despite this, the concept of selective uptake of an analyte of interest and the subsequent generation of a proper electrochemical signal opens an attractive pathway for sensor development (Merkoçi & Alegret, 2002).

There are excellent reviews and monographs of MIPs providing aspects of design, preparation, characterization and application (Alexander et al., 2006; Haupt, 2001; Sellergren, 2001). Different strategies have been investigated for the immobilization of MIPs on electrochemical transducers such as in situ synthesis on electrode surface by electropolymerization (Malitesta et al, 1999) or at a non-conducting surface by chemical grafting (Piletsky et al., 2000) .

The chemical grafting is usually prepared using two strategies "grafting-to" and "grafting-from" (Advincula et al., 2004; Pyun & Matyajaszewski, 2001). The "grafting to" technique involves tethering performed end-functionalized polymer chains to a suitable substrate. This technique often leads to low grafting density and low film thickness, as the polymer molecules must diffuse through the existing polymer film to reach the reactive sites on the surface. The steric hindrance for surface attachment increases as the tethered polymer film is thicker. To overcome this problem, the "grafting from" approach can be used and has generally become the most attractive way to prepare thick, covalently tethered polymer brushes with a high grafting density (Zhao & Brittain, 2000). The "grafting from" technique involves the immobilizing of initiators onto the substrate followed by in situ surface initiated polymerization to generate the tethered polymer brush.

The combination of imprinted films as the sensing element and an electrode as the transducer has been investigated by several groups (Gutierrez-Fernandez et al., 2001; Marx et al., 2004; Shustak et al., 2003). Some of them include molecular imprinting in sol-gel (Gupta & Kumar, 2008) or surface molecularly imprinted self-assembled monolayers (Li et al., 2006; Mirsky et al., 1999; Piletsky et al., 1999a) as strategies for the immobilization of MIPs on electrochemical transducers.

The surface tailoring by the use of molecularly imprinted polymers (MIPs) over a sol-gel matrix is an important strategy for the production of interfaces in which specific binding is improved by reducing non-specific binding (Patel et al., 2009a; Patel et al., 2009b).

This technology allows low temperature fabrication of a surface material starting either from a chemical solution or colloidal particles to produce an integrated network. Such network facilitates surface grafting of MIP, and the resulting hybrid possesses high glass-transition temperature with improved molecular specificity and selectivity.

Other systems, such as self-assembled monolayers (SAMs) and polymer brushes have recently attracted considerable attention due to their noble physicochemical surface property and ease of processing (Boyes et al., 2004). The modification of molecular recognition by SAMs, is based on the formation of rigid nanostructures organized around the template molecule by SAMs at the electrode surface and can be considered as a form of two-dimensional imprinting. The preparation of these systems involves the simultaneous adsorption of the template and mercaptan molecules at a metalic surface. The recognition is possible if specific interactions are developed to form a stable complex between the template and the alkylthiol chain forming the monolayer (Piletsky et al, 1999a). Polymer brushes refer to an assembly of polymer chains which are tethered by one end to a surface or interface. They have recently attracted considerable attention and there have been numerous studies to examine their structure and novel properties (Zhao & Brittain, 2000).

One way for MIPs deposition on the surface of different types of electrodes is the use electropolymerization techniques. Advantages of electropolymerization include the ability to coat very small or irregularly shaped electrode surfaces with a polymeric film, the ability to control film thickness based upon the amount of charge passed in the case of conducting films or by self-regulation in the case of non conducting films, and the ability to influence both the polymerization rate and the nature of the film via the applied potential use during electropolymerization. To prepare these molecularly imprinted layers on electrodes, such as polypyrrole (Ramanaviciene et al., 2006), overoxidized polypyrrole (Ozkorucuklu et al., 2008), polyaniline (Manisankar et al., 2008a) polyphenylendiamines (Liu et al., 2009; Malitesta et al, 1999), polyphenol (Panasyuk et al., 1999) or in some cases redox polymers such as metalloporphyrins (Gomez-Caballero et al., 2010; Mazzotta & Malitesta, 2010) have been used. The growth of these types of MIPs depends on the nature of the synthesized polymers. If the resulting polymer is conducting, the polymeric growth is practically unlimited, on the other hand, if the polymer is nonconducting, the growth would be self-limited obtaining very thin polymeric layers (10-100 nm) (Ulyanova et al., 2006).

Recently, conducting polymers have attracted attention as-sensitive layers in electrochemical sensors. These polymers are characterized by a high electrical conductivity and a good electrochemical reversibility, which justify their use as transducer in the fabrication of efficient electrochemical sensors. Thus, the objective of high specificity together with high sensitivity can be achieved by the combination of the concept of Molecularly Imprinted Polymers with the use of conducting polymers. In this context Pardieu et al. (2009) proposed the design of molecularly imprinted conducting polymers (MICPs) as a transducers for the selective and real time recognition of the herbicide atrazine. Two moieties are necessary for the building of such chemically functionalized, conducting polymer-based sensing layer, a molecular sensing unit- functional monomer -, together with a conjugated linker.

Electropolymerization has been successfully utilized for the preparation of electroactive and electroinactive polymers on a variety of conductive surfaces including, glassy carbon (Liu et al., 2006), graphite (Ozcan & Sahin, 2007; Weetall & Rogers, 2004) , gold (Feng et al., 2004; Liao et al., 2004), platinum (Xu & Chen, 2000) or indium tin oxide (Gao et al., 2007).

An electrochemical sensor based on molecularly imprinted polypyrrole membranes is reported by Xie et al. (2010a) for the determination of herbicide 2,4-dichlorophenoxy acetic acid (2,4-D). The sensor was prepared by electropolymerization of pyrrole on a glassy carbon electrode in the presence of 2,4-D as a template. The sensor can effectively improve the reductive properties of 2,4-D and eliminate interferences by other pesticides and

electroactive species. The method has been successfully applied to the determination of 2,4-D in environmental water samples, with recovery rates ranging from 92% to 108%.
Also, combinations of two or more functional monomers have been proposed, which originate in most cases copolymers that have higher recognition skills, in comparison with the polymers generated with single monomers. Electro-copolymerization of aniline with o-phenilendiamine was successfully used for the preparation of a metamitron selective voltammetric microsensor (Gomez-Caballero et al., 2007). The authors propose the modification of the surface of a carbon fiber microelectrode to create an MIP selective for the herbicide metamitron, employing the electrosynthesis as a technique for modifying the electrodic surface. The response of the synthesized microsensor was able to distinguish the metamitron from other closely related triazine herbicides as isomethiozin or metribuzin.
Electrosynthesis of MIPs can be carried out in aqueous or in organic media, but aqueous media are usually chosen to electrosynthesize imprinted polymers. The nature of the polymerization mixture generally depends on the solubility of the target analyte or the monomers. Gomez-Caballero et al. (2008) propose the synthesis of an MIP amperometric sensor that owned selectivity towards the pesticide 4,6-dinitro-*o*-cresol, placing particular emphasis on the fact that the methodology could also be used for all other dinitrophenolic pesticides. Because the template concentration was a key factor when imprinted sites had to be generated, in this work a semiorganic media was chosen to carry out the electrogeneration of the copolymer in order to favor the dissolution of the template molecules.
Although the amount of the imprinted sites increases with the increase of the imprinted membrane thickness, thick imprinted membranes could lead to slow diffusion of analytes to the recognition sites and to inefficient communication between the binding sites and transducers (Riskin et al., 2008). To further increase the amount of effective imprinted sites on the sensor surface, the simplest method is to use a higher electrode surface area (He et al., 2008) through the assembly of gold nanoparticles (AuNPs) at the surface of electrodes (Daniel & Astruc, 2004; Feng et al., 2008; Yu et al., 2003) due to its large specific surface area, good biocompatibility and high conductivity. Herein Xie et al. (2010b) discuss how the combination of surface molecular imprinting on a large surface area of a AuNP-modified glassy carbon electrode produces a high ratio of imprinted sites and, thus, provides an ultrasensitive electrochemical detection of organophosphate pesticides. The combination of surface molecular self-assembly with electropolymerization of aminothiophenol on a larger area of AuNP-modified electrode was expected to produce a high ratio of imprinted sites and to enhance the total amount of effective imprinted sites. The electrochemical sensor not only can strikingly improve the sensitivity and selectivity of pesticide chlorpyrifos analysis but also obtains good repeatability and, thus, can be potentially exploited for the detection of pesticide residues in the environment.
Choong et al. (2009) demonstrated that a free-standing carbon nanotube array serves as a high porosity 3D platform for the deposition of molecularly imprinted polypyrrol. The greatest advantage of this organised, 3D structure is that the thickness of the MIPPy film coated around each CNT can be adjusted accordingly to accommodate target molecules with different sizes.

2.1 Transducers

Apart from the recognition element, the other important part of the sensor is the transducer. In this chapter we will consider those transducers based on the measurement of a signal

caused by a change in properties of the system as a result of binding of the analyte with the MIP-transduction by capacitance, conductimetry and potentiometry- or measurement signal if the analyte undergoes electron transfer reaction - amperometry and voltammetry-.
MIPs based conductimetric (Sergeyeva et al., 1999a), voltammetric (Alizadeh, 2009) potentiometric (Prathish et al., 2007) and capacitive sensors (Gong et al., 2004) have been reported for determination of pesticides.
Despite the relatively simple transduction of the potentiometric signal, only a few sensing devices of this kind have been developed. All of them were based on the use of very thin membranes or films (Zhou et al., 2004). Prasad et al. (2007) report a new strategy to construct a potentiometric biomimetic sensor for direct, rapid and highly selective detection of atrazine. The stability, reusability and dynamic response time are analogous to conventional chemical sensors. The utility of the sensor was successfully tested for field monitoring of atrazine in ground waters. D´Agostino et al. (2006) developed a potentiometric sensor for the herbicide atrazine based on a molecularly imprinted polymeric membrane. The membrane was formed directly at the end of a small Teflon tube which was filled with a solution at constant composition, in contact with an internal reference electrode, as in a classical potentiometric cell for ion selective electrode. Atrazine is protoned, and as a consequence, positively charged in aqueous solution at sufficiently low pH. The combination of the positively charged species with the imprinted membrane should produce a variation of the membrane charge. An interesting characteristic of the potentiometric sensor is that a sort time, only a few seconds, is required to reach the equilibrium potential.
Liang et al. (2010) described a novel strategy for the selective and sensitive detection of the neutral specie chlorpyrifos, using polymeric membrane ISE which is based on a uniform-sized MIP.
Conductimetry is based on the current flow established by migration of ions of opposite charge, when an electric field is applied between two electrodes immersed in the electrolyte solution. Therefore, the development of an MIP-based conductimetric sensor, then, requires the preparation of the MIP as a membrane. In this sense Sergeyeva et al (1999a) developed an atrazine-sensitive conductimetric sensor using molecularly imprinted polymer membranes. These membranes were prepared by copolymerization of methacrylic acid and tri (ethylene glycol) dimetacrylate, in the presence of atrazine as a template. In order to improve the flexibility and mechanical stability of the membranes, oligourethane acrylate was added to the mixture of monomers. The effect of the membrane composition and the porogen concentration on the magnitude of the conductimetric responses was investigated.
Interfacial phenomena can be followed by changes in capacitance or impedance of the system. The requeriment is to have a totally pore-free, thin, dielectric film, usually on gold substrates. Panasyuk et al. (1999) used capacitive detection in conjunction with imprinted electropolymerized polyphenol layers on gold electrodes. In another work, an electropolymerized molecularly imprinted polymer as a selective receptor layer for the pyrethroid insecticide fenvalerate is reported (Gong et al, 2004). A capacitive chemical sensor for fenvalerate based on an electropolymerized molecularly imprinted polymer as sensitive layer was developed. To test the selectivity of the sensor for fenvalerate, an interference test was performed, including common pyrethroid insecticides such as fenpropathrin, deltamethrin, alphamethrin and cypermethrin.
Panasyuk-Delaney et al. (2001) applied the technique of grafting polymerization for the first time to artificial chemoreceptors based on molecularly imprinted polymers. The results

demonstrated the compatibility of capacitive detection with a chemically sensitive polymer layer obtained by grafting polymerization for the herbicide desmetryn.
Lower detection limits could be obtained by voltammetry and amperometry (Blanco-Lopez et al., 2003; Gutierrez-Fernandez et al, 2001; Lakshmi et al., 2006). These methods, which can be used for electrochemically active substances, are generally slow, being based on selective adsorption from the sample on the polymeric membrane, and quantification of the adsorbed analyte in a different solution.
Voltammetric and amperometric methods can be used to detect the rebinding of nonelectroactive substances too, by methods based on the competition with similar substances able to bind to the active sites on MIP but electrochemically active (Kroger et al., 1999). Amperometric sensors have continued to be the most popular largely due to their simplity, ease of production and the low cost of the devices and instruments.
Other electrochemical transduction methods for obtaining a signal indicating the rebinding of nonelectroactive substances are based on the change of the permeability of MIP membrane as a consequence of the recombination of the nonelectroactive substance (Piletsky et al., 1999b), obtaining conductivity sensors as reported for instance by (Sergeyeva et al., 1999b).
An interesting approach for the amperometric detection of the rebound analyte consists on the use of molecularly imprinted conducting polymers. The permeability change after the rebinding can be investigated also by using an electroactive marker able to diffuse through the membrane and to reach the conducting surface. Pesavento et al. (2009) presents the possibility of an amperometric sensor for non electroactive substances obtained by contacting glassy carbon or graphite electrodes with molecularly imprinted polymeric membranes. The idea was that the mobility of H^+ ions in acrylic membranes with cation exchange properties is probably high even in relatively thick membranes, and it is expected to be modulated by the rebinding of the template. To illustrate this concept, two template molecules were considered as representative target compounds, cyanuric acid and atrazine. They have an environmental interest, since atrazine is a widely used herbicide, and cyanuric acid is a product of the natural degradation of triazines.
The stage of electron transfer in voltammetry or amperometry leads to products, which may get fouled on the electrode surface, for this reason electrode cleaning solvents (Blanco-Lopez et al, 2003), a simple mechanical polishing (Andrea et al., 2001) or the use of disposable electrodes may be required.(Liang et al., 2005; Pellicer et al, 2010; Sode et al., 2003) .

3. Micro and nanostructured materials in electrochemical sensors and biosensors

The most important aspects to consider in the development of electrochemical sensors are the sensitivity, selectivity, long-term stability, response time, portability and low cost. So far, great efforts have been made to meet these needs through research in the development of new materials.
The electrochemical reactions of some pesticides have slow kinetics, causing fouling of the electrodes. This, affects the performance of the electrode during electrochemical measurements. In recent times this problem has been overcome by applying suitable modifiers such as hexadecane (Xu et al., 2002), clay (Manisankar et al., 2005a; Manisankar et al., 2006) stearic acid (Navaratne & Susantha, 2000) , crown sol gel film (Li et al., 2005a) ZrO_2 (Liu & Lin, 2005) and bismuth film (Du et al., 2008c) on the electrode surface, improving the performance.

Nafion film modification gave a better selectivity and mass transfer for parathion detection (Zen et al., 1999). Besides, this polymer film selectively preconcentrates the analyte of interest thus improving sensitivity and selectivity.

Metal and semiconductor nanoparticles in the construction of sensing devices have received considerable attention in recent years because of its unique electrocatalytic, chemical and electrical properties (Luo et al., 2006). Silver nanoparticles deposited on the glassy carbon electrode produced better electro catalytic activity and had higher reproducibility than bulky silver. Kumaravel & Chandrasekaran (2010) propose a new approach by electrochemical co-deposition of silver nanoparticles with a Nafion film on a glassy carbon electrode for the sensing of methyl parathion and parathion. The silver/Nafion co-deposited film offers better properties than silver or Nafion deposited individually. The experimental results showed that the nanosilver/Nafion composite electrode not only exhibited strong electro catalytic activity but also exhibited good reproducibility.

In addition to the modification of the electrode material, research in the field of electrochemical sensors has been developed in the area of miniaturization. Miniature sensors and detectors are becoming widespread devices in analytical laboratories. With this regard the replacement of classical graphite electrode or platinum by microelectrodes is atracting special interest. Microsized electrodes provided currents of lower intensity, greater relationship between the faradaic and capacitive current and reduced ohmic drop and their electrochemical answers change dramatically just by changing the diameter or the length of the electrode. The introduction of micrometer-sized electrodes has led to significant advances in studies of single-molecule detection. Related to this, Lopez de Armentia et al. (1999) proposed successfully the use of a carbon fiber microelectrode for the analysis of the herbicide metamitron.

