CHAPTER

I

CHAPTER I: INTRODUCTION

1.1 General introduction

1.1.1 Modern agriculture

In this century, great efforts have been made to improve agricultural productivity. Part of this development can be attributed to the use or to the application of sophisticated agricultural techniques involving extensive mechanization, advanced agricultural practices and the selection of more appropriate plant varieties. The extensive use of pesticides has also played an important role in the increase of the world food production. The pests infest all parts of the plant at all growth stages and can lead the yield losses from 20 to 50% thus lead the farmers using pesticides to protect their crops.

Among the different control measures such as cultural and traditional practices to minimize potential pests, mechanical such as light traps and sticky traps or physical removal of eggs, larvae and adult pests from the crops, biological and chemical methods, the farmers prefer chemical method of control pests because it gives quick results.

Pesticides are synthetic organic chemicals, that they are divided into classifications according to the target organisms are designed to control weeds in fields and lawn, unwanted plant (herbicide), harmful pests such as insects, worms, mites, (insecticide), fungi or microorganisms such as viruses or bacteria (fungicide) and different other used to control pests. (Dong *et al*, 2010). Even when applied in accordance with Good Agricultural Practices (GAP), they can leave residues, which can be detrimental to food

safety. The presence and bio-availability of pesticides in soil can adversely impact human and animal health, beneficial plants and soil organisms. Pesticides can move off-site contaminating surface and groundwater and possibly causing adverse impacts on aquatic ecosystem. (USDA Natural Resources Conservation Service, 1998).

Each pesticide has its own formulation in which the formulation is the chemical and physical form in which the pesticide is sold for use. The active ingredient (a.i.) is the chemical in the formulation that has the specific effect on the target organism. These active ingredients are transported into the target organism's circulation system via substances such as solvents, emulsifiers and buffers (United Nations Environmental Programme, 1992). The formulation improves the properties of the pesticides for storage, handling, application, effectiveness, or safety. Examples of formulated products are wettable powders and water-dispersible granules. A single pesticide is often sold in several different formulations, depending on use requirements and application needs (USDA Natural Resources Conservation Service, 1998).

1.2 Classification of pesticides

In the development of any pesticide, a particular mode of action such as cholinesterase inhibition, inhibition of cell division or immoderate stimulation of weed growth, is often conferred by the incorporation of functional groups into the structure of the chemical. Different groups of pesticides are formed based on the chemical nature of these functional moieties. The functional groups unique to each group of pesticides may confer the properties which affect:

- translocation through plants and soils, significant in terms of inclusion into plant material and potential for root uptake by current and sequential crops;
- Stability and residual time, which relates to their potential to be present at harvest;
- Extractability, related to the solubilities and volatility during production

The categorization of pesticides according to the type of pests controlled is illustrated in Table 1.1.

Table 1.1: Classification of the pesticides according to the type of pest control (Alloway, *et al*., 1997)

Pesticide	Function
Herbicide	**Control broad-leaved weeds and unwanted plants**
Insecticide	**Kills insects (biting, sucking) and other arthropods**
Fungicide	**Control fungal diseases such as rust**
Nematicide	**Kills nematodes**
Molluscicide	**Kills mollusks like snails and slugs**
Acaricide	**Kills ticks, mites and spiders**
Algicide	**Control algae in lakes, canals, water tank, swimming pool and other sites**
Rodenticide	**Kills rats and mice**
Biocide	**control microorganism**
Ovicide	**Kills eggs of insects and mites**
Miticide	**Kills mites that feed on plants and animals**
Antimicribial	**Kills microorganisms like viruses and bacteria**
Pheromone	**Biochemical used to disrupt the mating behaviour of insects**
Repellent	**Repels pests, containing insects and birds**

In addition, the chemical properties of pesticides have implications in the design of analytical methodology specific to their separation and detection (Sandra *et al*., 2004). All of chemical insecticides in use today are neurotoxicants and they act by poisoning the nervous system of target organisms.

1.2.1 Chlorinated chemicals

The organochlorine (OC) pesticides were extensively used from the mid 1940s to the mid 1960s in all aspects of agriculture and forestry, building and structural protection, and in human situations to control a wide variety of insect's pests. Nowadays, these groups of chemicals include chlorotriazoles function as systematic fungicides, and chlorotriazines which are broad-spectrum residual herbicides, used for pre and post emergence weed control.

Many organochlorines are very stable, which in addition with their translocation and absorption properties, are prone to bioaccumulation. The majority of these groups are sufficiently volatile and thermally stable to be amenable to gas chromatography. These groups present a specific feature which enables the unequivocal identification/ confirmation of these chemical species. It is the presence of at least one chlorine atom in the chemical structures. This feature provides distinctive isotope patterns in mass spectral analyses (MS). For example, simazine is a selective triazine herbicide used to control broad-leaved weeds and annual grasses. It acts to inhibit photosynthesis, and it is moderately persistent with an average field half-life of 60 days (Sandra *et al*., 2004).

1.2.2 Organophosphates

Organophosphorus (OP) insecticides were first synthesized in 1937 by German chemists as potential chemical warfare agents (Angelika & Rainer, 2001). This group of pesticides acts as potent cholinesterase inhibitors that generally have lower persistence and bioaccumulation compared to organochlorines, but is regarded as highly toxic. Organophosphates are one of the common classes involved poisoning of food because of the inhibition of acetyl-cholinesterase. Therefore, monitoring the trace levels of OPs in food is important for human health protection and environmental control.

Dimethoate, malation and acephate are examples of this group, which are polar and soluble in water. The presence of phosphorus atom which is the singular feature delineates them from the greater body of pesticides.

1.2.3 Pyrethroid insecticides

This is the newest class of insecticides, a group of chemicals that entered the market place in 1980 but by 1982 accounted for approximately 30% of worldwide insecticide use**.** Most pyrethroids are used in the control of agriculture, forestry, household, industrial, stored products, and veterinary pests in the integrated pest management (IPM) program of modern society (Angelika & Rainer, 2001).