During the last several years, a few research groups have been exploring different methodologies of manufacturing nanometer-sized disks, bands, cones and arrays of ultramicroelectrodes. Developments in the design and fabrication of ultramicroelectrodes offer considerable promises for advances in electrochemical sensors, being specially useful for environmental monitoring purposes (Draper et al., 1999). Another advantage of ultramicroelectrodes is that oftentimes no supporting electrolyte is necessary, owing to the favorable mass-transfer characteristics of tiny electrodes. Hence, it may be possible to use ultramicroelectrodes to measure analytes having very high redox potentials. In addition to ultramicroelectrodes, chemistry in miniature has also been carried out through chemical analysis on microchips. Microdevices on a micrometer scale have been fabricated using centimeter-sized chips comprised of glass, silicon or inert polymeric materials. As reactions can be completed effectively and quickly, analytical performance on a small scale is improved by means of speed and efficiency (Smirnova et al., 2008).

The advent of nanotechnology has led to great advances in detection strategies. Nanomaterials are attractive because they have conductivity, large surface area, chemical functionality and biocompatibility which make them particularly interesting for the development of electrochemical sensors and biosensors (Hernandez-Santos et al., 2002; Luo et al, 2006; Welch & Compton, 2006).

The predominant advantages of using electrodes modified with nanomaterials compared to typical macroelectrodes is their large effective surface area, increased mass transport, high catalytic activity, and the ability to exert control over the local environment at the electrode surface (Katz & Willner, 2004; Welch & Compton, 2006).

Carbon nanotubes CNTs have generated great interest in applications based on their field emission and electronic transport properties, their high mechanical strength, high-surface to volume ratio and their chemical properties (Baughman et al., 2002; Rao et al., 2001). The unique properties of CNTs make them extremely attractive for the task of electrochemical sensors (Luo et al., 2001; Luo et al, 2006; Zhao et al., 2002). Recently, it has been shown that CNTs can impart strong electrocatalytic activity, adsorption properties and minimization of surface fouling onto electrochemical devices (Andrews & Weisenberger, 2004; Salimi et al., 2007; Tu et al., 2005).

CNTs include both single-walled (SWCNTs) and multi-walled (MWCNTs) structures. SWCNTs comprise of a cylindrical graphite sheet of nanoscale diameter capped by hemispherical ends. The closure of the cylinder is result of pentagon inclusion in the hexagonal carbon network of the nanotube walls during the growth process. SWCNTs have diameters typically of 1 nm with the smallest diameter reported to date of 0.4 nm.

The MWCNTs comprise several to tens of incommensurate concentric cylinders of these graphitic shells with a layer spacing of 0,3-0,4 nm. MWCNTs tend to have diameters in the range 2-100 nm. These can be considered as a mesoscale graphite system, whereas the SWCNTs is truly a single large molecule.

The electronic, chemical and mechanical properties of CNTs can be tailored by replacing some of the carbon atoms with either boron or nitrogen. From the chemical point of view these doped structures would be more likely to react with donor or aceptor molecules, depending on the doping. Although not so far reported in electrochemical sensing systems, these B or N doped CNTs should merit future attention (Hsu et al., 2000; Kuo et al., 2008).

Functionalization of carbon nanotubes with various kinds of materials is gaining more attention as the different properties of the attached functionalities are required for specific applications (De la Torre et al., 2003; Rubianes & Rivas, 2003). Carbon composites have received considerable attention, thus, Wang & Musameh (2003) reported on a new and simple avenue for preparing effective CNT/Teflon composite- based electrochemical sensor and biosensors. The use of Teflon as binder for graphite particles has shown to be extremely useful for various electrochemical sensing applications. The resulting CNT/Teflon material brings new capabilities for electrochemical devices by combining the advantages of CNT and "bulk" composite electrodes. The accelerated electron transfer is coupled with minimization of surface fouling and surface renewability.

The use of multiwalled carbon nanotubes (MWCNTs) in composite materials is well established (Andrews et al., 2002; Manisankar et al., 2009; Sun et al., 2002a; Sun et al., 2002b). Li et al. (2005b) have also made a novel amperometric sensor for determination of the pesticide parathion from a multiwall carbon nanotubes/Nafion film-modified glassy-carbon electrode. Results suggest that the current response to the MWCNT/Nafion film electrode to parathion is highly sensitive and stable.

Carbon nanotubes are hydrophobic materials, rendering difficult the adhesion of metal deposits. In order to improve metal deposition onto nanotubes, two main approaches are possible: surface modification and sensitization activation. The former is associated with the oxidation of the nanotube surface, in order to create functional groups and increase metal nucleation (Sun et al., 2002c). The latter involves the generation of small nuclei to further promote metal deposits on carbon nanotubes (Ang et al., 2000; Liu et al., 2002). In this sense, Sun et al. (2003) show that it is possible to obtain very small Pt particles deposited onto multiwalled carbon nanotubes (MWCNTs) and that these metal particles are in electrical

contact, through the MWCNT, with the carbon backing, enabling the composite structure to be used as an electrode.

Also the atom transfer radical polymerization (ATRP) is a very powerful technique in functional macromolecular design and new material preparation, which has been applied successfully to the controlled functionalization of carbon nanotubes. Based on ATRP, some homopolymers and copolymers were grown on the surface of CNTs (Baskaran et al., 2004; Qin et al., 2004; Yao et al., 2003). Thus, such in situ surface initiating ATRP provides a remarkable route to tailor the structure and properties of the modified CNTs and a way to construct novel CNT-based hybrid nanomaterials (Kong et al, 2004).

Usage of conducting polymers as modifiers is a very promising field (Manisankar et al., 2002; Manisankar et al., 2004) because MWCNTs exhibit excellent electrocatalytic and adsorption properties (Ajayan, 1999) and conducting polymers exhibit preferential accumulation of analytes on bound surface functionalities (Manisankar et al., 2005b). In this sense it has been proposed a multiwalled carbon nanotubes modified glassy carbon electrode, covered with polyaniline and polypyrrole coating for the electrochemical reduction of the herbicide isoproturon, the insecticide voltage and the acaricide dicofol (Manisankar et al, 2008a) or of the insecticides cypermethrin, deltamethrin and fenvalerate (Manisankar et al., 2008b; Siswana et al., 2010) report that nanostructured nickel (II) phthalocyanine/multiwalled carbon nanotubes composite supported on a basal plane pyrolitic electrode, could serve as a viable platform for the sensitive electrocatalytic detection of the carbamate pesticide asulam. The selection for the nanoparticles of nickel (II) phthalocyanine is motivated by the envisaged enhanced electrocatalytic properties for high-surface area metallophthalocyanine nanoparticles species. By employing conducting polymer modified electrodes, the sensitivity is increased and the detection limit is considerably lowered (Manisankar et al, 2005b).

Among polymers selected to bond with CNTs, water-soluble polymers are very attractive because the functions of both the polymer and CNTs can be tailored to create one object and the so-prepared water-soluble nanocomposites have potential and versatile applications. So, Kong et al. (2005) proposed two kinds of water soluble anionic polyelectrolyte – polyacrylic acid(PPA) and poly(sodium-4-styrenesulfonate) (PPS), that were grafted onto the convex surfaces of multiwalled carbon nanotubes (MWCNTs) by "grafting from" approach. The process was conducted by the surface-initiating atom tranfer radical polymerization (ATRP) from the initiating sites previously anchored to MWCNTs. The grafting polymer amount can be efficiently controled by the feed ratio of monomer to MWCNT-supported macroinitiator (MWCNT-Br).

On the other hand the carbon nanotubes are excellent substrates for supporting metal nanoparticles. In particular the use of gold particles for the creation of electrochemical sensing devices is proved to be very promising. Although gold is a poor catalyst in bulk form, nanometer-sized gold nanoparticles can exhibit excellent catalytic activity due to their relative high surface area-to-volume ratio, and their interface-dominated properties, which significantly differ from their bulk counterparts (Shipway et al., 2000). Yu et al. (2003) focus their study on the electron-transfer characteristics of gold nanoparticles in polyelectrolyte multilayers, and the capacity of such films (with varying gold nanoparticle loadings) to act as electrochemical sensors.

Zhang et al. (2009) developed an electrochemical sensor based on electrodeposition of gold nanoparticles on a multi-walled carbon nanotubes modified glassy carbon electrodes. Their work was based on the synergistic properties that other authors had found for the

composite nanoparticles-nanotubes (Jiang & Gao, 2003). The composite structure increases electrocatalytic activities towards the reduction of the pesticide parathion. Due to the high surface area-to-volume ratios and good interface-dominated properties gold nanoparticles have been widely employed as catalysts (Qu et al., 2008; Shipway et al, 2000).

The remarkable specificity of biological recognition processes has led to the development of highly selective biosensing devices. A biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element –biochemical receptor- which is in direct spatial contact with a transducer element. Enzymes are still the most appropriate recognition elements because they combine high chemical specificity and inherent biocatalytic signal amplification.

Biosensor technology is well suited for field monitoring pesticides. Many studies are focused on enhancing the electrochemical properties of electrodes through the modification of the working electrode (Artyukhin et al., 2004) and improving the efficiency of enzyme immobilization (Katz & Willner, 2004; Saini et al., 2003). So, Zhang et al. (2008) described a controllable layer-by-layer self-assembly modification technique of multi-walled carbon nanotubes and poly(diallyldimethylammonium chloride) on glassy carbon electrode and introduced a controllable direct immobilization of acetylcholinesterase on the modified electrode.

Many of the electrochemical biosensors that have been developed for the detection of pesticides are based on the interaction with the enzyme acetylcholinesterase (AChE) (Du et al., 2007a; Du et al., 2007b; Kok & Hasirci, 2004)

In recent years, works are focused primarily on the oriented and site-specific immobilization of the enzyme, which has become crucial for the rational design of biosensors. Effective immobilization of the enzyme to a solid electrode surface still represents a great challenge for the fabrication of biosensors. General methods include direct physical adsorption onto a solid supporting matrix and a subsequent entrapment in different substrate materials (Sotiropoulou & Chaniotakis, 2005).

Some authors propose the use of organic conducting polymers as the enzyme-hosting matrix for biomolecules, due to its advantages of permitting a facile electronic charge flow through the polymer matrix, easy preparation, high conductivity and good stability (Njagi & Andreescu, 2007; Vidal et al., 2003). Recently, nanoparticles, specially the gold nanoparticles have been extensively used owing to their extraordinarily catalytic activity, good conductivity and biocompatibility (Willner et al., 2006). Also, gold nanoparticles and polymers can be assembled to act as an immobilization matrix of AChE. This resulting composite matrix exhibits a porous structure with large effective surface areas, good conductivity and high catalytic activity, which greatly facilitates electron-tranfer processes and the action of the immobilized AChE for organophosphorous pesticides (Gong et al., 2009)

Qu et al. (2010) propose as electrode modifier a layer-by-layer self-assembled acetylcholinesterase/dendrimers polyamidoamine-Au-carbon nanotubes configuration. The dendrimers polyamidoamine provides to electrode favorable configuration for the immobilization of AChE. This biosensor system was used successfully in pesticides sensing.

Usually these methods rely on enzyme immobilization directly onto the electrode surface. Although the proximity between the enzyme molecules and the electroactive surface provides a fast response of the biosensor, it can not overcome the biofouling of the electrode

surface, which would eventually lead to the deactivation of the biosensor or at least to the worsening of the electrochemical response. After a suitable chemical modification, for example with a polymeric membrane that could provide various functional groups, it is possible the selection of a more appropriate enzyme immobilization protocol. By using a membrane of acrylonitrile (AN) copolymer as a support for the enzyme immobilization, the need of constantly cleaning the electrode surface after each immobilization procedure, could be avoided. The main disadvantage of AN copolymer membranes comes from their hydrophobic and non-conducting properties. The referred disadvantage could be overcome by using carbon nanotubes as electron transfer mediators from the enzyme molecules to the electrode surface (Wang, 2005). The combination of the highly conductive and electrocatalytic behaviour of integrated multiwall carbon nanotubes membrane with the controlled site-specific enzyme immobilization results in a stable and sensitive sensor towards paraoxon (Ivanov et al., 2010).

In order to develop a biosensor for the detection of organophosphate pesticides, a novel acetylcholinesterase biosensor based on an enzyme immobilized in a membrane electrode was described (Sun et al. 2010). The electrode was modified with multiwall carbon nanotubes, and acetylcholinesterase was cross-linked with glutaraldehyde and then attached to chitosan membrane. During the detection, the enzyme membrane was fixed on the surface of multiwall carbon nanotubes/glassy carbon electrode with O-ring and thus the amperometric acetylcholinesterase-multiwall carbon nanotubes/glassy carbon electrode sensor was used for detecting organophosphate pesticides. The biosensor exhibited good reproducibility and stability and provided a new method for detecting organophosphate residues.

Du et al. (2008b) propose the immobilizatión of AChE onto a multiwalled carbon nanotube (MWCNTs) modified electrode prepared by controllable adsorption of the MWCNTs onto hexyl mercaptan self-assembled monolayers. After immobilization a stable biosensor was constructed for rapid determination of carbaryl. Likewise, they have reported the functionalization of carbon nanotubes (CNTs) by cyclodextrins. The amperometric biosensor based on the immobilization of AChE on MWCNTs-β-cyclodextrin composite modified glassy carbon electrode, has a high sensitivity for organophosphate pesticides (Du et al., 2010).

Likewise quantum dots (QDs) as cellular labeling in electrochemical bioassay were reported (Du et al. 2008a). The authors described a simple enzyme biosensor based on immobilized AchE on CdTe-QDs gold nanoparticles (GNPs) composite modified chitosan microsfere interface. This article analyzes the synergic effect between CdTe QDs to facilitate electron-transfer processes and the action of the chitosan as a matrix for the immobilization of AchE.

Recently, amperometric biosensors based on the immobilization of organophosphorus hydrolase (OPH) have gained much attention. An OPH-based biosensor for the detection of organophosphorus was developed by using mesoporous carbon (MC) and carbon black (CB) as anodic layer. The MC/CB/glass carbon layer exhibited an enhanced amperometric reponse relative to a carbon nanotube-modified electrode (Lee et al., 2010).

4. Enantioselective electrochemical sensors

The chirality is a very important concept in the field of pesticides, whose significance has been recognized in relation to the relative biological activity of the individual enantiomers (Hegeman & Laane Remi, 2002) and their use as biological tracers has been also investigated (Bidleman & Falconer, 1999a; Bidleman & Falconer, 1999b)

The movement in the biosphere of chiral pesticides and their residues can be traced through determinations of enantiomeric excesses. Transport phenomena, volatilization, leaching, atmospheric deposition and reactions, abiotic hydrolysis or photolysis do not alter the enantiomeric excess. However, the metabolism of pesticides by microorganisms and enzymes in higher animals can alter it. Therefore the determination of enantiomeric excess indicates the biological degradation of these products and can report the origin of pesticides in ecosystems.

The introduction of different types of enantioselective sensors and biosensors increases the reliability of the assays as the enantiomer can be determined without prior separation, directly from the matrix with only dissolution and dilution step being involved. Many systems have been reported for the molecular recognition of both enantiomers in an injection analysis system using two amperometric biosensors (Stefan et al., 2000a) or with a potentiometric enantioselective membrane electrode for the S-enantiomer assay, and with an amperometric biosensor for the R-enantiomer assay (Stefan et al., 2000b)

Enantioselective immunosensors are the only type that may be considered to be enantiospecific because the antibody is not reacting with the other enantiomer.

One of the most critical tasks in molecular recognition of enantiomeric pairs is the selection of the chiral selector or the enzyme that favors only the reaction with the enantiomer that must be selected (Gübitz & Schmid, 2004).

Certain chiral selectors can form inclusion complexes or *host-guest*, in which the analyte-molecule guest- is spatially included inside of a host-ligand-molecule. Among these chiral ligands cyclodextrins and crown ethers can be considered as the most representative ones (Maier et al., 2001; Shahgaldian & Pieles, 2006) . The functionalization of cyclodextrins often improves their enantioselectivity when the grafted groups are able to interact with some families of guests (Bellia et al., 2009).

Another relevant fact to be considered regarding the development of enantioselective sensors is the selection of the best matrix for the electroactive material. The most employed matrices are the carbon paste electrode or polymer based structures.

Also MIPs have promising prospects in chiral discrimination (Mahony et al., 2005; Maier & Lindner, 2007; Yan & Ramström, 2004). Most authors propose for this purpose, the synthesis of MIPs by non-covalent bonds.

Recently electrosynthesized polymers such as poly(dopamine) (PDA) and poly(*o*-phenylendiamine) (P*o*-PD) have been prepared to form a novel well-defined structure MIP at an electrode. The designed copolymer film could be used for the recognition of the enantiomer, as a capacitive sensor (Ouyang et al., 2007)

On the other hand some authors have focused their studies on the "gate effect" which is a phenomenon where solute permeability of the molecularly imprinted polymer membrane changes with specific binding with the template (Piletsky et al, 1999b). The "gate effect" is probably caused by a morphological change in the polymer matrix due to specific binding with the template, similar to the "induced fit" of the natural receptors. Sekine et al. (2007), have evaluated chiral discrimination of phenyl-alanine anilide (PAA) in organic solvents by an electrochemical method using a L- or D- PAA imprinted polymer grafted on an indium-tin-oxide electrode. The results indicate that the gate effect of MIP can discriminate between the template and its analogue by stereochemical structure.