1.2.4 Urea chemicals

Phenyl-substituted ureas are used extensively in agriculture as selective herbicides, mainly for pre and post emergence and they act by inhibiting photosynthesis. Commonly used substituted ureas are linuron and diuron, which have low residual action and persistence. Solubility of this group in water and the polarities

and chemical distinction of the ureas are features which may be exploited in clean-up methodology and using chromatographic products respectively.

Application of gas chromatogtaphy to analyze phenylurea pesticides is not suitable due to these compounds are thermally unstable and rapidly degrade to isocyanates and amines.

1.2.5 Carbamate derivatives

The carbamates are N-substituted esters of carbamic acid and act as cholinesterase inhibitors that confer insecticidal activity. Their effects are generally less intense than the organophosphates and they have low persistence in the environment. Solubility of the carbamates can vary quite dramatically. Although the likelihood of the co-extraction of different carbamates will vary with varying representatives of this chemical class, the potential to co-extract with concretes and absolutes is high. This is particularly true for the carbamate pesticides commonly used carbaryl.

The majority of N-substituted carbamates are thermally unstable and therefore not amenable to gas chromatography. However, liquid chromatography is more suited to the analysis of this chemical type.

1.2.6 Dithiocarbamates

Dithiocarbamates are pesticides used to control fungal diseases such as rust. They are non-systematic, contact fungicides that remain on the surface of the plants until degraded or washed off with rain or abrasion. Dithiocarbamates are heat labile and degrade to a number of products including ethylenethiourea (ETU), which is soluble in water and readily absorbed and metabolised in plant. Dithane is a dithiocarbamate

fungicide whose active ingredient is mancozeb. Mancozeb rapidly and spontaneously degrades to ETU in the presence of water and oxygen.

1.2.7 Pesticides with acidic moieties

This class of broad leaf weed killers include a large range of carboxylic acid herbicides. Some of the pesticides are applied in chemical formulations as esters, which decompose to the acidic form under alkaline or acidic conditions. Translocation of the acidic pesticides takes place in the roots of treated plants.

The acidic forms of this class of pesticide, however, are water soluble which confers a physical parameter on which clean-up protocols may be designed. The parent esters of pesticides with acidic moieties follow the same considerations as previously discussed for the organochlorines. They are directly amenable to GC and elute in the same time frame as many of the oxygenated sesquiterpenes. Dicamba is a benzoic acid herbicide. It can be applied to the leaves or the soil to control annual and perennial broadleaf weeds.

1.2.8 Quaternary nitrogen herbicides

The most frequently pesticides of this class such as paraquat and diquat are used for broadleaf weed control. They are quick acting, non-selective; contact poisons, which are also translocated through the plant. Both pesticides are very soluble in water and insoluble in hydrocarbons. The quaternary ammonium herbicides are not amenable to GC. LC is limited usually requiring the inclusion of ion-pair reagents or the analytes must be derivatised (Sandra *et al*., 2004).

1.3 Effects of Pesticides on Soil Quality

The capacity of the soil to filter, degrade, buffer, immobilize and detoxify pesticides is a function or quality of the soil. The presence and bio-availability of pesticides in soil give adverse impact on human and animal health, and beneficial plants and soil organisms. Pesticides can move off-site contaminant surface and groundwater and possibly causing adverse impacts on aquatic ecosystems as well.

1.4 Pesticide mode of action

Mode of action refers to the mechanism by which the pesticide kills or interacts with the target organism. Two different modes of action refering to pesticides are as follows:

- Contact pesticides kill the target organism by weakening or disturbing the cellular membranes; death can be very rapid.

- Systemic pesticides must be absorbed or ingested by the target organism to disturb its physiological or metabolic procedures; usually they act slowly.

How effective the pesticides are at killing the target organisms (efficacy) depends on the properties of the pesticide and the soil, formulation, agricultural management, application method, environmental or weather conditions, characteristics of the crop, and the nature and behaviour of the target organism.

1.5 Fate of pesticides in the Environment

Perfectly, a pesticide stays in the treated area long enough to produce the desired effect and then degrades into harmless materials. Three primary modes of degradation occur in soils as follows:

- Biological - breakdown by micro-organisms
- Chemical - breakdown by chemical reactions, such as hydrolysis and redox reactions.
- Photochemical - breakdown by ultraviolet or visible light

The rate at which a chemical degrades is expressed as the half-life. The half-life is the amount of time taken for half of the pesticide to be converted into other compounds, or its concentration is half of its initial level. The half-life of a pesticide depends on soil type, its formulation, and environmental conditions (e.g. moisture and temperature).

Other procedures that influence the fate of the chemical include plant uptake, soil sorption, leaching and volatilization. If pesticides move off-site (e.g., wind drift, runoff and leaching), they are considered to pollutants. The potential for pesticides to move off-site depends on the chemical properties and formulation of the pesticide, soil properties, rate and method of application, pesticide persistence, frequency and timing of rainfall or irrigation, and depth to ground water.

1.6 Retention of pesticides in the soil

Retention refers to the ability of the soil to hold a pesticide in place and not allow it to be transported. Adsorption is the primary process of how the soil retains a pesticide and is defined as the accumulation of a pesticide on the soil particle surfaces. Pesticide adsorption to soil depends on both the chemical properties of the pesticide (i.e., water solubility, polarity) and properties of the soil (i.e., organic matter and clay contents, pH, surface charge characteristics, permeability). For most pesticides, organic matter is the most important property which of soil controls the degree of adsorption.

In the most cases, the degree of adsorption is described by an adsorption distribution coefficient (K_d), which is mathematically defined as the amount of pesticide in soil solution divided by the amount adsorbed to the soil.

1.7 Pesticide toxicity

The toxicity level of a pesticide depends on the deadliness of the chemical, the dose, the length of exposure, and the route of entry or absorption by the body. Pesticide degradation in soil generally results in a reduction in toxicity; however, some pesticides have breakdown products (metabolites) that are more toxic than the parent compound. Pesticides are classified according to their potential toxicity to humans and other animals and organisms, as restricted-use (can only be purchased and applied by certified persons who have had training in pesticide application), and general use (may be purchased and applied by any person).

Risk of using pesticides is the potential for damage or the degree of risk involved in using a pesticide under given set of conditions (USDA Natural Resources Conservation Service, 1998).

Hazard depends on the toxicity of the pesticide and the amount of exposure to the pesticide and is usually showed by the following equation:

Hazard = Toxicity × exposure

Acute toxicity of a pesticide includes the chemical's capability to cause hurt to a human or animal from a single exposure, usually of short period.

Acute toxicity has been measured as the concentration of a toxicant which is the active ingredient needs to kill 50% of the animals in a test population. This measure is often explained as the LD_{50} (lethal dose 50) or the LC50 (lethal concentration 50). The lower the LD_{50} value of a pesticide product, the greater its toxicity to human and animals.

Several types of toxicity related to pesticides are as follows:

- Toxicity of fungicide

The severe toxicity of fungicides to human is often low considerable; by which they can be annoying to the skin and eyes. Chronic exposures to low concentrations of fungicides are adverse for human health. One of the common cases of human fungicide poisonings was due to the consumption of seed grain. Nowadays, to prevent these kinds of poisoning, fungicide treatment contains a brightly coloured dye to clearly exhibit that the seed has been treated.

- Toxicity of herbicide

Normally, herbicides have a less acute toxicity to humans because the physicology of plants is too different than that of human. Neverthless, there are exceptions; many of them are often strong acids, esters, amines and phenols, so can be dermal irritants. Prolonged inhalation often causes dizziness and ingestion sometimes cause vomiting, a burning sensation in the stomach, and muscle twitching.

- Toxicity of insecticide

Insecticides cause the largest number of pesticide poisoning in the United States. Acute exposure to organophosphate and carbamate insecticides is often one of the most serious pesticide poisonings. Organophosphate insecticides contain chlorpyrifos, dimethoate, diazinon, malathion, disulfoton, methyl pathion and ethyl parathion. The carbamate compounds include carbaryl, carbofuran, methomyl and oxamyl. Organophosphates and carbamates prevent the enzyme chlonisterase causing commotion of the nervous system. In advanced poisoning, the victim is pale, sweating and frothing at the mouth. The pupils are tight and insensitive, other signs such as changes in heart rate, mental confusion, muscle weakness, and coma. The victim may die if not treated.

1.8 Maximum Residue Level (MRL) and Legislation

A Maximum Residue Limit (MRL) is the maximum concentration of a pesticide residue legally permitted in a food or feed commodity. An MRL is assessed as representing the maximum residue level expected to be found in a foodstuff or feed if a pesticide is applied according to good agricultural practice or GAP (FAO, Rome, 2002). Many control authorities have established maximum residue limits (MRLs) or tolerances to protect the environment and consumer health. Due to consumer awareness of potentially hazardous pesticide residues in foods, international trade issues, regulatory requirements, risk assessment and other reasons, monitoring of food items for pesticide residues is often conducted in government and private contract labs worldwide. Maximum Residue Limit (MRL) symbolizes the maximum amount of pesticide residue that may be anticipated in a food commodity when a product is used according to Good Agricultural Practices (GAP), as certified by the PMRA on mid 1950s. They are established after a dietary assessment illustrates that they are satisfactory.

The Codex Alimentarius Commission (Codex) is an international body created by FAO and WHO in 1963 to develop food standards, guidelines and related texts. MRLs for the purposes of international trade were first recommended in 1966 by the Joint FAO/WHO (Food and Agriculture of the United Nations/ World Health Organization) Meeting on Pesticide Residues (JMPR) to Codex. An MRL can be thought of as comprising three parts, a residue definition, a sample definition (size and component to be analysed, equivalent to 'the commodity') and a numerical value (MacLachlan *et al*., 2010). MRLs are necessary when there is a probability of pesticide residues occurring on treated crops or in commodities from animal fed these harvests. MRLs apply for

domestic and imported food. MRLs are legitimately established under the food and drug regulations of the FDAA, and under the FDA the Canadian Food Inspection Agency (CFIA) is responsible to control domestic and imported foods and performing enforcement actions to avoid the sale of food including immoderate residues.

In 1963, the Codex Alimentarius Commission was made by FAO and WHO to improved guidelines and food standard programmes. The main purpose of this program is to protect the health of the consumers and to ensure fair trade practices in the food trade, and to promote coordination of all food standards work undertaken by international and non-governmental organizations (NGOs).

The emphasis on great productivity in the agricultural part and the food industry in 1970s has moved towards greater worry to satisfy the requirements of consumers as considerations the quality and safety of products. In addition, the European Commission decided to establish severe safety standards across the whole food chain, since 1990s, and this white pare published in 2000. Adopted at the end of 2002, regulation EC 178/2002 is the linchpin of the new legislation governing food safety, forming the bases of the new approach. It officially established the European Food Safety Authority along with a standing committee on the food chain and animal health. The main goals of this permanent organization are as follows:

- Protect human and health
- Protect human interests including equitable trade in food practices
- attain free movement of food in the community
- Harmonizes regulations related to food and animal feed. (Gwin, 2003).

1.9 Pesticides selected for this study

In this study, a multiclass of pesticides with different structures and two chemical uses (insecticides and herbicides) such as, dimethoate, carbaryl, simazine, atrazine, terbuhylazine, diuron and malathion were selected among different groups of compounds containing organophosphates, carbamates, triazines and phenylureas respectively.

1.9.1 Dimethoate

Dimethoate is a widely used organophosphorus insecticide to kill mites and insects systematically and on contact (Pesticide management information programme, New York, 1993). It is also used as residual wall spray in farm buildings for houseflies. Dimethoate has been administered to livestock for control of bottle flies. It is moderately toxic by ingestion, inhalation, and dermal absorption. Dimethoate has a low persistence in the soil environment. Soil half-lives of 4-20 days have been reported for this compound. Dimethoate will be broken down faster in moist soils because soil microorganisms rapidly break it down. In water, it is neither expected to adsorb to sediments or suspended particles nor to bioaccumulate in aquatic organisms. The half-life in river water was 8 days, with disappearance possibly due to microbial action or chemical degradation (Howard, 1991).