Another alternative would be the association of MIPs with macrocyclic host molecules, to improve the selectivity and the sensitivity particularly in complex liquid phases (Dickert & Hayden, 1999). Molecules such as cyclodextrins have been thoroughly studied as hosts for

organic compounds of low molecular weight (Shahgaldian & Pieles, 2006). These hosts have hydroxyl functional groups that can be used to couple covalently in the polymer matrix. On the other hand crown ethers are macrocyclic polyethers consisting of a number of oxygen atoms, which form a plane, bound by chains of two carbons, some of which contain acid groups. The ring creates a cavity capable of forming complexes with alkali and alkaline earth cations as well as protonated primary amines. The primary amine can penetrate into the cavity forming hydrogen bonds with oxygen atoms, however the enantioseparation comes from the interaction between the substituents of the asymmetric centers and the ring by acid groups by hydrogen bonds and/or electrostatic interactions (Kim & Spivak, 2003).

New MIPs were developed by (Piletsky et al. 2005) for the enantiomers of phenylalanine using functional monomers such as bis-acryloyl β-CD and 2-acryloylamido-2-methyl-1-propanesulfonic acid. The roles of the hydrophobic interacting cyclodextrin and the electrostatic interacting sulfonic acid monomers were examined.

The synthesis of optically active polymers has attracted considerable interest for application as chiral sensors (Shuangyan et al., 2004). These studies have been largely focused on optically active polypyrrole, which is electrochemically deposited on electrodes of platinum or glassy carbon (Liang et al, 2005; Schwientek et al., 1999). There are also many studies that propose the use of optically active polyaniline (Kane-Maguire & Wallace, 2010) .This can be generated via two routes, by doping of base emeraldine with (+) o (-) camphorsulfonic acid (HCSA) or by the enantioselective electropolymerization of aniline in the presence of these chiral acids (+) or (-) CSA. The observed macroasymmetry of the polyaniline so generated was rationalised in terms of the polyaniline chain adopting a preferred one–sense helical screw maintained by the dopant anions via electrostatic and hidrogen bonding (Li & Wang, 2003; Reece et al., 2001). The major limitation of conducting polymer processing arises from their insolubility in most common solvents. The formation of coloidal dispersions is an attractive alternative route to solution processing. Conducting polymer colloids can be produced by electrochemical oxidation of monomers in the presence of a steric stabiliser. Colloids produced electrochemically are formed by intercepting the polymer deposition on the electrode surface utilising hydrodinamic control. This is facilitated by the presence of a steric stabiliser in solution that coats the insoluble polymer upon formation, preventing deposition. The electrochemical approach is advantageous in that the polymer properties can be altered by accurate control of the oxidation potential during polymerisation. This technique also allows a wide range of dopants to be incorporated into the polymer to give different properties. For example, proteins can be incorporated into conducting polymers whilst retaining their biological integrity (Barisci et al., 1999).

Aboutanos et al. (1999) reported the preparation of chiral polyaniline-camphorsulfonic acid colloids using silica as the dispersant in the absence of a steric stabiliser, whereas Caramyshev et al. (2007) proposed the micelar synthesis of chiral conducting polyaniline in presence de dodecylbenzenesulfonic acid using an enzyme as a catalyst for aniline polymerization. Okuno et al. (2002) also proposed a polypyrrole colloid that had been prepared electrochemically and overoxidized at +1.5 V to create a complementary cavity for recognition of molecules structurally similar to the dopant.

Likewise Huang et al. (2008) successfully prepared polypyrrol nanowires by electrochemical polymerization on a platinum electrode, in which chiral camphorsulfonic acid molecules acted as both the dopant and pseudo-template. The enantioselective sensoring and uptaking for corresponding chiral phenylalanine were confirmed.

On the basis of the undertaken studies it is clear the need to give a step forward in the field of chiral sensing and it is very likely that future trends will be focused on the development of new matrices based on the molecular imprinting technique itself or combined to nanomaterials.

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15

Organophosphorus Pesticides Determination by Electrochemical Biosensors

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1. Introduction

According to the definition given by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA, 2008), a pesticide is any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest (insects, mice and other animals, unwanted plants, fungi, microorganisms such as bacteria and viruses, and prions). Considering their chemical structure, the pesticides are organophosphorus, carbamates, organochlorines, and pyrethroid ones (U. S. EPA, 2009). Currently, more than 30 % of the registered pesticides in the world market (Hill, 2003) and about 45 % of those registered with U. S. Environmental Protection Agency (EPA) (Roger & Dagnac., 2006) are organophosphorus (OP).

The organophosphorus pesticides, because of their high toxicity, fast biodegradation, low bioaccumulation, and broad target spectrum are extensively used in the agricultural and veterinary practices for protection of field and fruit crops, and for parasites control in domestic animals. However, their intensive and indiscriminate application, as well as their high acute toxicity generated risks to man and his environment. The resulting public concern created a demand for the development of reliable, sensitive, simple and low-costing methods for their fast " *in field*" detection.

In this work are reviewed the principles of the emerging electrochemical biosensors based methods for organophosphorus pesticides determination during the last decades, as methods of choice for "*in situ*" and "*on line*" application. Two main analytical techniques are considered, involving respectively the direct enzyme transformation of the pesticide and its enzyme activity inhibition effect, both followed by the conversion of the signal produced by the interaction between the bioreceptor and the analyte, into electrical one. The advantages and the drawbacks of each of them are discussed. The recent trends in the development of electrochemical biosensors for OP pesticides quantification, including nanomaterials transducer modification and genetic engineering of the biological recognition element are revised. Special attention is paid to the electrochemical biosensors based methods application for OP pesticides residues detection in food and in the environment.

2. The electrochemical biosensors

The biosensors are analytical devices, products of the current progress in biotechnology and material science, in concert with the modern principles of transduction of the chemical information. They are considered, due to their selectivity and specificity, as a very promising variety of chemical sensors. The electrochemical biosensors in particular use a biological recognition element retained in direct spatial contact with an electrochemical transducer (Thévenot et al., 1999) to obtain an analytically useful signal by coupling biochemical and electrochemical interactions.

The biorecognition elements or bioreceptors, according to the biochemical event, are of two main types (Thévenot et al., 1999): biocatalytic and biocomplexing (bioaffinity based). The biocatalytic ones include enzymes, whole cells (bacteria, fungi, eukaryotic cells or yeasts) or cell organelles and particles (mitochondria, cell walls), and tissues (plant or animal tissues). The biocomplexing receptors are mainly antibodies, biomimetic materials, cell receptors, and nucleic acids.

The applied electrochemical transduction mode (Thévenot et al., 1999) is commonly potentiometric or amperometric one.

The potentiometric determinations are based on the measurement of the emf of a galvanic element, constituted by an indicator and a reference electrode. The potential of the indicator electrode depends on the analyte concentration, according to the Nernst equation, while the potential of the reference electrode remains constant. The exponential character of the relationship between the potential of the indicator electrode and the analyte concentration defines the wide dynamic concentration range of the determinations (3-4 decades), but also the low accuracy and precision of the method.

The amperometry involves the measurement, at a constant potential, of the current response of an indicator electrode, as a function of the concentration of the present electroactive specie. The amperometric detection presents several advantages: (i) controlling the process through the electrode potential; (ii) high sensitivity and precision of the determinations; (iii) linear calibration plot.

The retention of the biological component of the biosensor in contact with the transducer is performed by its immobilization. The biorecognition element being usually an enzyme, the term "enzyme immobilization" was defined at the First Enzyme Engineering Conference held at Hennicker, NH, USA, in 1971. It describes "enzymes physically confined at or located in a certain region or space with retention of their catalytic activity and which can be used repeatedly and continuously" (Powel, 1996). This method ensures several issues as the effective use of the enzyme and its stabilization, the localization of the interaction, the prevention of product contamination, etc. (D'Souza, 1982).

Biorecognition elements immobilization is achieved applying various techniques including (Thévenot et al., 1999): entrapment behind a membrane, entrapment within a polymeric matrix, entrapment within self-assembled monolayers, covalent bonding, bulk modification of entire electrode material (carbon paste or graphite epoxy-resin), etc.

The biosensors are self-contained (Thévenot et al., 1999), simple to handle and able to provide information in real time, without or with a minimum sample preparation (Andreescu & Marty., 2006). These performances, in concert with their sensitivity, selectivity and low cost, make them suitable for "*in field*" and "*on line*" analysis, and an excellent complement to the expensive and time-consuming classical analytical techniques.

3. OP pesticides determination by electrochemical biosensors

3.1 Acylcholinesterases based sensors for OP pesticides determination

The acylcholinesterases (acetylcholinesterase EC 3.1.1.7 and butyrylcholinesterase EC 3.1.1.8) are enzymes, belonging to the class of the hydrolases. Acylcholinesterases based sensors exploit their ability to catalyze the following reactions:

$$\text{R-choline} + H_2O \rightarrow \text{choline} + \text{R-COOH} \quad (1)$$

or

$$\text{R-thiocholine} + H_2O \rightarrow \text{thiocholine} + \text{R-COOH} \quad (2)$$

where R is usually an acetyl or butyryl moiety.

Acetylcholinesterase (AChE) demonstrates a high specificity toward acetylcholine, while butyrylcholinesterase (BuChE) is less specific and hydrolyses a number of choline esters, including acetylcholine. Apart from the natural substrates, acylcholinesterases catalyze the hydrolysis of the synthetic thiocholine esters, too (reaction 2).

OP pesticides determination using acylcholinesterases based sensors involves enzyme inhibition, according to the following mechanism (Aldridge, 1950):

$$\underset{\text{enzyme}}{EH} + \underset{\text{OP pesticide}}{(OR)_2P(=O)-X} \rightleftharpoons \left[EH.(RO)_2P(=O)-X\right] \longrightarrow \underset{\text{phosphorylated enzyme}}{HX + (RO)_2P(=O)-E} \quad (3)$$

The resulting reduction in reagents consumption and products release is correspondingly detected applying electrochemical techniques and is correlated to the OP pesticides concentration.

These inhibition based acylcholinesterases sensor for OP quantification have been extensively reviewed (Andreescu & Marty, 2006; Anzai, 2006; Jaffrezic-Renault, 2001; Noguer et al., 1999; Prieto-Simón et al., 2006; Rodriguez-Mozaz et al., 2004; Solé et al., 2003a; Solé et al., 2003b; Tran-Minh, 1985; Turdean et al., 2002). Attention has been paid to two main types of them, according to the transduction mode: the potentiometric and the amperometric ones.

The potentiometric sensors detect the pH shift resulting from the decrease, in the presence of OP pesticides, of the acid release during the enzyme catalyzed hydrolysis of the choline esters (reaction 1). The detection is performed in a single step, using a range of pH-sensitive transducers, varying from the traditional pH glass electrodes (Tran-Minh, 1985) to the ion-selective field effect transistors (ISFET) (Yuqing Miao et al., 2010). The equipment is simple and includes commercially available devices. Drawback of the method, apart of the non-linearity of the biosensor response and the related error of the determination is the increased response time. It varies from 2 to 10 min (Nikol'skaya & Evtyugin, 1992) in dependence of the time needed to reach the equilibrium at the interface between the biosensor and the solution, the measurements being performed under no current flow conditions. In addition, the sensitivity of the analyses of OP pesticides is lower in comparison to that performed using amperometric biosensors (Solé et al., 2003).

The amperometric acylcholinesterases based sensors of first generation take advantage of the following reactions sequence, illustrated in Fig. 1:

$$\text{R-choline} + H_2O \xrightarrow{\text{ChE}} \text{choline} + \text{R-COOH} \quad (1)$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{betaine} + 2H_2O_2 \quad (4)$$

$$2H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (5)$$

or

$$O_2 + 4e^- + 2H_2O \rightarrow 4\,OH^- \quad (6)$$

where ChE is acylcholinesterase and ChO is choline oxidase.

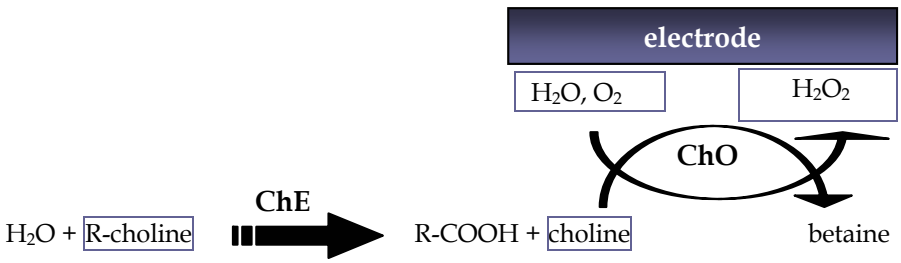


Fig. 1. Inhibition based amperometric biosensor of first generation

R-choline hydrolysis catalyzed by the acylcholinesterases (reaction 1) does not involve electroactive species. Thus, the process has been coupled with the choline oxidase catalyzed betaine oxidation (reaction 4). The current of the oxidation of the produced H_2O_2 (reaction 5) or the current of the reduction of the consumed O_2 (reaction 6), is registered as a sensor response.

The drawbacks of the sensors of first generation are: (i) the sophisticated design, as two enzymes have to be integrated; (ii) the need of optimization of the experimental conditions and the kinetics of the process to ensure the linearity of the biosensor response as a function of the analyte concentration; (iii) the possible interferences at the potential of H_2O_2 oxidation (+0.6 V vs. SCE); (iiii) the fluctuations in the oxygen concentration.

The formation of the analytical signal of the amperometric acylcholinesterases based sensors of second generation results from the combination of the following biochemical and electrochemical reactions:

$$\text{R-thiocholine} + H_2O \xrightarrow{\text{ChE}} \text{thiocholine} + \text{R-COOH} \quad (2)$$

$$\text{thiocholine} \rightarrow \text{dithio-bis-choline} + 2H^+ + 2e^- \quad (7)$$

The enzymatic hydrolysis of the thiocholine esters (reaction 2) generates products able to be easily oxidized. The current of thiocholine oxidation is recorded as a sensor response (reaction 7). Fig. 2 illustrates the occurring processes.

The advantages of the amperometric biosensors of second generation are: (i) the simple detection principle implicating direct thiocholine oxidation and the use of a single enzyme; (ii) the simple biosensor construction, the system being monoenzymatic one.

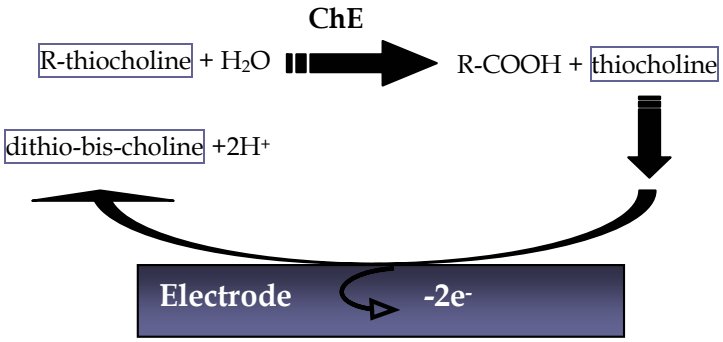


Fig. 2. Inhibition based amperometric biosensor of second generation

The main problems come from: (i) the spontaneous hydrolysis of the thiocholine esters, leading to overestimation of the anodic current response; (ii) the passivation of the platinum anodes by the sulfur-containing compounds; (iii) the high potential of thiocholine oxidation (+0.8 V vs. SCE) at conventional metal and graphite transducers (Martorell et al., 1994; Marty et al., 1992; Marty et al., 1993; Marty et al., 1995; Sužnjević et al., 1985) as a cause of possible interferences.

The non-enzymatic thiocholine hydrolysis could be reduced by dilution in a NaCl (0.09 %, w/v) solution and storage in ice (Andreescu & Marty, 2006).

Potential lowering could be achieved applying an alternative route to obtain response-generating electroactive species, comprising the following stages: (i) acylthiocholine enzymatic hydrolysis (reaction 2); (ii) chemical reduction of the produced thiocholine (reaction 8) using an appropriate electron mediator; (iii) electrochemical mediator regeneration (reaction 9):

$$\text{R-thiocholine} + H_2O \xrightarrow{\text{ChE}} \text{thiocholine} + \text{R-COOH} \quad (2)$$

$$2\text{thiocholine} + M_{ox} \rightarrow \text{dithio-bis-choline} + M_{red} \quad (8)$$

$$M_{red} \rightarrow M_{ox} + 2e^- \quad (9)$$

(M_{ox} and M_{red} are the oxidized and the reduced forms of the mediator M).

The redox potential of the mediator determines the potential of the working electrode. The current of its oxidation is recorded as a sensor response.

This detection principle was exploited using acylcholinesterases sensors, including electrodes chemically modified with mediators such as phtalocyanines (Harlbert & Baldwin, 1985; Hart & Hartley, 1994; Skladal, 1991), Prussian blue (Ricci et al., 2004), tetracyanoquinodimethane (Kulys & D'Costa, 1991; Martorell et al., 1997), ferrocene (Evtugyn et al., 1996), etc. The redox reaction (8) could be performed in a homogeneous phase, too (Neufeld et al., 2000; Ovalle et al., 2009).