Physical properties of dimethoate:

Pesticide class	Organophosphorous pesticides
Molecular formula	$C_5H_{12}NO_3PS_2$
IUPAC name	0,0-dimethyl S-methylcarbamoylmethyl phosphorodithioate
CAS number	60-51-5
Molar mass	229.26 g mol^{-1}
Appearance	Grey-white crystalline solid
Water solubility	2.5×10^4 mg L^{-1} at 21°C
Solubility in other solvents	Methanol, cyclohexane, diethyl ether, hexane, xylene
Melting point	143-145°C
Vapour pressure	1.1 mPa at 25°C
Log K_{ow}	0.704
(K_{ow}: Octanol/Water Partition)	

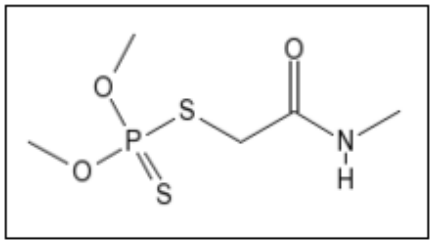

Figure 1.1: Molecular structure of dimethoate

1.9.2 Malathion

Malathion is a non-systematic, widespread organophosphate insecticide. It was one of the earliest organophosphate insecticides developed in 1950. It is suited for the control of sucking and chewing insects on fruits and vegetables, and is also used to control mosquitoes, flies, household insects, animal parasites, and head and body lice (Gallo *et al*., 1991).

Malathion is slightly toxic via the oral toute, with reported oral LD_{50} values of 1000 mg kg^{-1} to greater than 10,000 mg kg^{-1} in the rat and 400 mg kg^{-1} to greater than 4000 mg kg^{-1} in the mouse (Kidd *et al*., 1991). Malathion has a low persistence in soli with reported field half-lives of 1-25 days. Degradation in soil is rapid and related to the

degree of soil binding. If released to the atmosphere, malathion will breakdown rapidly in sunlight, with a reported half-life in air of about 1.5 days. In raw river water, the half-life is less than a week, whereas malathion remained stable in distilled water for 3 weeks (Howard *et al*., 1991).

Physical properties of malathion:

Pesticide class	Organophosphorous pesticides
Molecular formula	$C_{10}H_{19}O_6PS_2$
IUPAC name	2-(dimethoxyphosphinothioylthio) butanedioic acid diethyl ester
CAS number	121-75-5
Molar mass	330.358 g mol^{-1}
Appearance	Clear or amber liquid at room temperature
Water solubility	145 mg L^{-1} at 21°C
Solubility in other solvents	Miscible with most organic solvent
Melting point	285°C
Vapour pressure	5.3 mPa at 30°C
Log K_{ow}	2.75

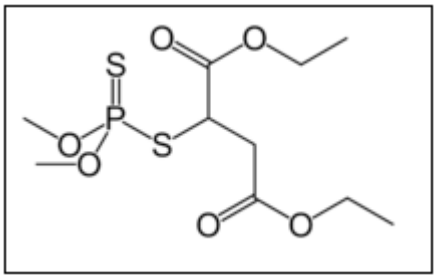

Figure 1.2: Molecular structure of Malathion

1.9.3 Carbaryl

Carbaryl is a wide spectrum carbamate insecticide which over 100 species of insects on citrus fruit, cotton, lawns, nuts, ornamental, shade trees, and other crops, as well as on poultry, livestock, and pets. It is moderate toxic. It can produce adverse effects on human by skin contact, inhalation or ingestion. The symptoms of acute toxicity are typical of the other carbamates. The oral LD50 of carbaryl ranges from 250

µg g^{-1} to 850 µg g^{-1} in rats and from 100 to 650 µg g^{-1} in mice. Carbary has a low persistence in soil and its degradation in the soil is mostly due to sunlight and bacteria action. In surface water, carbaryl is broken down by bacteria and through hydrolysis. It has a half-life of about 10 days at neutral pH. The metabolites of carbaryl have lower toxicity to humans than carbaryl itself (Smith, 1993).

Physical properties of carbaryl:

Pesticide class	Carbamate pesticides
Molecular formula	$C_{12}H_{11}NO_2$
IUPAC name	1-naphthyl methylcarbamate
CAS number	63-25-2
Molar mass	201.22 g mol^{-1}
Appearance	Solid with variety of colour from colourless to white or gray
Water solubility	40 mg L^{-1} at 21°C
Solubility in other solvents	Acetone, cyclohexane, dimethyl sulfoxide
Melting point	137.5°C
Vapour pressure	< 5.3 mPa at 25°C
Log K_{ow}	1.80

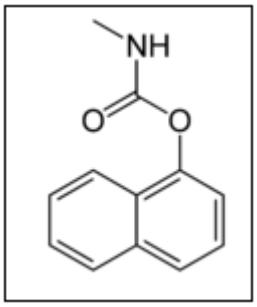

Figure 1.3: Molecular structure of carbaryl

1.9.4 Simazine

Simazine is a pre-emergence, s-triazine herbicide used to control of broad-leaved and grassy weeds on a variety of deep-rooted crops. Simazine may be released into the environment via effluents at manufacturing sites and at points of application where it is employed as herbicide. Since simazine is not a listed chemical in the toxics release

inventory, data on releases during its manufacture and handling are not available. If released to water, simazine is not expected to adsorb to sediment and suspended particulate matter, or volatilize. Persistence depends upon many factors including degree of algae and weed infestation. Simazine residues may persist up to 3 years in soil under aquatic field conditions. Dissipation of simazine in pond and lake water was variable, with half-lives ranging from 50 to 700 days. If released to soil, the mobility of simazine will be expected to vary from slight to high in soil-types ranging from clay soils to sandy loams soils, respectively based upon soil column, soil thin-layer chromatography, and K_{oc} experiments. Therefore, it may leach to groundwater. If released to the atmosphere, simazine is expected to exist almost entirely in the particulate phase. Vapour phase reactions with photochemically produced hydroxyl radicals in the atmosphere may be important (estimated half-life of about 2.8 hr). Photolysis may be an important removal mechanism in the atmosphere (National Primary Drinking Water Regulations, EPA 2003).