Fig. 3 illustrates the measurements carried out using an acetylcholinesterase based amperometric sensor of second generation applying two different strategies. The first one (Fig. 3A), consists in the registration of the amperometric biosensor response to OP pesticide additions in the presence of acetylthiocholine with a constant concentration. The second one (Fig. 3B) involves biosensor current response recording for increasing acetylthiocholine

concentrations before and after the incubation of the enzyme with the pesticide. The latter method is suggested for the determination of slow acting inhibitors (Tran-Minh, 1985).

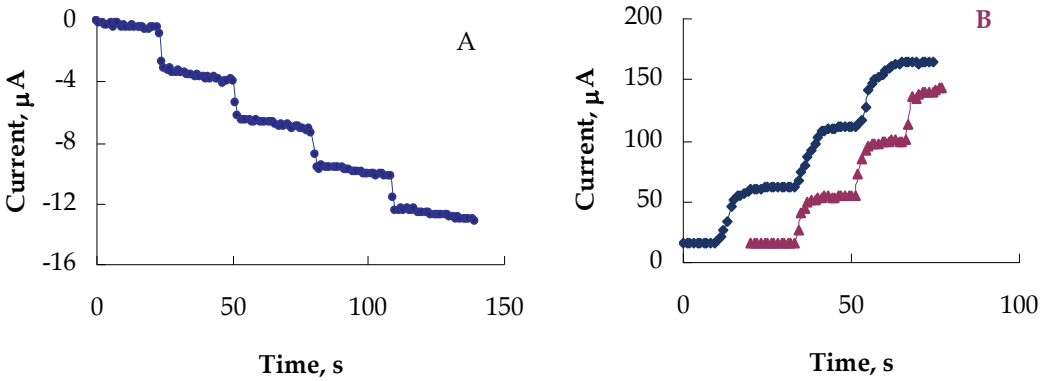


Fig. 3. Amperometric response of an acetylcholinesterase based sensor to: (A) increasing chlorofos concentrations (1.0 to 4.0 mmol L^{-1}, with an increment of 1.0 mmol L^{-1}) and a constant acetylthiocholine concentration (0.2 mmol L^{-1}); (B) increasing acetylthiocholine concentrations (0.2 mmol L^{-1}, 0.4 mmol L^{-1} and 0.6 mmol L^{-1}) before and after 30 min enzyme incubation with chlorofos (4.0 mmol L^{-1}). Applied potential +0.80 V vs. Ag, AgCl, pH 7, 26°C ± 0.5°C, and electrode rotation speed of 1000 rpm. Membrane fraction of rat brain was used as a biorecognition element and source of AChE.

The great advantage of the inhibition based acylcholinesterases sensors for OP pesticides quantification is their sensitivity. The major drawbacks are due to the fact that the determination is not direct, as well as to the lack of selectivity and the need of enzyme reactivation/regeneration.

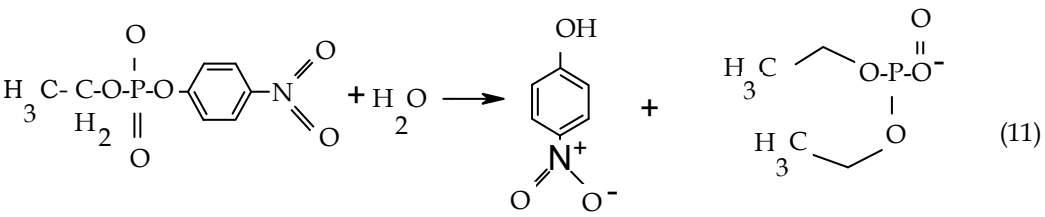
3.2 Organophosphorus hydrolase based sensors for OP pesticides determination

Organophosphorus hydrolase (OPH, EC 3.1.8.1) is a bacterial enzyme, catalyzing the hydrolysis of a range of OP pesticides (paraoxon, parathion, coumaphos, diazinon, dursban, methyl parathion, etc.), according to the following reaction:

$$\underset{\text{OP pesticide}}{RO\text{-}P(=O(S))(OR)\text{-}X} + H_2O \xrightarrow{\text{OPH}} RO\text{-}P(=O(S))(OR)\text{-}OH + HX \qquad (10)$$

The hydrolysis involves a pH change, as well as electroactive species generation, thus allowing the development of potentiometric and amperometric sensors for OP pesticides quantification (Anzai, 2006; Chough et al., 2002; Lei et al., 2007; Mulchandani et al., 2001a; Mulchandani et al., 2001b; Prieto-Simón et al., 2006; Rodriguez-Mozaz et al., 2004; Wang et al., 2003).

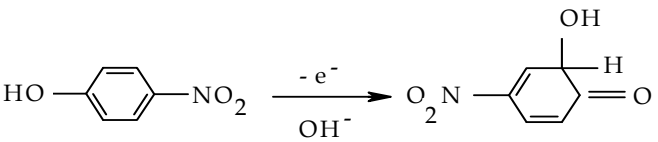
For instance, OPH catalyzed hydrolysis of parathion, methyl parathion, paraoxon, fenitrothion, etc. yields 4-nitrophenol (reaction 11):



(11)

paraoxon 4-nitrophenol diethyl phosphate

The current of 4-nitrophenol oxidation, proportional to the OP pesticide concentration, is recorded as a biosensor response (reaction 12):



(12)

4-nitrophenol

The OPH based sensors for OP pesticides determination allow their selective quantification, applying a simple measurement procedure. However the reported detection limit (Mulchandani et al., 2006) is higher than that reached using acylcholinesterases based sensors. Another disadvantage represents the complex, long-lasting, and expensive procedure for OPH extraction and purification, performed in specialized microbiological laboratories (to note that this enzyme is not commercially available) (Prieto-Simón et al., 2006).

4. Electrochemical biosensors for OP pesticides determination in food and in the environment

The World Health Organization (WHO), the Food and Agricultural Organization of the United Nations (FAO), the Codex Alimentarius Commission, the EU Commission, and the U. S. Environmental Protection Agency (EPA) are among the principal organizations enacting the allowable pesticide residues levels in food, drinking water and environmental samples. The European Council Directive 98/83/EC on the quality of water intended for human consumption (Council Directive 98/83/CE) for instance, 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. EPA 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. 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).

The electrochemical biosensors for OP pesticides determination, despite of their analytical potential, have found until now a limited application for real samples analysis. Some relevant data are presented in Table 1.

Pesticide	LOD	Sample	Reference
Diazinon	35 ppb	Spiked soil	Kumaran & Morita, 1995
Trichlorfon	0.1 μmol L^{-1}	Water	Wan et al., 2000
Parathion	10 ng mL^{-1}	Spiked river water	Sacks et al., 2000
Paraoxon	0.5 μg L^{-1}	Spiked river water	Bachmann et al., 2000
Paraoxon	1 nmol L^{-1}	Potato	Simonian et al., 2001
Paraoxon	0.6-0.8 μmol L^{-1}	Spiked river water	Jeanty et al., 2002
Paraoxon	2 μg kg^{-1}	Orange juices	Schulze et al., 2002
Paraoxon	2 μg kg^{-1}	Peach pap	Schulze et al., 2002
Paraoxon	2 μg kg^{-1}	Baby food	Schulze et al., 2002
Paraoxon-ethyl	10 nmol L^{-1}	Soil samples	Dzyadevych et al., 2003
Paraoxon-methyl	0.5 μmol L^{-1}	Soil samples	Dzyadevych et al., 2003
Trichlorfon	0.3 μmol L^{-1}	Soil samples	Dzyadevych et al., 2003
Paraoxon	0.1 μg L^{-1}	Grapes	Boni et al., 2004
Pirimiphos-methyl-oxon	10 nmol L^{-1}	Wheat and apple	Crew et al., 2004
Pirimiphos-methyl	38 ng mL^{-1}	Durum wheat	Del Carlo et al., 2005
Paraoxon	1.20 μg L^{-1}	Milk	Zhang et al., 2005
Paraoxon	1 μg L^{-1}	Milk	Yang et al., 2005
Paraoxon	0.5 nmol L^{-1}	Water	Joshi et al., 2005
Parathion	9-10.3 μg L^{-1}	Spiked water	Pedrosa et al., 2008
Chlorpyriphos-oxon	2 μg L^{-1}	Waters	Hildebrandt et al., 2008
Chlorpyriphos-oxon	2 μg L^{-1}	Beverages	Hildebrandt et al., 2008
Dimethoate	$5.6x10^{-4}$ ng mL^{-1}	Chinese cabbage	Ning Gan et al., 2010

Table 1. OP pesticides determination in real samples, applying electrochemical biosensors

5. Recent trends in the development of electrochemical biosensors for OP pesticides determination

Two main strategies to improve electrochemical biosensors performances emerged during the recent years: nanomaterials transducer modification and genetic engineering of the biological recognition element.

The nanotechnological approach in electrochemical biosensors development (Balasubramanian & Burghard, 2006; Eftekhari, 2008; Gorton, 2005; Guo & Wang, 2007; Kerman et al., 2008; Kumar, 2007; Luo et al., 2006; Merkoçi & Alegret, 2005; Merkoçi, 2009; Pumera et al., 2007), takes advantage of the electrocatalytical properties of the nanostructures, their action as electron transfer mediators or electrical wires, large surface to volume ratio, structural robustness, and biocompatibility. Therefore, it yielded the following chief issues: electrode potential lowering, enhancement of the electron transfer rate with no electrode surface fouling, sensitivity increase, stability improvement, and interface functionalization.

Various nanomaterials used as acetylcholinesterase immobilization matrices in electrochemical biosensors for organophosphorus pesticides determination, along with biosensors performance characteristics such as sensitivity, linear dynamic range, and detection limit are evaluated and summarized in the review work of Periasamy et al. (Periasamy et al., 2009). As demonstrated, the nanomaterials transducer modification confers long storage stability of the biosensors, and enables OP pesticides detection in the nanomole – picomole range.

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. These performances are achieved through appropriate site-directed mutagenesis ensuring increased biorecognition element affinity for the target analyte favoring the accessibility of the active site, enhanced electron transfer, and oriented or more stable immobilization (Campàs et al., 2009; Lambrianou et al., 2008).

Genetically modified enzymes are extensively used in inhibition based biosensors for OP pesticides determination (Bachmann & Schmid, 1999; Bucur et al., 2005; Marques et al., 2005; Nunes et al., 2001; Valdés-Ramírez et al., 2008), allowing attaining LOD as low as 10^{-17} M (Sotiropoulou et al., 2005).

6. Conclusion

In the recent decades, new methods for OP pesticides determination were developed, taking advantage of the unique analytical performances of the electrochemical biosensors. They made possible the reliable, fast, sensitive, simple and low-costing, "*on line*", "*on site*", and in real time pesticides quantification. This review gives a survey on the state of the art of organophosphorus compounds analysis using enzyme-based electrochemical sensors, pointing out on their advantages, drawbacks, real samples application and characteristics improvement.

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Mercaptobenzothiazole-on-Gold Organic Phase Biosensor Systems: 4. Effect of Organic Solvents on Organophosphate and Carbamate Pesticide Determination

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1. Introduction

With an ever increasing demand for agricultural products delivered over shorter periods, we have seen the use of agricultural chemicals such as pesticides increasing in order to effectively control the damaging effects of harmful micro-organisms and insects. This has also resulted in an increase of usage of insecticides that are classified as cholinesterase (ChE) inhibitors such as organophosphates (OPs) and carbamates (CMs), to protect various crops such as bulbs, cereals, fruits, vegetables, cotton, peanuts, soybean, potato, sugar cane, coffee, alfalfa and pasture from deleterious effects. These pesticides have low environmental persistence and are highly effective as insecticides, but some exhibit potential dose-related acute and chronic toxicity in human beings by acting on the inhibition of ChE activity, followed by the accumulation of acetylcholine at cholinergic receptor sites thereby excessively stimulating the cholinergic receptors. This poses serious health effects for humans if water, processed food, fruits and vegetables with high concentrations of OPs and CMs are consumed. In children and infants it can lead to serious health effects such as as eye pain, abdominal pain, convulsions, respiratory failure, paralysis and even death (Kim *et al.*, 2007; Liu *et al.*, 2008; Sbai *et al.*, 2007; Skladal *et al.*, 1997).

The toxicity of OPs and CMs are caused by their ability to bind irreversibly to the catalytic serine residue in acetylcholinesterase (AChE), which leads to inhibition of AChE that prevents nerve transmission by blocking the breakdown of the transmitter choline (Ch) (Somerset *et al.*, 2009; Liu *et al.*, 2008; Kim *et al.*, 2007; Du *et al.*, 2007; Kim *et al.*, 2000).

Since a high degree of toxicity is assigned to organophosphate (OP) and carbamate (CM) pesticide compounds, the rapid detection of these toxic chemicals in environmental samples have become increasingly important (Sbai *et al.*, 2007; Tapsoba *et al.*, 2009; Valdés-Ramírez *et al.*, 2008).

The standard methods that have been traditionally used for pesticide detection include gas chromatography (GC) with different detectors (e.g. mass spectrometry in GC-MS), high performance liquid chromatography (HPLC) and HPLC coupled with mass spectrometry (HPLC-MS) or UV detection (HPLC-UV). However, despite the precision and accuracy of these methods, analyses are restricted to laboratory facilities, are time-consuming and expensive due to its analytical cost, limiting the operation of these instruments to highly qualified laboratory personnel (Somerset *et al*., 2009; Valdés-Ramírez *et al*., 2008; Kim *et al*., 2007; Gong *et al*., 2009; Caetano and Machado, 2008).

Therefore, the need to find rapid, simple and sensitive methods for the detection of organophosphate (OP) and carbamate (CM) pesticide compounds are growing. Biosensors are considered an ideal alternative analytical tool for pesticides quantification, since they offer good selectivity, fast response, miniature size, and reproducible results. The use of amperometric AChE biosensors based on the inhibition of the AChE enzyme have shown satisfactory results for the analysis of various matrices (Frasco *et al*., 2006; Gong *et al*., 2009; Ion *et al*., 2010).

In a previous paper, the application of a mercaptobenzothiazole-on-gold biosensor system for application to organophosphorous and carbamate pesticides determination has been reported. The activity of the AChE immobilized in the biosensor construction was measured by amperometry based on the detection of thiocholine produced in the enzymatic hydrolysis of acetylthiocholine as substrate. This paper further report the results obtained for the biosensor analysis of selected OPs and CMs. The aim of this work was to improve the detection limit of these pesticides with an AChE biosensor, applied to various water miscible organic solvents. The results obtained for the operation of the constructed biosensor in various aqueous-organic media are reported.

2. Materials and methods

2.1 Reagents and materials

Several reagents consisting of aniline (99%), potassium dihydrogen phosphate (99+%), disodium hydrogen phosphate (98+%) and diethyl ether (99.9%) were obtained from Aldrich, Germany. The acetylthiocholine chloride (99%) was obtained from Sigma, Germany. The mercaptobenzothiazole (MBT), acetylcholinesterase (AChE, from Electrophorus electricus, EC 3.1.1.7; ~ 850 U/mg), acetylcholine chloride (99%) and acetone (>99.8%, pestanal) were obtained from Fluka, Germany. The hydrogen peroxide (30%) and the organic solvents ethanol (99.9%, absolute grade), acetonitrile (99.9%, pestanal grade) were purchased from Riedel-de Haën, Germany. The potassium chloride, sulphuric acid (95%), and hydrochloric acid (32%) were obtained from Merck, South Africa. Organophosphorous pesticides used in this study include chlorpyrifos, malathion and parathion-methyl. Carbamate pesticides include carbaryl, carbofuran and methomyl. The pesticide standards used in this study were purchased from Riedel-de Haën, Germany. Platinum (Pt) wires as counter electrodes were obtained from Sigma-Aldrich, South Africa. Alumina micropolish and polishing pads that were used for the polishing of the working electrode were obtained from Buehler, IL, USA (Somerset *et al*., 2009; Somerset *et al*., 2010a).

2.2 Instrumentation

A BAS-50/W electrochemical analyser with BAS-50/W software (Bioanalytical Systems, Lafayette, IN, USA) was used for all electrochemical protocols and data collection. Either

cyclic voltammetry (CV), Oysteryoung square wave voltammetry (OSWV), differential pulse voltammetry (DPV) or time-based amperometric modes were employed. A conventional three electrode system was employed. The working electrode was a gold disc electrode (diameter: 1.6 mm; area: 2.01 x 10^{-2} cm^2; Bioanalytical Systems, Lafayette, IN, USA). Silver/silver chloride (Ag/AgCl – 3 M NaCl type) was used as the reference electrode and a platinum wire was used as auxiliary electrode (Somerset *et al.*, 2010a).