Physical properties of simazine:

Pesticide class	s-triazine herbicides
Molecular formula	$C_7H_{12}ClN_5$
IUPAC name	6-chloro-*N*,*N'*-diethyl-1,3,5-triazine-2,4-diamine
CAS number	122-34-9
Molar mass	201.657 g mol^{-1}
Appearance	White crystalline powder
Water solubility	Insoluble (5 mg/L water)
Solubility in other solvents	Methanol, chloroform, diethyl ether,
Melting point	225-227°C
Vapour pressure	0.000810 mPa at 20°C
Partition Coefficient:	1.9600
Adsorption Coefficient (Kd):	130
Log K_{ow}	2.18

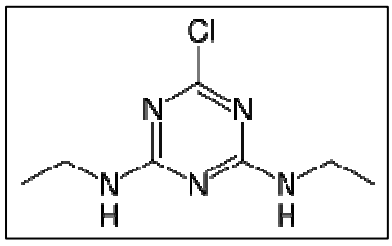

Figure 1.4: Molecular structure of simazine

1.9.5 Terbuthylazine

Terbuthylazine is an algicide, microbicide and microbiostat used to control slime-forming algae, fungi, and bacteria. It is registered for use in commercial and industrial water cooling systems, and in residential and commercial ornamental ponds, fountains and aquaria. Terbuthylazine generally has relatively low acute toxicity. It is mildly to moderately irritating to the eyes, and slightly irritating to the skin. Terbuthylazine is stable to hydrolysis, and to aqueous photolysis. It degrades very slowly under aerobic aquatic conditions, and will persist under most aquatic conditions. Terbuthylazine is practically nontoxic to birds on an acute and sub-acute dietary basis. However, it is moderately toxic to both cold and warm water fish, slightly toxic to aquatic invertebrates, and highly toxic to estuarine/marine invertebrates from acute exposures. Terbuthylazine is expected to be phytotoxic to aquatic plants because it belongs to the triazine family (which includes many herbicides), is released to waterways, and dissipates slowly in the environment (Prevention, Pesticides And Toxic Substances, EPA, 1995).

Physical properties of terbuthylazine:

Pesticide class	s-triazine herbicides
Molecular formula	$C_9H_{16}ClN_5$
CAS name	6-chloro-N-(1,1-dimethylethyl)-N'-ethyl-1,3,5-triazine-2,4-diamine
CAS number	5915-41-3
Molar mass	229.710 g mol^{-1}
Appearance	White crystalline powder
Water solubility	11.5 mg mL^{-1}
Solubility in other solvents	Acetone 41.3, Ethanol 15.0, Toluene 10.4, n-Octanol 12.5, Ethylene glycol 2.36 (g/L at 20°C)
Melting point	178-179.3°C
Adsorption Coefficient:	530
Log K_{ow}	3.21

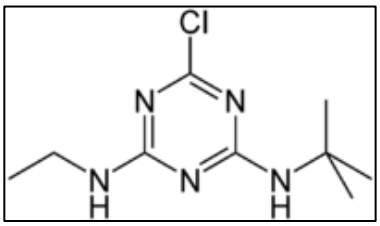

Figure 1.5: Molecular structure of terbuthylazine

1.9.6 Atrazine

Atrazine is a widely used s-triazine herbicide for control of broadleaf and grassy weeds. Atrazine may be released to the environment through effluents from manufacturing facilities and through its use as an herbicide. Atrazine was the second most frequently detected pesticide in EPA's National Survey of Pesticides in Drinking Water Wells. Microbial activity possibly accounts for significant degradation of atrazine in soil. The effect of atrazine on these organisms seems to be negligible. Photo-degradation and volatilization are of little significance under most field conditions. Atrazine was completely hydrolyzed within 34 days at extreme pHs. Alkaline hydrolysis proceeds twice as rapid as acidic hydrolysis. Based on the K_{oc} values for soils, atrazine is expected to maintain a high to medium mobility class in soils. Reactions with photochemically produced hydroxyl radicals in the atmosphere may be important, with reports of an atmospheric half-life of about 2.6 hr at an atmospheric concentration of 5×10^5 hydroxyl radicals per cu cm (National Primary Drinking Water Regulations, EPA 2003).

Physical properties of atrazine:

Pesticide class	s-triazine herbicides
Molecular formula	$C_8H_{14}ClN_5$
IUPAC name	2-chloro-4-(ethylamine)-6-(isopropylamine)-1,3,5-triazine-2,4-diamine
CAS number	1912-24-9
Molar mass	215.68 g mol^{-1}
Appearance	Colourless solid
Water solubility	0.07 g mL^{-1}
Solubility in other solvents	
Melting point	175°C
Log K_{ow}	2.34

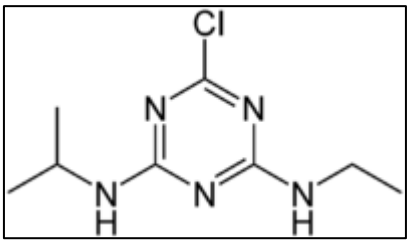

Figure 1.6: Molecular structure of atrazine

1.9.7 Diuron

Diuron is a systemic substituted phenylurea herbicide. Diuron is easily taken up from soil solution by the root system of plants and rapidly translocated into stems and leaves by the transpiration system, moving primarily via the xylem. Diuron primarily functions by inhibiting the Hill reaction in photosynthesis, limiting the production of high-energy compounds such as adenosine triphosphate (ATP) used for various metabolic processes. Diuron is a broad-spectrum residual herbicide registered for pre-emergent and post-emergent control of both broadleaf and annual grassy weeds. Diuron also has widespread use in non-agricultural applications, especially industrial and rights of way uses, where often in combination with other herbicides it provides total vegetation control. These applications include along fence lines, pipelines, powerlines,

railway lines, roads, footpaths; in timber yards and storage areas; and around commercial, industrial and farm buildings, electrical substations, and petroleum storage tanks. It has some use as an algaecide in ornamental ponds, fountains, and aquaria, but not natural water bodies (U.S, EPA, 2004a, b). Consequently diuron is both mobile and relatively persistent, and is therefore prone to off-site movement in surface runoff, and migration to ground water. Diuron is moderately to highly persistent in soils. The commonly reported average field dissipation half-life is 90 days, although such half-lives are typically highly variable. Phytotoxic residues generally dissipate within a season when applied at low selective rates. At higher application rates, residues may persist for more than one year (Kidd & James, 1991).

Physical properties of Diuron:

Pesticide class	Sulfunyl urea herbicides
Molecular formula	$C_9H_{10}C_{l2}N_2O$
IUPAC name	3-(3,4-dichlorophenyl)-1,1-dimethylurea
CAS number	330-54-1
Molar mass	233.10 g mol^{-1}
Appearance	Colourless solid
Water solubility	42 mg L^{-1}
Melting point	158°C
Vapour pressure	6.9×10^{-8} mm Hg (at 25°C)
Adsorption Coefficient:	418-460
Log K_{ow}	2.68

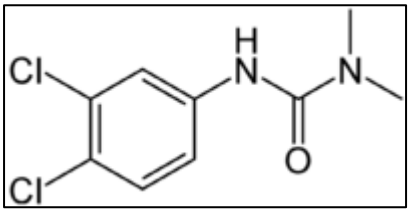

Figure1.7: Molecular structure of diuron

1.10 Introduction to oil samples

1.10.1 Olive oil

Olives (*Olea europaea*; family *Oleaceae*) have been cultivated for thousands of years in the countries surrounding the Mediterranean Sea. Today olives are commercially produced in Spain, Italy, France, Greece, Tunisia, Morocco, Turkey, Portugal, China, Chile, Peru, Brazil, Mexico, Angola, South Africa, Uruguay, Afghanistan, Australia, New Zealand, and California. The Mediterranean area produces 93% of the olive production. Currently there are some 800 million olive trees being cultivated. California is the only state where olives are grown commercially in US. Over 90% of the olive production is used to make olive oil. A Franciscan missionary planted the first olive tree in California in 1769 at a Franciscan mission in San Diego. Olives are not edible, green, or ripe, and must be treated with lye and/or cured in brine or dry salt before being edible. They contain about 20% oil. Olives must be processed to remove the bitter glycoside oleuropein, before they are edible, so they are usually first treated with lye and then pickled.

Olive oil is the oil extracted from the olive fruits of the olive trees. It is the only oil that can be consumed because it has been freshly pressed from the fruit. It is commonly used in cooking, cosmetics, pharmaceuticals, and soaps and as a fuel for traditional oil lamps. Olive oil is considered as an essential foodstuff because its composition is rich in monounsaturated fatty acids and antioxidants. All these characteristics have increased the demand for this commodity throughout the world. In order to satisfy the increasing demand and provide new alternatives to consumers, other countries in Asia such as Iran and Turkey are currently producing olive oil.

There are several types of olive oil which are available in the market around the world. The different types of olive oil are listed below:

1. Extra Virgin Olive Oil

Extra virgin olive oil is the best quality and most expensive olive oil. It is produced from the first pressing of the fresh handpicked olive fruits by the use of physical means without chemical treatment. It has the highest amount of antioxidants compared to other types of olive oil. An olive oil to qualify as an extra virgin should have less than 1% acidity and it should be cold.

2. Virgin Olive Oil

Virgin olive oil is slightly inferior to extra virgin olive oil. It can be obtained after pressing the oil by mechanical means, without processed or refined. It has a slightly higher level of acidity than extra virgin olive oil that is approximately 1.5% to 2.0%.

3. Refined Olive Oil

Refined olive oil has been treated by the use of chemical treatment and physical filters to neutralize strong tastes free fatty acids. It has mostly lower quality than virgin oil. Refining virgin olive oil eliminates the high acidity level.

4. Olive Oil

This type of olive oil is made by blending refined olive oil with virgin olive oil in certain proportions to retain some of its aroma and taste. Olive oils that are sold as "Light" or "Extra Light" all fall under this category (Boskou, 1996).

1.10.2 Palm oil

Oil palm (*Elaeis guineensis*) is grown extensively in Southeast Asia and Equatorial Malaysia is not only one of the leading countries in exporting palm fruit, but also is the largest exporter of palm oil in the world. According to the World Bank and the Asian Development Bank, Malaysia is the world's second largest palm oil producer (Ong & Goh, 2002). Palm oil is derived from the flesh of the palm fruit (mesocarp), while palm kernel oil is derived from the seed or kernel of the fruit. The palm oil obtained from the mesocarp of the palm fruit is widely used in various food products, such as margarines, shortenings, cooking oils, confectionery fats, and vanaspati without or with only minimal modification of palm oil composition, as well as in non food products such as oleochemicals, soaps, and biodiesel. It is the largest edible oil by dominating 25% of total global oils and fats production in 2007 and has been perceived as the most promising feedstock for biodiesel production (Malaysian Palm Oil Council, MPOC, 2007).

1.11 Thesis objectives

The objectives of this study are as follows:

1. To evaluate sample treatment methods for the development of multiresidue pesticides in palm oil and olive oil using LC-QTOF-MS with an electrospray interface operated in positive ionization mode.
2. To validate the methods chosed before in terms of recovery (trueness), repeatability (within sequence precision), reproducibility (precision among sequences), detectability (lowest calibrated level), linearity (R^2), and matrix effects (%ME) before application to the real samles of palm oil and olive oil.

CHAPTER

II

CHAPTER II: LITERATURE REVIEW

2. Review of analytical methods and pesticide formulations for pesticides analysis in food matrices by chromatography-based techniques

2.1 Sample preparation and extraction techniques

Food is a complex non-homogenous mixture of a wide range of chemical substances that makes it hard to isolate and determine the analyte of interest. Scientific knowledge about chemical contamination of food has grown considerably in recent years. Until fifteen years ago, this area of research was considered relatively young. Since then, this area of science has continued to develop, in particular becoming an established part of regulatory reviews of food safety across the world. Analysis of pesticides in food matrices is a difficult task, because of the complexity of the matrix and the low concentrations at which these compounds are usually present. Despite employing of advanced techniques of separation and identification and powerful instrumental techniques such as chromatography instruments with mass spectrometry detection, the risk of interference increases with the complexity of the matrix studied, so sample preparation prior to instrumental analysis is necessary. Qualitative and quantitative analysis require a procedure of sample preparation.