2.3 Preparation of mercaptobenzothiazole on gold electrode

Gold disc electrodes were carefully prepared for the biosensor construction. Prior to use, gold electrodes were first polished on aqueous slurries of 1 μm, 0.3 μm and 0.05 μm alumina powder. After thorough rinsing in deionised water followed by acetone, the electrodes were etched for about 5 minutes in a hot 'Piranha' solution {1:3 (v/v) 30 % H_2O_2 and concentrated H_2SO_4} and rinsed again with copious amounts of deionised water. The polished electrodes were then cleaned electrochemically by cycling the potential scan between - 200 and + 1500 mV (vs. Ag/AgCl) in 0.05 M H_2SO_4 at the scan rate of 40 $mV.s^{-1}$ for 10 min or until the CV characteristics for a clean Au electrode were obtained. The platinum (Pt) counter electrode was regularly cleaned before and after synthesis and in between synthesis and analysis. This involved flaming the Pt electrode in a Bunsen burner until it was white hot, followed by rinsing with copious quantities of deionised water.

The cleaned Au electrode was then immersed into an ethanol solution containing 10 mM of mercaptobenzothiazole (MBT) for 2 hours, thereby coating a self-assembled monolayer (SAM) of MBT on the gold electrode. This was followed by rinsing the SAM electrode extensively with ethanol and water and storing it in 0.1 M phosphate buffer (pH 7.2) for later use (Somerset *et al.*, 2010a).

2.4 Preparation of Au/MBT/PANI modified enzyme electrode

After preparation of the Au/MBT electrode, a polymer film layer of polyaniline (PANI) was coated on the SAM-modified electrode. A three electrode arrangement was set up in a sealed 10 ml electrochemical cell. Polyaniline (PANI) films were prepared by electropolymerisation from a 0.2 M aniline solution dissolved in 1 M hydrochloric acid (HCl) onto the previously prepared Au/MBT-modified electrode. The aniline/HCl solution was first degassed by passing argon (Ar) through the solution for approximately ten minutes and keeping the Ar blanket during electropolymerisation. Initial optimisation of the potential window for electropolymerisation was performed. The electropolymerisation was performed by scanning the potential repeatedly between – 200 mV and +1200 mV, at a scan rate of 40 mV/s (vs. Ag/AgCl) for 20 cycles. The Au/MBT-polyaniline modified electrode was then rinsed with deionised water and used as the working electrode in subsequent studies. The electrode will be referred to as Au/MBT/PANI for the gold-MBT-PANI modified electrode.

Following the electropolymerisation of a fresh PANI polymer film on an Au/MBT electrode, the Au/MBT/PANI electrode was transferred to a batch cell, containing 1 ml argon degassed 0.1 M phosphate buffer (pH 7.2) solution. The PANI polymer film was then reduced at a potential of – 500 mV (vs. Ag/AgCl) for approximately thirty minutes, until a steady current was achieved. Electrochemical incorporation of the enzyme acetylcholinesterase (AChE) onto the Au/MBT/PANI electrode was then performed. This involved the addition of 60 μL of AChE to the 0.1 M phosphate buffer (pH 7.2) solution.

After enzyme incorporation the Au/MBT/PANI/AChE bioelectrode was arranged vertically and then coated with a 2 μL drop of poly(vinyl acetate) (PVAc) solution (0.3 M), prepared in acetone, and left to dry for 1 min. The resulting Au/MBT/PANI/AChE/PVAc biosensor was then ready for pesticide analysis (Somerset *et al.,* 2010a).

2.5 Inhibitory studies of AChE-based biosensors in the presence of pesticide inhibitors

A new Au/MBT/PANI/AChE/PVAc biosensor was prepared each time a new organophosphorous or carbamate pesticide was studied, including each time a new concentration of the OP and CM pesticides was evaluated. The electrochemical cell consisted of Au/MBT/PANI/AChE/PVAc bioelectrode, platinum wire and Ag/AgCl as the working, counter and reference electrode, respectively. A 1 ml test solution containing 0.1 M phosphate (0.1 M KCl, pH 7.2) solution was degassed with argon before any substrate was added and after each addition of small aliquots of 0.01 M acetylthiocholine (ATCh). Inhibition plots for each of the OP and CM pesticides detected were obtained using the percentage inhibition method. The following procedure was used. The biosensor was first placed in a stirred 1 ml of 0.1 M phosphate (0.1 M KCl, pH 7.2) solution (anaerobic conditions) and multiple additions of a standard acetylthiocholine (ATCh) substrate solution was added until a stable current and a maximum concentration of 2.4 mM were obtained. This steady state current is related to the activity of the enzyme in the biosensor when no inhibitor was present. This was followed by incubating the biosensor in anaerobic conditions for 20 min with a standard pesticide phosphate buffer-organic solvent mixture. This was followed by multiple additions of a standard ATCh substrate solution (anaerobic conditions), to a fresh 1 ml of 0.1 M phosphate (0.1 M KCl, pH 7.2) solution (anaerobic conditions) and multiple additions of a standard acetylthiocholine (ATCh) substrate solution was again added, until a stable current was obtained. The maximum concentration of acetylthiocholine (ATCh) was again 2.4 mM. The percentage inhibition was then calculated using the formula (Albareda-Sirvent *et al.,* 2001; Sotiropoulou and Chaniotakis, 2005; Wilkins *et al.,* 2000):

$$I\% = \frac{I_1 - I_2}{I_1} X100 \quad (1)$$

where $I\%$ is the degree of inhibition, I_1 is the steady-state current obtained in buffer solution, I_2 is the steady-state current obtained after the biosensor was incubated for 20 min in phosphate buffer-organic solvent mixture.

Cyclic, square wave and differential pulse voltammetric measurements were performed after each addition of ATCh up to a maximum concentration of 2.4 mM. Cyclic voltammetry (CV) was performed at a scan rate of 10 $mV.s^{-1}$ by applying a linear potential scan between – 400 mV and + 1800 mV (vs. Ag/AgCl). For some experimental runs the anodic difference square wave voltammogram (SWV) was collected in an oxidation direction only by applying a linear potential scan between – 400 mV and + 1800 mV (vs. Ag/AgCl), at a step potential of 4 mV, a frequency of 5 Hz, and a square amplitude of 50 mV.

The anodic difference differential pulse voltammogram (ADPV) was collected in an oxidation direction only by applying a linear potential scan between – 400 mV and + 1800

mV (vs. Ag/AgCl), at a scan rate of 10 $mV.s^{-1}$ and a pulse amplitude of 50 mV. The sample width, pulse width and pulse period were 17 ms, 50 ms and 200 ms, respectively (Somerset *et al.*, 2010a).

2.6 Assessment of organic solvent influence on biosensor operation

The response of the Au/MBT/PANI/AChE/PVAc biosensor was further evaluated in the presence of various organic-aqueous solvent mixtures. The biosensor response was first measured in a 0.1 M phosphate buffer, KCl (pH 7.2) solution, in the presence of a fixed concentration of ATCh. The biosensor was thereafter incubated for 20 minutes in an aqueous-solvent mixture or the pure organic solvent. The response of the Au/MBT/PANI/AChE/PVAc biosensor was then again measured in a 0.1 M phosphate buffer, KCl (pH 7.2) solution, in the presence of a fixed concentration of ATCh. The results for the calibration curves obtained after successive addition of the substrate ATCh to 0.1 M phosphate buffer, KCl (pH 7.2) solutions before and after incubation of the AChE biosensor in the polar organic-aqueous solvent mixtures were then evaluated and characterised (Somerset, 2007b).

3. Results and discussion

3.1 Effect of polar organic solvents on amperometric behaviour of biosensor

Research conducted by other researchers has shown that organic solvents can induce extensive changes in the activity and specificity of an enzyme. This is due to the enzyme's structure and reactivity that depends on several non-covalent interactions in the biocatalyst, which includes hydrogen bonding, ionic, hydrophobic, and van der Waals interactions. Enzymes have further evolved to maintain their structural stability in aqueous medium, but organic solvents are known to disrupt the abovementioned forces of interaction in the enzyme, causing changes in the kinetic and thermodynamic behaviour of the enzyme. Any changes that occur in solvent hydrophobicity, dielectric constant and water content of the reaction medium, affect the ability of enzymes to use their free energy of binding with a substrate, leading to changes in substrate specificity and reactivity. The solvents media that can be used for biosensing can be classified into two groups, i.e. anhydrous organic media and water-containing media. When anhydrous organic media are employed, it refers to pure solvents or a mixture of pure organic solvents that may be polar or non-polar in nature (Iwuoha *et al.*, 1997; Dordick, 1992).

Contrary, water-containing organic media consist of micro-aqueous systems, water-organic solvent mixtures, water and immiscible organic solvent biphasic systems and reverse micellar solutions. The term micro-aqueous reaction media are associated with the non-polar organic solvents that are immiscible with water. Enzymes generally require essential water of hydration for activity, therefore it is essential that non-polar solvents be saturated with water before they are used as reaction media for biosensing. These systems will contain a water content that is very insignificant when compared to the water content of organic solvents, and it depends on the ability of the solvent to absorb water. Since the so-called anhydrous aqueous systems require a minimum amount of water for enzyme activity, it is more suitable to refer to such a system as a micro-aqueous solution. Polar organic solvents can be used as systems that contain some amount of water. The hydration of polar solvents

ensures that the flexibility, structure and local dielectric constant of the enzyme redox site environment, stay as much as possible, unaltered. If the effect of increased solvent polarity occurs, it will weaken the electrostatic forces in the enzyme, which will lead to water partitioning out of the enzyme into the bulk solvent. For this reason, biosensors exhibit much greater reactivity in the presence of polar organic solvents that contain some amount of water (Iwuoha *et al.*, 1997; Chatterjee and Russell, 1992; Borzeix *et al.*, 1992; Somerset, 2010b).

3.2 Voltammetric characterisation in a 90% aqueous-organic solvent mixture

In Figure 1 the individual results obtained for the inhibition of the AChE enzyme in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in a 90% water-organic solvent mixture is shown.

From the results shown in Figure 1 it was observed that the highest decrease in the catalytic activity of AChE was observed when the biosensor was exposed to acetonitrile, while the

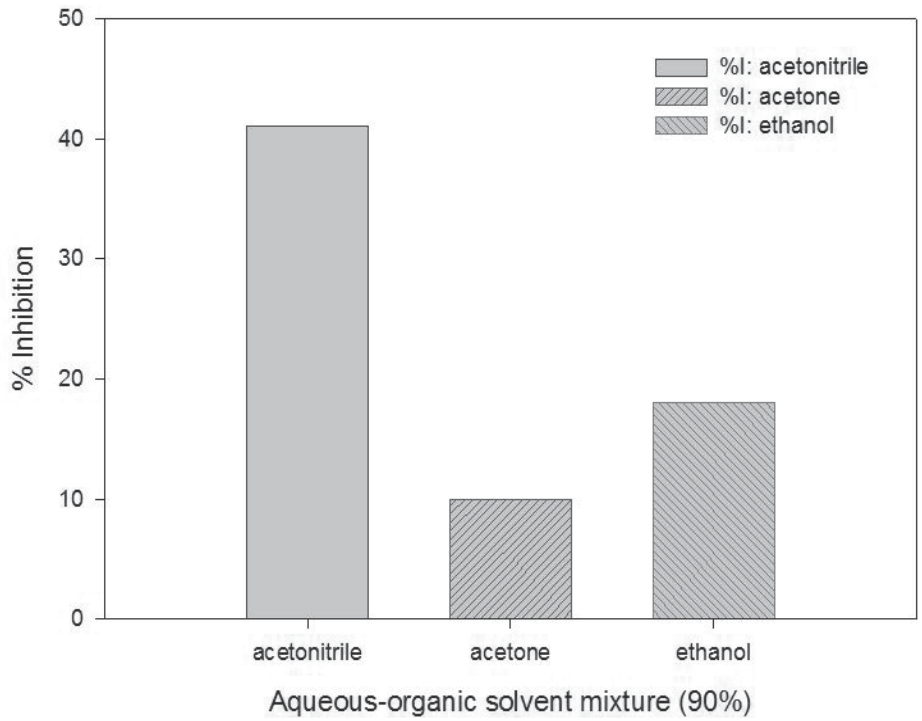

Fig. 1. Results obtained for the inhibition of the AChE enzyme activity in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in 3 different 90% water-organic solvent mixtures. The maximum ATCh substrate concentration was 2.0 mM during the biosensor evaluation.

lowest decrease was observed in acetone. Results on the use of organic solvents in biocatalysis research have shown that enzymes have a high activity in hydrophobic solvents that have a log *P* value greater than 4. On the other hand, enzyme activity will be low in hydrophilic solvents with a log *P* value less than 2, where *P* is the octanol/water partition coefficient of a specific organic solvent. The log *P* values of the polar solvents used in this study are - 0.33, - 0.23 and - 0.24 for acetonitrile, acetone and ethanol respectively. The results for the log *P* values of the three solvents shown in Figure 1 are less than 2, therefore it was expected that the AChE activity will not be high since the solvents are hydrophilic. The results obtained thus indicate that the effect of adding 10% water to keep the active centre of the AChE enzyme hydrated during the biosensor studies, improved the enzyme activity. The best results of 10% inhibition of the AChE catalytic activity was obtained for acetone (Iwuoha *et al.* 1997; Konash and Magner, 2006; Somerset, 2010b).

Analysis of the voltammetric results (not shown here) for the inhibition studies on a 90% acetone-aqueous organic solvent mixture, revealed that relatively similar anodic peak data was observed after exposure of the AChE biosensor to the solvent mixture. In the case of the cyclic voltammetry (CV) results, a good decrease in the anodic current at a potential of approximately + 1250 mV (vs. Ag/AgCl) was observed. Further evaluation of the differential pulse voltammetry (DPV) responses of the Au/MBT/PANI/AChE/PVAc biosensor, confirmed the anodic peak results at + 1195.3 mV (vs. Ag/AgCl). Although a slight shift in the peak potential (E_p) occurred, the magnitude of the anodic current was relatively similar (Somerset, 2010b).

3.3 Voltammetric characterisation in a 95% aqueous-organic solvent mixture

The AChE enzyme activity in the Au/MBT/PANI/AChE/PVAc biosensor was then studied in a 90% water-organic solvent mixture, with the results shown in Figure 2.

The results shown in Figure 2 indicate that higher percentage inhibition results were obtained for the evaluation of the AChE enzyme activity in 95% water-organic solvent mixtures. The inhibition effect was the highest for acetonitrile used as solvent, with the decreasing order of inhibition as acetonitrile > acetone > ethanol for the 3 solvent mixtures evaluated. The results further indicate that the decrease of the water content to 5% in the aqueous-organic solvent mixtures, result in a considerable reduced AChE enzyme response for the constructed biosensor. When 5% water-organic solvent mixtures were used, the lowest inhibition observed was 10% for acetone, while for the 10% water-organic solvent mixtures, the lowest inhibition observed were 33% for ethanol. This can also be attributed to the fact that less water was used in the water-organic solvent mixtures under discussion, indicating that the hydration of the enzyme influences the catalytic ability of the enzyme.

In Figure 3 the differential pulse voltammetric (DPV) responses for the inhibiting effect of a 95% ethanol-aqueous mixture on the enzyme AChE activity in the Au/MBT/PANI/AChE/PVAc biosensor is shown. A shorter potential window between + 600 and + 1100 mV is shown in order to highlight the effect of the inhibition observed.

Evaluation of the results shown in Figure 3 clearly shows the inhibiting effect of the 95% ethanol-aqueous organic solvent mixture on the catalytic activity of the enzyme AChE, with the decreasing anodic peak current observed. At a potential of approximately + 847.6 mV (vs. Ag/AgCl) the difference in anodic current was of 2 orders magnitude after exposure to the ethanol-aqueous organic solvent mixture with reduced water content, compared to the results in the previous section (Somerset, 2010b).

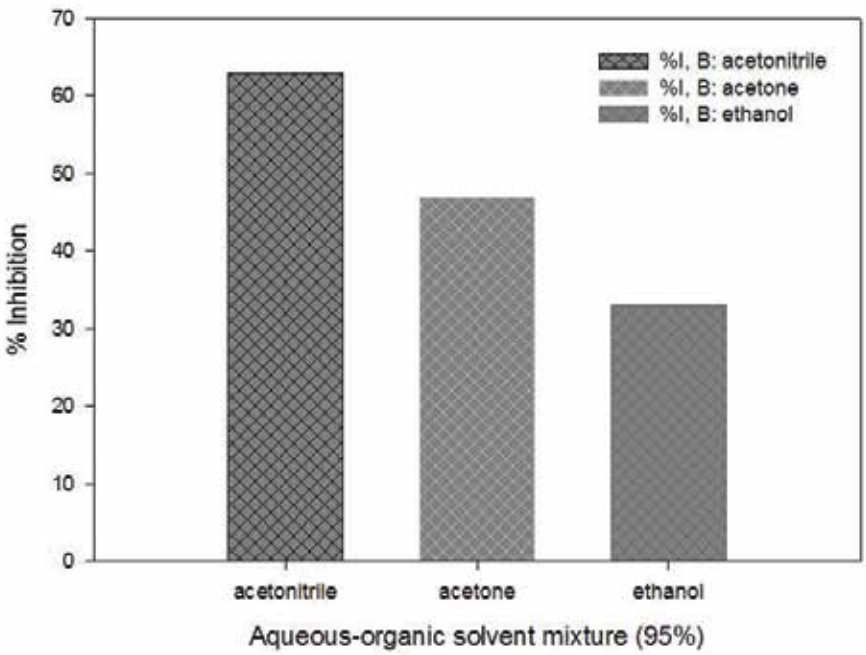

Fig. 2. Results obtained for the inhibition of AChE enzyme activity in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in 3 different 95% water-organic solvent mixtures. The maximum ATCh substrate concentration was 2.0 mM during the biosensor evaluation.