In the past decades, a great advancement has been made in order to achieve efficient separation of analyte from a sample matrix with high selectivity and sensitivity. Different extraction methods are employed, consisting of solvent extraction (SE) from

solids and liquid-liquid extraction (LLE) from solutions. The solvents may be organic liquids, supercritical fluids or superheated liquids. Alternatively, the liquid extractant may be bonded to a support material. Selectivity can be achieved by altering the extraction temperature and pressure, by the choice of extraction solvent or liquid, and the control of pH and additives such as ion-pair reagents. Poor sample treatment or roughly prepared extract will invalidate the total analysis and will make it impossible to gain a valid result even by use of the most powerful separation method. Therefore, a correct sample preparation can be economically valuable as well as analytically important.

One of the goals in the routine monitoring of pesticide residues by regulatory and private contract laboratories is to attain quick sample turnaround time and high sample throughput. In addition to being fast, useful methods must also achieve high quality results for a wide scope of analytes and matrices, have excellent robustness for routine use, meet low detection limits, and be affordable, simple to perform, environmentally friendly, and safe (Koesukwiwat *et al*., 2010).

The extraction step is the least evolved part of most analytical procedures. Extraction procedures adopted in many standardized analytical methodologies for determining contaminants in food, in particular products from food-producing animals, are labour-intensive and solvent consuming. In order to obtain satisfactory analyte recovery, efforts to isolate the compound(s) of interest include repeated extractions of the analytes from the biological matrix, use of fresh solvent each time, centrifugation, and pooling of the supernatants. This part of the analytical protocol requires the use of relatively large volumes of toxic, expensive, and flammable solvents, and the subsequent need to evaporate and dispose of the solvent. In many cases, the combination of sample and

solvent produces emulsions that may decrease the extraction efficiency and lengthen the time the analyst needs to complete the procedure. As the generally employed organic solvents do not selectively extract the targeted compounds and tedious and time-consuming cleanup procedures are needed to partially isolate analytes from the matrix components. (Bojialli *et al*., 2007a)

During the last decades, several modern techniques have been purposed to reduce sample handling and toxic waste, consequently to maximize recovery of the analytes and minimize the accompanying interferences by the use of appropriate extraction and clean-up procedures. After the extraction steps, the analytes of interest are obtained in an organic or aqueous solution, which then requires concentration or additional clean-up. The extract can then be treated similar to liquid samples. Liquid samples can be handled directly such as quick, easy, cheap, effective, rugged, and safe (QuEchERS) procedure (Lesueur, *et al*., 2008), or instrumentally-based heating or agitating of sample such as pressurized liquid extraction (PLE) (Soler *et al*., 2006), microwave assisted extraction (MAE) (Papadakis *et al*., 2006), ultrasonic extraction (USE) (Jianfeng *et al*., 2008), supercritical fluid extraction (SFE) (Aguilera *et al*., 2003), or by solvent–solvent extraction methods or sorption methods such as solid-phase extraction (SPE) (Zhou *et al*., 2006a,b), solid-phase microextraction (SPME) (Raposo *et al*., 2007), headspace-solid phase micro-extraction (HS-SPME) (Mmualefe *et al*., 2009) and stir-bar-sorptive extraction (SBSE) (Juan *et al*., 2004). The use of solid sorbent material to extract analytes from a solution was developed in the 1980s and is now widely applied to many matrices, including food. A sorbent with strong affinity towards some target analytes will retain and concentrate those compounds from the sample solution. Many sorbents are specifically suited for the extraction of different analytes with various degrees of

selectivity such as (SPE), (SPME), (SBSE) and matrix solid-phase dispersion (MSPD). These methods offer both advantages and disadvantages and so the application of any one of those depends on the properties of analyte and analytical problems. Some of these methods are described in the next sections.

Solid samples are usually prepared by grinding, mixing, agitating, stirring, chopping, crushing, pressing and pulverizing directly or after drying followed by solvent or liquid extraction. In most of cases, sample homogenization with an organic solvent often mixed with water is achieved by using a homogenizer, blender or sonicator (Tadeo *et al*., 2000; Pico *et al*., 2000). After the extraction steps the analytes of interest are obtained in an organic or aqueous solution, which then requires concentration or additional clean-up.

2.1.1 Solvent extraction procedures

2.1.1.1 Solvent extraction (SE)

Solvent extraction (SE), which may be followed by solid-phase extraction (SPE) is still the most widely used technique, mainly because of its ease of use and wide ranging applicability. The extraction process varies slightly, depending on whether the sample is liquid or solid. Analysis of liquid samples usually requires fewer pre-treatment steps in comparison with analysis of solid samples because of their liquid state. Occasionally, very little sample preparation may be required if the liquid is sufficiently free from matrix interferences, for example dilution with water or filtration (Lambropoulou, *et al*., 2007).

A number of solvents have been used for this purpose and the most common include ethyl acetate (EtAc) (Blasco *et al*., 2005; Tanaka *et al*., 2007; Ferrer *et al*., 2007; Min *et al*., 2008), acetone (Rissato *et al*., 2004; Coscolla *et al*., 2008), acetonitrile (MeCN) (Hercigova *et al*., 2005; Serodio *et al*., 2005; Chen et al., 2007; Zhao *et al*., 2008; Amvrazi *et al*., 2008; Garcia *et al*., 2010), methanol (MeOH) (Juan *et al*., 2004; Nozal *et al*., 2005; Sergi *et al*., 2007; you *et al*., 2007), dichloromethane (DCM) (Soler *et al*., 2006; Sanchez *et al*., 2008; Ravelo *et al*., 2008), n-hexane (Cheng *et al*., 2009; Fujita *et al*., 2009) and diethyl ether (Yoshioka *et al*., 2004). However, it is important to match the polarity of the solvent to the solubility of analyte, and the addition of non-polar, water immiscible solvents such as DCM or n-hexane to the different polarity solvents to obtain the proper viscosity and modified solvent for extraction.