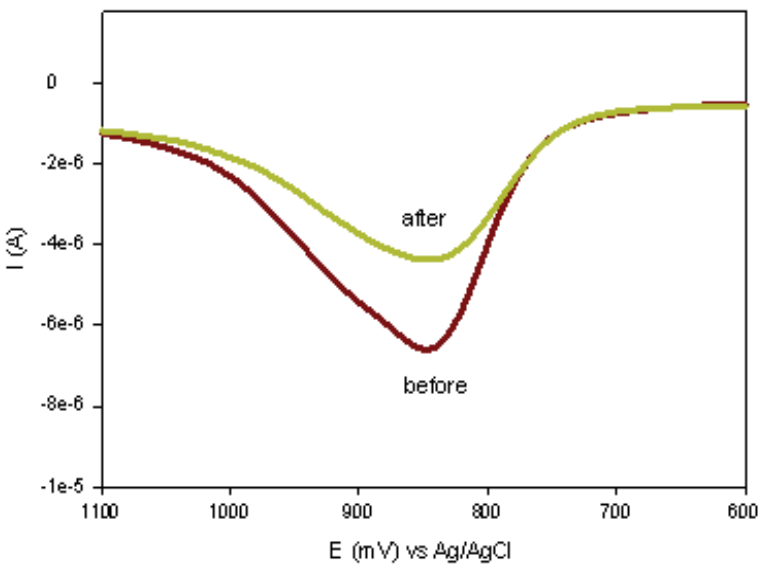

Fig. 3. Results for the DPV responses (before and after) of the Au/MBT/PANI/AChE/PVAc biosensor in a 0.1 M phosphate buffer, KCl (pH 7.2) solution in the presence of a 2 mM ATCh solution.

3.4 Voltammetric characterisation in pure organic solvent

The third investigation focussed on the use of the pure organic solvent, compared to the aqueous-organic solvent mixtures with 5% and 10% water content. Figure 4 shows the individual results obtained for the inhibition of AChE activity in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in the 3 different pure organic solvents.

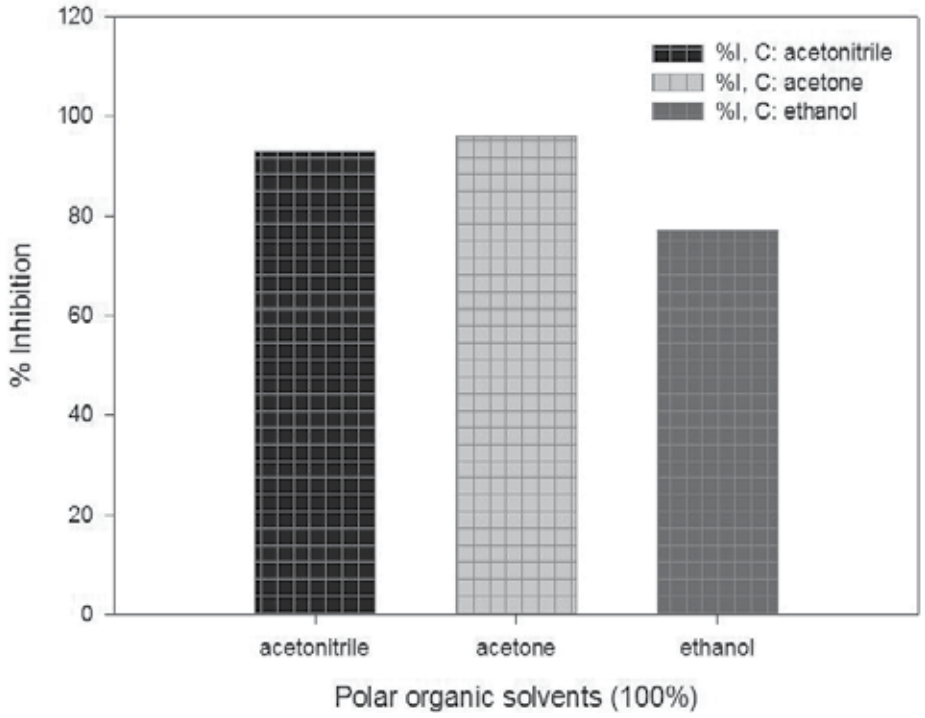

Fig. 4. Inhibition results for AChE enzyme activity in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in the pure organic solvents, with the ATCh substrate concentration at 2.0 mM.

The results obtained for the exposure of the enzyme to pure organic solvents are shown in Figure 4. Analysis of the results has shown that the highest inhibiting effect on the AChE enzyme activity was experienced when the enzyme was exposed to the pure organic solvents. The individual inhibition results obtained were 93% in acetonitrile, 96% in acetone and 77% in ethanol. Comparison of these results to that obtained for the 90% and 95% aqueous-organic solvent mixtures in the previous sections, indicate a definite decrease in AChE enzyme activity in the absence of water. In the case of ethanol as polar organic solvent, it was observed that the best results were obtained in using this solvent, therefore the Au/MBT/PANI/AChE/PVAc biosensor assay of the OP and CM pesticides were further evaluated using this solvent.

A summary of the results obtained for the percentage inhibition investigation of the AChE enzyme activity in different aqueous-polar solvent mixtures, are shown in Table 1.

Polar organic solvent	Log *P*	% Inhibition of enzyme, AChE, in different solvent mixtures		
		90% aqueous-solvent mixture	95% aqueous-solvent mixture	100% pure solvent
acetonitrile	- 0.33	41	63	93
acetone	- 0.23	10	47	96
ethanol	- 0.24	18	33	77

Table 1. Summary of results obtained for the percentage inhibition investigation of the AChE enzyme activity in the different aqueous-polar solvent mixtures investigated.

The results obtained in Table 1 indicate that aqueous-solvent mixtures of acetone and ethanol with water gave the best results and the smallest degree of inhibition of the AChE biosensor and are in line with the investigations reported by Evtugyn *et al.* (1998) on the presence of water and in the enzyme's active centre (Somerset, 2010b).

3.5 Inhibition results for standard samples evaluated

Following the study of the AChE activity in the different aqueous-polar organic solvents, inhibition data were collected for each of two organophosphorus and carbamate pesticide standard samples, respectively. The results obtained for the inhibition studies are shown in Table 2.

Biosensor inhibition results					
		Organophosphates		Carbamates	
[pesticide], ppb	- log [pesticide]	% I (chlorpyrifos)	% I (malathion)	% I (carbaryl)	% I (methomyl)
0.60	0.222	43.42	31.96	18.07	36.15
1.00	0.000	51.71	41.55	25.30	44.80
2.00	-0.301	60.00	48.90	36.09	56.20
5.00	-0.699	67.49	57.43	48.16	63.84
7.00	-0.845	73.80	67.50	58.97	72.37
10.00	-1.000	80.61	74.59	67.70	79.59

Table 2. Percentage inhibition results obtained for six different concentrations of two organophosphorus and carbamate pesticide standard concentrations investigated, using the Au/MBT/PANI/AChE/PVAc biosensor.

Analysis of the results show in Table 2, indicate that the highest percentage inhibition (%I) of 81% was obtained for chlorpyrifos at a concentration of 10.0 ppb, while the lowest %I of 18% was obtained for carbaryl at a concentration of 0.6 ppb.

Inhibition plots for the results shown in Table 2 were plotted and are shown in Figure 5. The inhibition plots were obtained using the percentage inhibition method described in section 2.5.

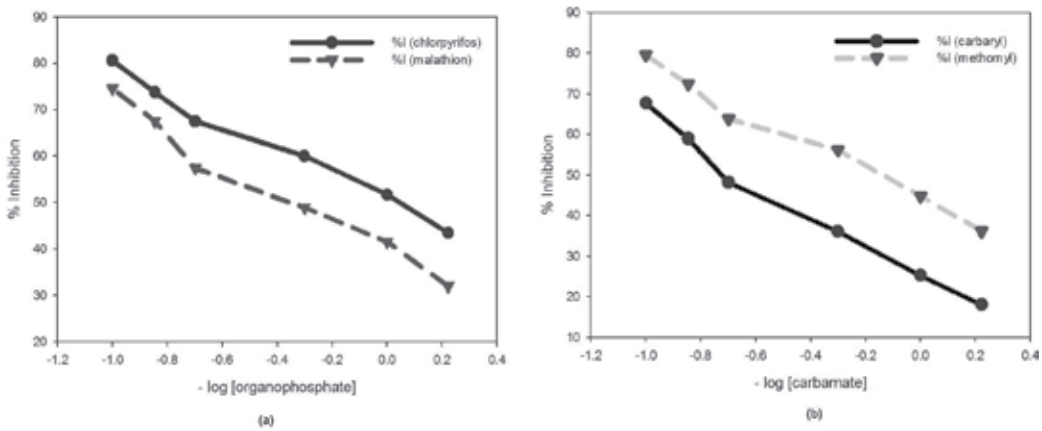

Fig. 5. Graphs of percentage inhibition vs. – log [pesticide] results for two different organophosphorous pesticides in (a) and two different carbamate pesticides in (b). Results were obtained with the use of the Au/MBT/PANI/AChE/PVAc biosensor for six different pesticide concentrations evaluated.

Analysis of the results in Figure 5 (a) indicate that in the case of the organophosphorous pesticides, higher percentage inhibition results were obtained for chlorpyrifos compared to malathion over the 6 different concentrations evaluated. For the results in Figure 5 (b) it was observed that carbaryl had higher percentage inhibition results compared to methomyl, in evaluation of the carbamate pesticides. Comparison of the results for the four different pesticide samples investigated, shows that the highest overall percentage inhibition was obtained for the chlorpyrifos OP pesticide evaluated.
The data of the inhibition plots were further analysed in order to obtain results for the sensitivity, detection limits and regression coefficients that are shown in Table 3.

Pesticide	**Organophosphorous pesticides**		
	Sensitivity (%I/decade)	Detection limit (nM)	Regression coefficient (R^2)
Chlorpyrifos	-26.68	0.028	0.997
Malathion	-35.24	0.189	0.998
Pesticide	**Carbamate pesticides**		
	Sensitivity (%I/decade)	Detection limit (nM)	Regression coefficient (R^2)
Carbaryl	-21.92	0.880	0.996
Methomyl	-21.04	0.111	0.995

Table 3. Results for the different analytical parameters calculated from the inhibition plot data of the Au/MBT/PANI/AChE/PVAc biosensor detection of standard OP and CM pesticide solutions (n = 2).

The results in Table 3 indicate that the lowest sensitivity was obtained for methomyl as pesticide, with the sensitivity of carbaryl relatively close to that result. A very good

sensitivity was also obtained for chlorpyrifos and this pesticide delivered the lowest detection limit of 0.028 nanoMolar (nM). Comparison of the detection limits for the four OP and CM pesticides evaluated have shown that the detection limit decreases from carbaryl > malathion > methomyl > chlorpyrifos. The application of the Au/MBT/PANI/AChE/PVAc biosensor delivered very good results for analysis of the pesticides in a 90% aqueous-ethanol solvent mixture.

Furthermore, the detection limits of the Au/MBT/PANI/AChE/PVAc biosensor also compare favourably with the detection limits of 1.91×10^{-8} M for paraoxon and 1.24×10^{-9} M for chlorpyrifos ethyl oxon obtained in 5% aqueous-acetonitrile solvent mixture, obtained with the SPCE/PVA-SbQ/AChE biosensor constructed by Dutta *et al.* (2008).

4. Conclusions

The results described in this chapter have successfully demonstrated the construction and use of an Au/MBT/PANI/AChE/PVAc thick-film biosensor for the detection of organophosphorous and carbamate pesticides in various polar organic solvents of acetonitrile, acetone and ethanol. This study has also shown that self-assembled monolayers can be applied in thick film biosensor construction and that the poly(vinyl acetate) film does not interfere with the PANI-AChE electrocatalytic activity towards thiocholine. Furthermore, very good detection limits for the standard OP and CM pesticide standard samples were obtained with the Au/MBT/PANI/AChE/PVAc biosensor. Application of the constructed biosensor to aqueous-polar solvent mixtures of acetone and ethanol with water gave the best results and the smallest degree of inhibition of the AChE enzyme activity. The results also clearly indicated that the presence of water has a considerable effect on the functioning of the AChE-biosensor, highlighting the importance of the presence of water in the enzyme's active centre, particularly for organic phase enzyme electrodes. The results for the detection limit values for the individual organophosphate pesticides were 0.028 nM (chlorpyrifos) and 0.189 nM (malathion). The detection limit values for the individual carbamate pesticides were 0.880 nM (carbaryl) and 0.111 nM (methomyl).

5. Acknowledgement

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17

An analytical Task: A miniaturized and Portable μConductometer as a Tool for Detection of Pesticides

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1. Introduction

Biological background: Chloride ion sensing is important in many fields such as clinical diagnosis (Huber et al., 1998; Jiang et al., 1998) environmental monitoring (Huber et al., 2000; Martin & Narayanaswamy, 1997; Montemor et al., 2006) and various industrial applications (Babu et al., 2008; Badr et al., 1999). Considering the fact that chloride channels play crucial role in physiological processes it is not surprising that missregulation of chloride ion transport by these channels can cause serious disorders. Cystic fibrosis is a disease in which gene encoding of a protein, called cystic fibrosis transmembrane regulator, which functions as a chloride channel in epithelial membranes, is mutated and thus its function is altered (Ratjen & Doring, 2003). Besides the importance of monitoring chloride ions in patients with this disease, monitoring chloride ion in the environment is needed.
Building industry issue: Chloride ions content in concrete plays important role in the quality of reinforced concrete, as these ions induce depassivation of the steel rebar and initiation of the corrosion process leading to degradation of the structure. Chloride ions in concrete come from cement, aggregate materials and water used for creating concrete, or by diffusion of chloride ions from outside of the structure through water pores in the concrete. Determination of chloride ions in materials for concrete is thus necessary (Junsomboon & Jakmunee, 2008; Montemor et al., 2006). The content of chloride ions in waters is also monitored well (Hahn, 2005; Sebkova, 2003).
Environmental issue: Besides, according to proverb "God created 90 elements, man around 17, but Devil only one – chlorine", it is obvious that this element is also a component of substances very harmful to the environment, which are called pesticides. Organophosphate, carbamate and organochlorinated compounds belong to the most widely used organic pesticides. Principal attention of many scientists has been paid to organochlorinated hydrocarbons, which affect the nervous and respiratory system and, unfortunately, persist in the environment. In developed countries their use is strictly regulated, whereas new

technologies to remove their residues are searched for (Francova et al., 2004; Chroma et al., 2002; Chroma et al., 2003; Kucerova et al., 1999; Kucerova et al., 2001; Leigh et al., 2006; Sudova et al., 2007; Villacieros et al., 2005). Nevertheless, in developing countries there is no restriction on the use of these substances. Therefore monitoring of environment pollution by pesticides is still concern in both developed and developing countries (Fig. 1).

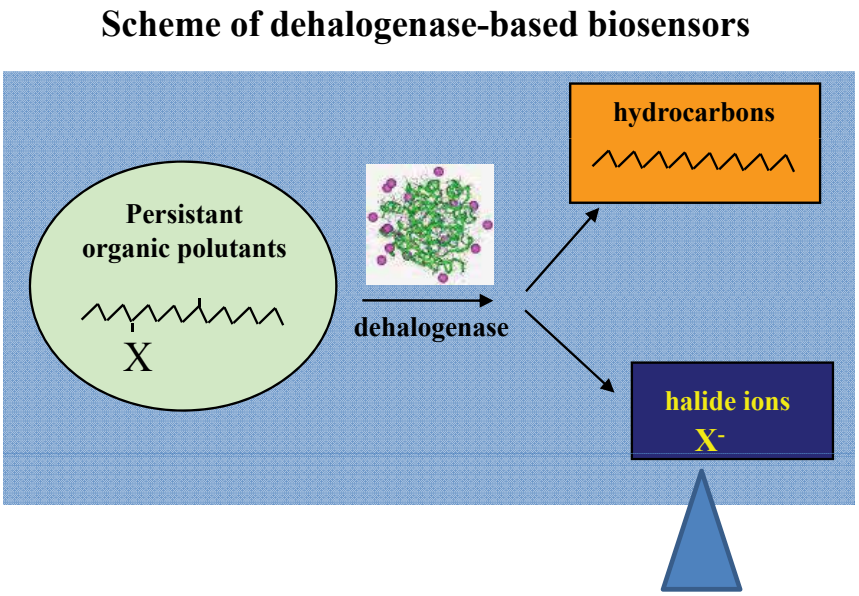

Fig. 1. Scheme of dehalogenase-based biosensors. Dehalogenase is able to cleave persistent organic pollutant (POP) into hydrocarbon and halide. The halide ion is subsequently detected by the appropriate detector.

Analytical task: Development of low cost and sensitive methods for the determination of pesticides in the environment is needed (Andrade-Eiroa et al., 2002; Heinisch et al., 2005; Heinisch et al., 2004; Taylor & Grate, 1995; Wegner et al., 2005). For direct detection of these molecules hyphenated techniques such gas or liquid chromatography with mass spectrometry (Alder et al., 2006; Hercegova et al., 2007; Hernandez et al., 2005; Lacorte & Barcelo, 1996; Lambropoulou & Albanis, 2007; Popp et al., 1997; Rodriguez-Mozaz et al., 2007; Sancho et al., 2006), capillary electrophoresis (Boyce, 2007; Ravelo-Perez et al., 2006), thin layer chromatography (Sherma, 2007) coupled with various detectors are used. These methods are often time-consuming, demanding on high-cost instrumentation and most of all, lack any no possibility of miniaturization. However, recently discovered enzymes called haloalkane dehalogenases (Fig. 1) are involved in biochemical pathways enabling bacteria to utilize halogenated compounds via releasing halogen ion from the molecule of halogenated hydrocarbon (Bosma et al., 2002; Damborsky & Brezovsky, 2009; Jesenska et al., 2005; Nagata et al., 1997; Nagata et al., 2005; Pavlova et al., 2009; Prokop et al., 2003). Haloalkane dehalogenases are key enzymes in the degradation of synthetic haloalkanes that occur as soil pollutants (Janssen, 2004). The reaction mechanism of haloalkane dehalogenases was initially clarified by detailed crystallographic and site-directed mutagenesis analyses of DhlA from *Xanthobacter autotrophicus* GJ10 (Janssen, 2004). A catalytic triad (i.e., nucleophile-

histidine-acid) is essential for the reactions catalyzed by members of the α/β-hydrolase family. Amino acid residues for the catalytic triad of LinB were proposed to be D108, H272, and E132 on the basis of a site-directed mutagenesis analysis (Hynkova et al., 1999). A catalytic pentad is composed of a catalytic triad and two hydrogen bond-donating residues providing halide stabilization (Janssen, 2004). One out of the two residues involved in the halide binding, i.e., tryptophan localized directly next to the nucleophilic aspartate, is invariable. The second halide-stabilizing residue is represented by a tryptophan residue in DhlA (W175) or an asparagine residue in DhaA (N41) and LinB (Bohac et al., 2002; Nagata et al., 2007). This feature can be used for suggesting appropriate biosensors which detect released chloride, as shown by Mikelova et al. (Mikelova et al., 2008) and Murthy et al. (Murthy et al., 2010). Numerous analytical methods for chloride ions in a variety of samples have been developed, such as ion chromatography (Jeyakumar et al., 2008; Pereira et al., 2008), near-infrared spectrometry (Wu & Shao, 2006), spectroscopy (Philippi et al., 2007), light scattering (Cao & Dong, 2008), ion-selective electrode method (Babu et al., 2008; Junsomboon & Jakmunee, 2008; Kumar et al., 2006; Shishkanova et al., 2007), turbidimetric method (Mesquita et al., 2002) and flow based methods coupled with different detectors (Bonifacio et al., 2007; Junsomboon & Jakmunee, 2008; Pimenta et al., 2004; Trnkova et al., 2008). The main aim of this paper was to propose, fabricate and test new conductometer for the fast and sensitive detection of chloride ions. Moreover, we coupled the conductometer with haloalkane dehalogenase, enzyme able to cleave chlorinated chemicals, in order to detect pesticides (Fig. 2).

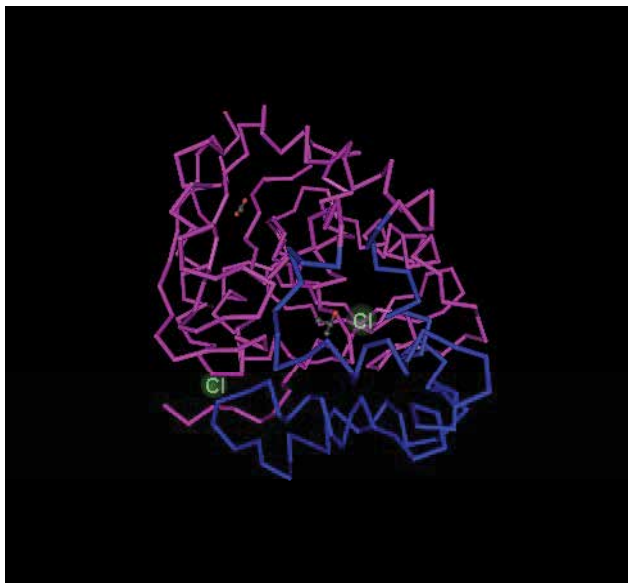

Fig. 2. Structure of haloalkane dehalogenase mutant Dha15 (I135fC176Y) from *Rhodococcus rhodochrous;* PDB ID: 3FWH, Gavira JA, Stsiapanava A, Kuty M, Dohnalek J, Lapkouski M, Kut I, Smatanova, 2009/1/18 . The figure prepared using the program Cn3D of the National Center for Biotechnology Information.

2. Experimental section

2.1 Chemicals, material and pH measurements

The chemicals used were purchased from Sigma Aldrich Chemical Corp. (USA) in ACS purity unless noted otherwise. The stock standard solutions were prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at -4 °C. Working standard solutions were prepared daily by dilution of the stock solutions. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany). Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified using Millipore RG (Millipore Corp., USA, 18 MΩ) – MiliQ water.

2.2 Designing the μconductometer

During the design process of the microchip, attention was paid to characteristics of the measurement method and the goal was to design such a microsystem structure as would use the advantages of this method. A block scheme is shown in Fig. 3A. The comb-like electrodes on the sensor are connected to a bridge of switches which allow to change polarity of the bias current flowing through the sensor controlled by CLK phase. Four subranges of the sensor currents were designed to induce sensor voltage drop on the sensor from 10 mV to 100 mV. Te offset compensation designed allows to compensate not just the opamp (operational amplifier) offset but also to eliminate the parasitic voltage drop at sensor switches.

2.3 Fabrication of the new μconductometer and sensors

The screen-printed comb-like Pt electrodes were fabricated as sensing part of the sensor (Fig. 3B). The screen-printed working electrode was fabricated using standard thick-film cermet paste on an alumina substrate with dimension 25.4 × 7.2 mm. The paste used for leads and contact pads was AgPdPt based paste type ESL 9562-G (ESL Electroscience, UK). The working electrode was fabricated from carbon paste BQ-221 (DuPont, USA). Isolation was made from dielectric paste ESL 4913-G (ESL Electroscience, UK). Prior to measurement with electrically modified sensors, an optical analysis of platinum layer on the sensor was carried out. The measurements were performed on a confocal laser microscope LEXT (Olympus, Germany). The electrode was connected to ASIC microchip performing bipolar pulse technique, which was implemented into new conductometric instrument controlled by microcontroller including A/D and D/A converters and UART circuits assuring USB data loading (Fig. 3C). Moreover, impedance precise analyzer Agilent 8492A (EIS, USA) controlled by LabView program was utilized for Electrochemical Impedance Spectroscopy and calibration of the fabricated instrumentation.

2.4 Enzyme

Haloalkane dehalogenase (E.C. 3.8.1.5.) was donated by Prof. Jiri Damborsky from Loschmidt Laboratories, Faculty of Science, Masaryk University, Brno, Czech Republic. Briefly about these enzymes: haloalkane dehalogenases make up an important class of enzymes that are able to cleave carbon-halogen bonds in halogenated aliphatic compounds. There is a growing interest in these enzymes because of their potential use in

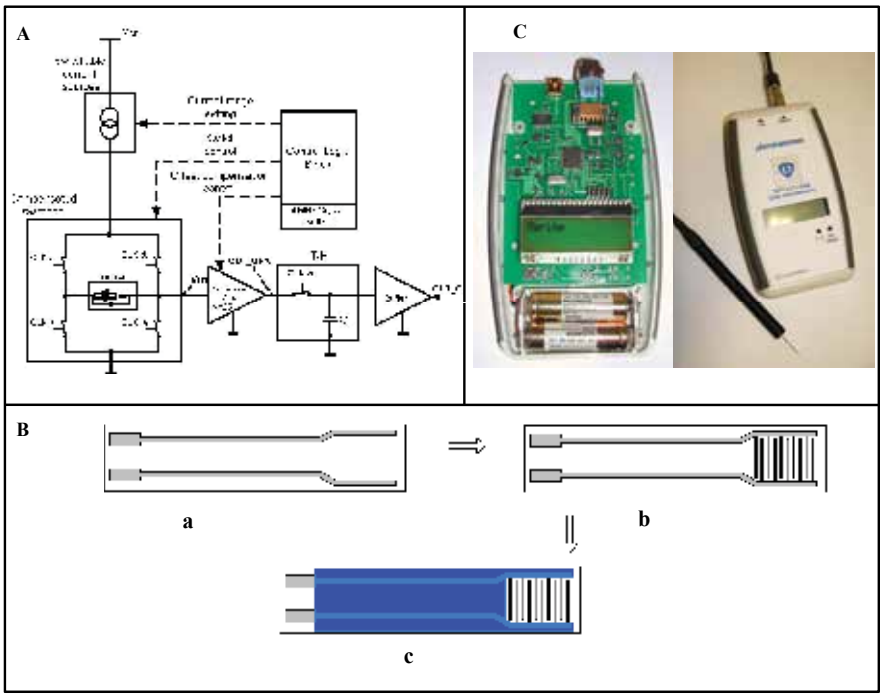

Fig. 3. Home made μconductometer. (A) A block scheme of the bipolar technique implemented into the microchip. (**B**) Fabrication of the sensor: a: conductive layer, b: electrodes, c: double covering layer. (**C**) Hand-held instrumentation with the chip designed (ASIC).

bioremediation, as industrial biocatalysts, or as biosensors. Structurally, haloalkane dehalogenases belong to the α/β-hydrolase fold superfamily (Ollis et al., 1992). Without exception, haloalkane dehalogenases contain a nucleophile elbow (Damborsky, 1998), which is the most conserved structural feature within the α/β-hydrolase fold. The other highly conserved region in haloalkane dehalogenases is the central β-sheet. Its strands, flanked on both sides by α-helices, form the hydrophobic core of the main domain that carries the catalytic triad Asp-His-(Asp/Glu). The second domain, consisting solely of α-helices, lies like a cap on top of the main domain. Residues at the interface of the two domains form the active site. Whereas there is significant similarity in the catalytic core, the sequence and structure of the cap domain diverge considerably among different dehalogenases. The cap domain is proposed to play a prominent role in determining substrate specificity (Kmunicek et al., 2001; Pries et al., 1994b). A reaction mechanism for haloalkane dehalogenase has been proposed on the basis of x-ray crystallographic (Verschueren et al., 1993), site-directed mutagenesis (Pries et al., 1995; Pries et al., 1994a), and kinetic (Schanstra & Janssen, 1996; Schanstra et al., 1996) studies with the haloalkane dehalogenase of Xantobacter autotrophicus GJ10 (DhlA). Catalysis proceeds by the nucleophilic attack of the carboxylate oxygen of an aspartate group on the carbon atom of the substrate, yielding displacement of the halogen as halide and formation of a covalent alkyl-enzyme intermediate. The alkyl-enzyme intermediate is subsequently hydrolyzed by a water molecule activated by the histidine. A catalytic acid (Asp or Glu) stabilizes the charge developed on the imidazole ring of the histidine during the hydrolytic half-reaction.

2.5 Enzymatic experiment

Enzyme haloalkane dehalogenases were dissolved (7.6 g/mL) in phosphate buffer 0.1 mM with pH 7.6. the Dissolved 1-chloro-hexane (as pesticide substitute) in the phosphate buffer was added to enzyme solution and tempered 1 hour at 38°C according to (Mikelova et al., 2008), which ensured all hexane molecules to enter into reaction with enzyme. Samples of this analyte were dropped into buffer with 5 min intervals.

3. Results and discussion

Primarily, a attempt to test our hypothesis on suggesting conductivity-based biosensor for detection of pesticides was made. In this experiment, we used WTW conductometer (Wissenschaftlich-Technische Werkstätten - WTW, Weilheim, Germany). The measurements were carried out with InoLab Cond 730 coupled with conductivity cell TetraCon 325. The instrument was firstly used for detection of various concentrations of chloride ions. The logarithmic calibration curve is shown in Fig. 4A. The dependence was linear with R^2 above 0.9. Further, we were interested in the issue whether the instrument could be able to detect changes in resistance depending on addition of haloalkane dehalogenase (500 nM) and its substrate as 1-chlorohexane (H_3C-$(CH_2)_5$-Cl) (Fig. 4B). It clearly follows from the results obtained that the additions of the substrate with different volumes have detectable influence on the resistance. Moreover, we investigated dependence of the resistance on time of the reaction. The results obtained are shown in Fig. 4C. Almost all substrate was cleaved up to 30 min. It can be concluded that coupling of conductometer and haloalkane dehalogenase can be used for detection of pesticides.

3.1 µConductometer

In the following experiments, we aimed at suggesting, fabricating and testing such a home-made conductometer for similar purposes, as would have better parameters than InoLab Cond 730 coupled with conductivity cell TetraCon 325. The bipolar pulse technique was originally described in 1970 for measurements with conventional electrodes (Johnson & Enke, 1970). Nowadays, the technique is applied with microelectrode systems (Pumera et al., 2006). The first integration of the technique into microchip came in 2002 in our laboratories using AMIS technology. A new microchip with improved parameters was designed and fabricated in 2006. To detect chloride ions we used newly designed instrument carrying out bipolar pulses (Fig. 3). The method of bipolar pulse consists of applying two consecutive voltage pulses of equal amplitude and pulse width, but of opposite polarity, to a cell and then measuring the cell current precisely at the end of the second pulse. The method directly measures conductance as in the AC technique of measurements. With this measurement we can reach 0.01% accuracy if the pulse widths of both pulses are equal to within 1% and the double layer voltage is kept to less than 1% of the applied voltage (Johnson & Enke, 1970). Another advantage of bipolar pulse measurements is the elimination of capacitance as the double layer capacitance and the faradaic capacitance. The width of pulses should be determined to minimise the error caused by non-capacitive part of the impedance. The instrument measurement and calibration using resistor was performed with United Kingdom Accreditation Service calibration certificate. The error at range 3 and 4 is below 1 %, at range 2 below 1.4 % (Fig. 5). The lowest range error is much higher because of a circuit fault on the microchip. This lowest range is not necessary for our measurements.

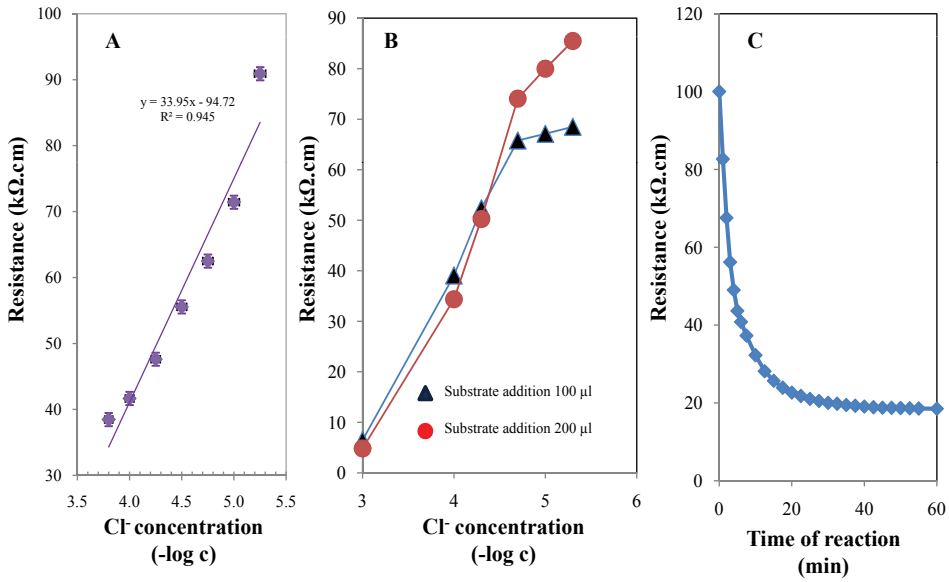

Fig. 4. WTW measurements. (**A**) Dependence of resistance on logarithmic concentration of chloride ions. (**B**) Dependence of resistance on two additions (100 and 200 µl) of the substrate (1-chlorohexane). (**C**) Dependence of resistance on time of reaction of haloalkane dehalogenase (500 nM) with 1-chlorohexane (5 µM).

3.2 Fabrication and characterization of sensors coupled to the µconductometer

We used screen-printed comb-like Pt electrodes as a sensor. The goal was to design the microsystem structure and during the design process of the microchip attention was paid to characteristics of the measurement method. The comb-like electrodes on the sensor are connected to a bridge of switches which allow to change polarity of the bias current flowing through the sensor. Four sub-ranges of the sensor currents were designed to induce sensor voltage drop on the sensor from 10 mV to 100 mV. The offset compensation designed allows to compensate not just the opamp (operational amplifier) offset but also to eliminate the parasitic voltage drop at sensor switches.

Prior to measurement with electrically modified sensors, an optical analysis of platinum layer on the sensor was carried out. The measurements were performed on a confocal laser microscope LEXT. It uses a laser for the screening of a surface point by point. Superimposing of measured data provides sharp images with magnification of up to 14 400 × with a resolution of 0.12 µm. In addition, this device allows easy measurement of surface relief. The anode of the electrode showed in Fig. 6A was galvanized (the comb at the top of the figure), but the cathode was not galvanized (the comb in the middle of the figure). the galvanized platinum was black, unlike the printed platinum, which had a silver-grey colour. To better assess the surface, measurements with higher magnification were needed. These measurements showed that the deposited layers were homogeneous and smooth (Fig. 6B). Under more careful treatment of the observed figures, it was possible to identify scales that originated on the surface. They hold tight, because the whole sensor was rinsed thoroughly with demineralized water after galvanization.

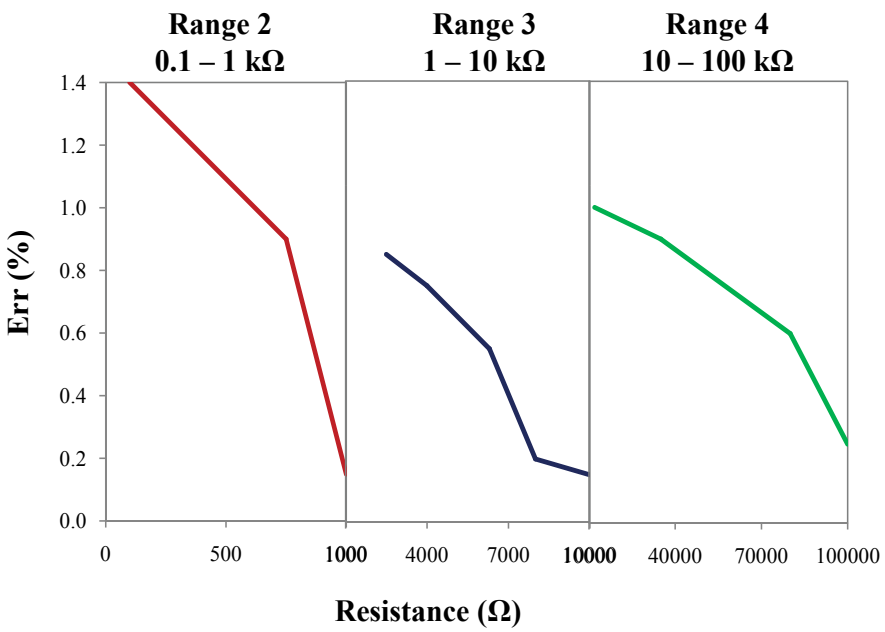

Fig. 5. Home made µconductometer. Errors of resistance measurements at three ranges measured by a µconductometer.

By contrast, a non-galvanized electrode surface is completely different. There is a large grain size and porosity of the surface evident (Fig. 6C). After galvanizing the surface porosity is not seen. The flakes, which were seen in the previous figure, are probably only bright point on the surface, as can be seen from the centre of Fig. 6D. To improve the equality of electrode surface ultrasound was used during galvanizing. After starting the electrolysis at the plated surface hydrogen begins to develop and this hydrogen evolution hinders the whole process. Ultrasonic vibration releases gas bubbles from the surface and the deposited layers are cleaner and smoother. Therefore, we used this procedure for the preparation of electrodes in the following part of the experiment.

3.3 Characterization of the µconductometer and fabricated sensors

The characterization of screen-printed sensor has been done under chlorides ions measurements using electrochemical impedance spectroscopy and NaCl analyte (from 100 nM to 1 mM). It was found that electrodes with two fingers showed instability and very poor reproducibility with standard deviation of measurement higher than 45 %. It confirms the results described in (Jacobs et al., 1995), where the drawback causing this error is well explained. Multiple numbers of fingers are eligible for precise and reproducible measurement. The reproducibility was increased by galvanic plating of black platinum on the total sensing area of electrodes. The diffusion part (Warburg impedance) becomes significant with increasing concentration of conductive ions because of very small interelectrode distance of the electrode structure. The phosphate buffer with concentration of 1 mM was found as optimal for measurement according to measured conductivity (resistance) about 2 kΩ. The sensor sensitivity as rate R/c (c - concentration of NaCl) was

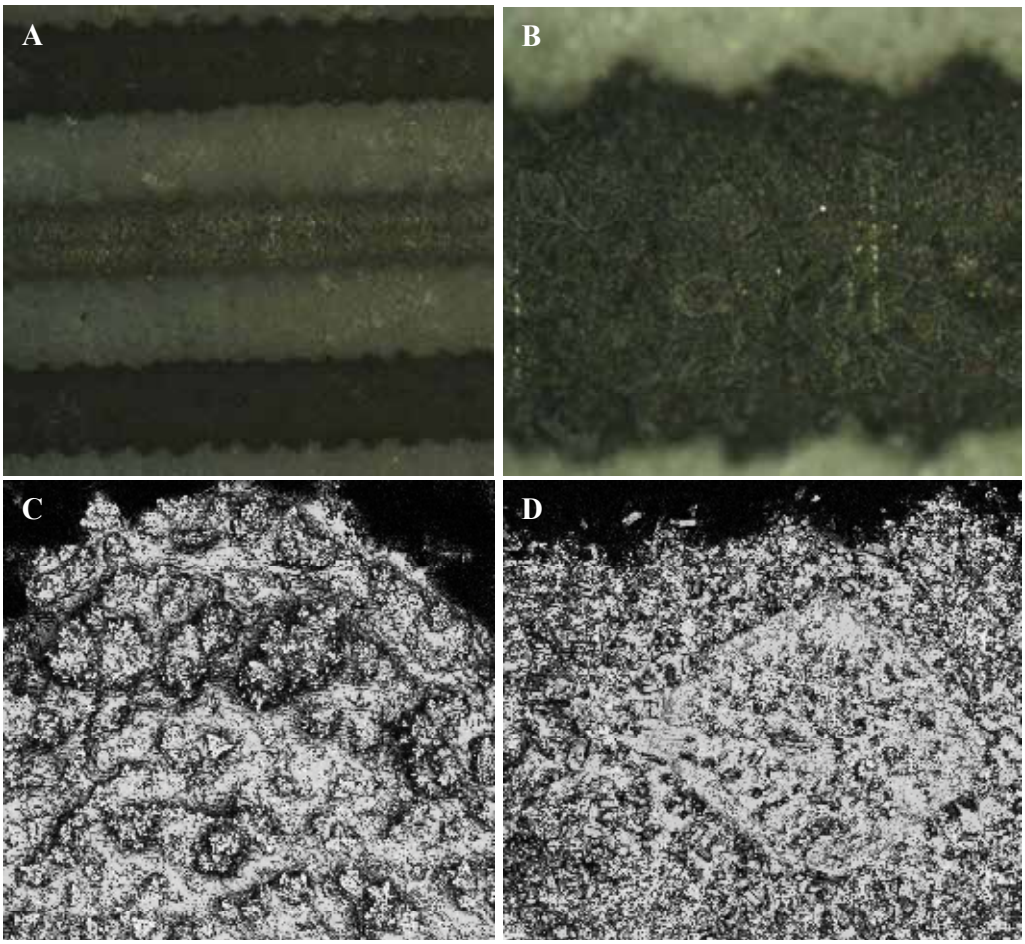

Fig. 6. Home made μconductometer. Comparison of surfaces of (A) galvanized and (B) non-galvanized electrodes. Detail galvanized electrode surface. Surface detail of (C) non-galvanized electrode and (D) galvanized electrode.

determined from calibrating curve for 20 kHz measuring sinusoidal signal and NaCl concentration changes in buffer. Determined sensitivity -2.2 $\Omega \cdot \mu M^{-1}$ (R^2=0.995) was obtained by recalculating to chloride ions.

3.4 Detection of pesticides

Many species of chemotrophic bacteria contain enzymes (dehalogenase) which are able to release halides from the molecule of halogenated hydrocarbon. Haloalkane dehalogenase (E.C. 3.8.1.5.) can be used to cleave hydrolytically the carbon-halogen bond forming corresponding alcohol, halide anion and a proton (Janssen et al., 1994). These enzymes can be utilized for detection of pesticides made of chlorinated hydrocarbons. The halide released by the enzyme can be detected by using an electrochemical detector, as it was shown above. It is known that the enzymatic activity can be affected by many factors like buffer composition, pH, ionic strength and temperature. To detect the halogenated hydrocarbons,

the haloalkane dehalogenase LinB from bacterium *Sphingobium japonicum* UT26 was used (Nagata et al., 1997). The experimental conditions for the highest activity of this enzyme described by Nagata et al. had to be modified in our experiments, because the presence of glycine buffer (pH 8.6) used for measurement of kinetic properties of LinB is not suitable for electrochemical analysis due to high background signal and reduced sensitivity of the electrochemical response. The glycine buffer was therefore replaced with for borate buffer pH 7.6. Under these experimental conditions, the enzymatic release of chloride from chlorinated hydrocarbon 1-chlorohexane (1 mM) was investigated. Detection of cleaved chloride ions was carried out with two instruments: i) a commercial R-C meter Agilent 4284A and ii) a home-made μconductometer as mentioned above.

To verify the correctness of theoretical assumptions on which the measurement is based, it was necessary to carry out specific measurements. The enzyme itself is very conductive and the addition of this biologically active compound to the buffer at a concentration, which ensures efficient progress of reaction, resulted in a decrease of resistance down to 150 Ω. The decrease in conductivity caused by the chlorid anions themselves, which are released from the pesticide, is not so great because a pesticide is supplied in solution in very small quantities. The measurability of the pesticide concentration in the solution would thus be easily questionable, as already mentioned increase in conductivity could be described as one caused only by the enzyme, rather than pesticides. Therefore, we carried out measurements in an environment of pure buffer to which the enzyme was added and, then, a substance representing the pesticide 1-chlorohexane.

The main obstacle consists in the measurement of enzyme activity with 1-chlorohexane. It is necessary to maintain a high temperature of the solution throughout the reaction for relative long time (about 50 minutes). Moreover, it was necessary to close the entire reaction system due to volatility of 1-chlorohexane. The possible release of 1-chlorohexane into the environment could significantly decrease its concentration in reaction solution. To test μconductometer, three concentrations of 1-chlorohexane, 500 nM, 5 and 50 μM to evaluate the detectable range and two concentrations of the enzyme (100 and 500 nM) to test the hypothesis of the enzyme as a catalyst depending on the quantity were chosen.

The measured course (marked with pink colour, Fig. 7) means a solution having a concentration of the enzyme (500 nM) and 1-chlorohexane (50 μM) and was supposed to verify the accuracy of measurements. It follows from the results obtained (part II) that the addition the enzyme caused a decrease of resistance of 161 Ω. Further decrease in resistance (39 Ω) in part III of the Figure was caused by chloride ions released from 1-chlorohexane. The second course (marked in violet, Fig. 7) is measured with the same concentration 1-chlorohexane, but with five fold lower concentration of added enzyme (100 nM). The purpose of the measurements was to demonstrate that the same amount 1-chlorohexane can be cleaved as a substantially smaller amount of the enzyme and thus the decrease in resistance by the enzyme itself can be avoided. The decrease in resistance caused by the enzyme is 33 Ω and the decrease in resistance caused by chlorine ions released from 1-chlorohexane was 17 Ω. The difference compared to the chlorine-based decrease in the first measurement with the higher concentration of the enzyme is probably caused by lower concentration of the enzyme. This presumption is also supported by lower rate of reaction because the curve is not as steep as in the previous case. The last two curves (marked in blue and red, Fig. 7) represent the situation when we added the enzyme as 500 nM, but concentrations of 1-chlorohexane were much lower (500 nM and 5 μM). The Decrease in the resistance of the solution after adding the enzyme was 149 Ω, after adding 1-chlorohexane was 8 and/or 10 Ω for 500 nM and 5 μM of 1-

chlorohexane, respectively. The results are in good agreement with those measured by WTW instrument (Fig. 4). Moreover, bipolar pulse measurements have a greater sensitivity compared to WTW instrument opening a window for other applications.

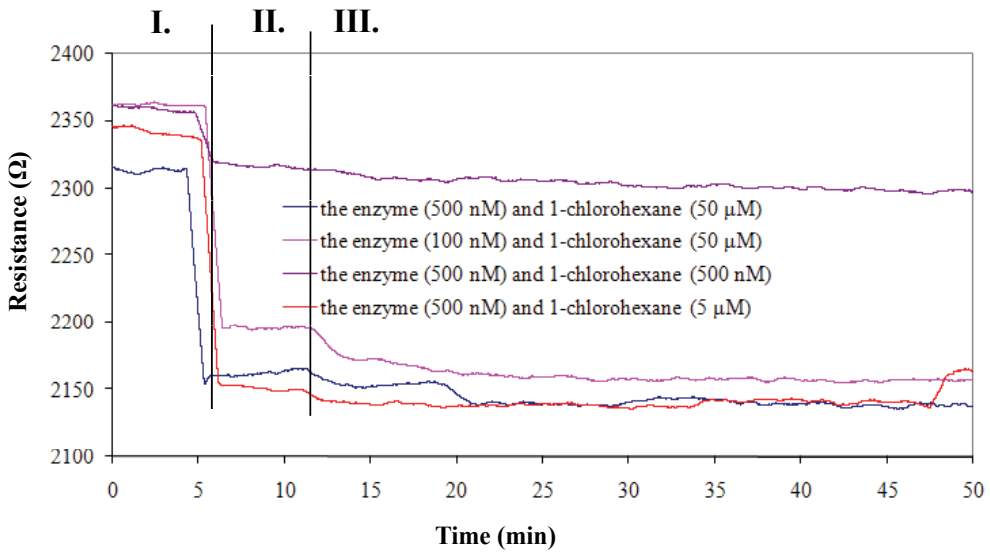

Fig. 7. Home made μconductometer. Dependence of resistance of various solutions consist of the enzyme and 1-chlorohexane on the time of the reaction.

Further, we calibrated the entire system. The measured calibration curve for the solution enzyme-1-chlorohexane with changing 1-chlorohexane concentration given on the x-axis has the following slope-intercept equation: y = 5.84 x (concentration of the enzyme – 100 nM; concentration of 1-chlorohexane – 500 nM, 1 μM, 5 μM, 10 μM and 50 μM). Subsequently, we plotted the dependence of resistance on the concentration of the enzyme itself with the following slope-intercept equation: y = 0.268 x (concentration of the enzyme – 10 nM, 100 nM, 500 nM and 1,000 nM). When comparing the difference between measurements with addition of pure enzyme and enzyme-1-chlorohexane, slopes of both curves were different. This means that despite the very high conductivity change caused by the enzyme itself we are able to detect reaction with 1-chlorohexane, because with the increasing concentration of cleaved 1-chlorohexane resistance decreases rapidly. Moreover, we compared the results obtained with those obtained with Agilent 8492A and WTW and we found that they instrument were in good agreement. Moreover we found a high positive correlation (r = 0.954) between results obtained.

4. Conclusion

A biosensor is an analytical device for the detection of an analyte that combines a biological component with a physicochemical detector component. Simple biosensors are of great development and would be considered as analytical tools in 21st century, which could be used for both diagnostic and environmental monitoring purposes (Bidmanova et al., 2010; Cagnin et al., 2009; Caruana & Howorka, 2010; Ferreira et al., 2009; Gawel et al., 2010;

Kurzawa & Morris, 2010; Lee et al., 2010; Libertino et al., 2009; Murthy et al., 2010; Nayak et al., 2009; Ronkainen et al., 2010; Roy & Gao, 2009; Scarano et al., 2010; Vikesland & Wigginton, 2010; Yoo & Lee, 2010). The main advantage of these tools is their rapidity. This feature is very important for operational decisions in the event of natural or other emergencies. In this study, simple μconductometer was fabricated and tested. the results of comparison of the Agilent 8492A and WTW instrument with bipolar pulses technique confirm that this new instrumentation gives similar results. The resistance discrepancy for EIS was determined 2 Ω indicating detection limit about 1 μM of chloride ions. The new instrumentation reaches a discrepancy of 4 Ω determining detection limit about 2 μM of chloride ions released by reaction between enzyme and pesticide. Based on this, we were able to determine detection limit for pesticide as 100 nM.

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