Roos and co-workers first reported the use of EtAc and sodium sulfate in a multi-residue extraction procedure to eliminate the liquid-liquid partition (LLP) step. They used size exclusion chromatography (SEC) as a clean-up procedure for the analysis of organochlorine and organophosphorus pesticides, fungicides and chlorobiphenyls from fats, fish oils, vegetables, fruits, cereals and liver. The use of a 10-mm i.d. SEC column provided the same limits of determination as those attainable with commercial systems but requires only 15% of the amount of solvents normally used (Roos *et al*., 1987) whereas Holstege *et al*. (1994) modified the method by the addition of acetone, methanol or ethanol in EtAc in order to increase the polarity of the solvent system. The ethyl acetate method is also named the on-line extraction method because they omit a separate LLP step.

Cheng *et al*. (2007) studied the application of several organic solvents such as MeOH, chloroform, acetone-n-hexane (1:1 v/v), DCM-MeOH (9:1 v/v) and

chloroform-MeOH (1:1 v/v) for extracting triazines from sheep liver, at 70°C for 10 min. According to the obtained recoveries, the recovery of analytes was decreased when chloroform was used due to the emulsification procedure during extraction. However, the highest recoveries were obtained when MeOH was used as the extracting solvent.

Among the solvents mentioned above, MeCN as the extracting solvent has some advantages because MeCN is polar and soluble in water. In addition, it can furnish sufficient extraction for polar and non-polar pesticides from non-fatty foods due to its hydrophobic property. When MeCN is employed as the extracting solvent, the extracts have only a small quantity of co-extractives and facilitate direct analysis by LC-MS or LC-MS-MS whereas using other solvents such as DCM the extracts contain larger amounts of co-extractives. Hence, it is possible to use MeCN in the analyses of pesticide residues in complex matrices with different mixture of ingredients and interferences. The special properties of MeCN make it the solvent of choice in the QuEChERS technique.

Zhao *et al*. (2008) compared four organic solvents such as MeCN, methanol, ethanol and acetone for the optimal selection. In their experiments, LC-grade MeCN and MeOH were used directly, but analytical-grade ethanol and acetone were used after redistillation to remove the impurities. It was found that MeCN gave the best elution performance for the analysis of s-triazine herbicides, giving better separation and good regular peak shape.

Yoshioka *et al*. (2004) used the lower boiling point of diethyl ether (DEE) instead of EtAC because evaporation of DEE is easier by rotary evaporator at lower temperature. DEE was employed as extracting solvent for the analysis of post harvest fungicides, phenylphenol, diphenyl (DP), thiabendazole (TBZ), and imazalil (IMZ) in citrus fruits.

However, care must be taken when using DEE because it is flammable due to its low ignition point and it tends to form explosive proxides. Many extractions have also been performed using medium-polarity solvents such as acetone (Rissato *et al*., 2004; Gabaldón *et al*., 2007) and DCM (Sanchez *et al*., 2008; Ravelo *et al*., 2008) but DCM is a carcinogenic.

On-column liquid-liquid extraction method (OCLLE) (based on classical LLE principle, but assisted by inert solid support), has been tested by Pirard et al. for the analysis of different pesticides in honey using LC-MS-MS. OCLLE combines the advantages of LLE, SPE and SPME. In LLE technique, MeCN was used as the extracting solvent whereas in OCLLE, after agitating the samples with hexane and MeCN, the solution was re-extracted with MeCN. The recoveries were between 71% and 90%. Results proved that extraction by OCLLE can be efficient for a wide range of pesticides and nearly independent of their polarities (pirard *et al*., 2007).

The solvent mixture such as MeCN saturated with n-hexane (Geovania *et al*., 2008), MeOH-water (Fuentes *et al*., 2007), acetone-n-hexane (Wang *et al*., 2007; Iglesias *et al*., 2008), DCM-MeOH (Melo *et al*., 2005; Smalling *et al*., 2008) and MeCN saturated with petroleum ether (García-Reyes *et al*., 2006; García-Reyes *et al*., 2007a) have been also used. Because the use of low polarity solvents such as EtAc and DCM increases the extracts polarity prior to LC analyses, hence some or the whole eluate is evaporated before injection to the LC or is dissolved in a high polar solvent such as MeCN (Cheng *et al*., 2008), isooctane (Karazafiris *et al*., 2008), acetone (Min *et al*., 2008), mixtures of MeCN with water (Garcia *et al*., 2006; Garcia *et al*., 2007_b) or ultra pure water (Goto *et al*., 2006). SPE, dispersive solid-phase extraction (d-SPE) (Lehotay *et al*., 2005a, b; Leandro *et al*., 2005; Ferrer *et al*., 2005; Diez *et al*., 2006; Banerjee

et al., 2007; Kmellar *et al*., 2008; Dong *et al*., 2008; Garrido *et al*., 2008; Hernandez-Borges *et al*., 2009; Mezcua *et al*., 2009), and classic solvent extraction (SE) (Amvrazi *et al*., 2008; Jianfeng *et al*., 2008; Chen *et al*., 2009), are the methods that most commonly used for this purpose, although gel-permeation chromatography (GPC) (Shuling *et al*., 2007) and SBSE (enrichment and clean-up) (Amvrazi *et al*., 2008) have also been employed successfully.

In the analysis of fatty samples such as fish, after extraction with appropriate solvent, low temperature clean up is often performed before SPE clean up (Chen *et al*., 2009). In this case, the extracted solution is collected and stored in the freezer at – 24 ºC for 20 min to freeze lipids. After filtration to remove frozen lipids, the filtered extract is concentrated prior to SPE clean up procedure. Although SE methods have some drawbacks such as laborious, expensive, and have numerous problems to evaporate large volumes of toxic eluent and consequently time-consuming, these methods are accepted and popular for sample preparation due to having advantages like simplicity, robustness and efficiency. The advent of new modified SE methods in sample treatment resulted in the decline of organic solvent consumption, more effective extraction and on-line adaptation connecting directly instruments giving high extraction yield. Some of these techniques are described in the next sections.

2.1.1.2 QuEChERS

QuEChERS is a quick and convenient replacement for LLE which offers a great quality results with less labour-intensive sample preparation steps and low consumption of solvent and glassware. QuEChERS stands for quick, easy, cheep, effective, rugged and safe and is the newest-generation method for the analysis of pesticide residues in

food matrices. These characteristics are evident in its name (Anastassiades *et al*., 2003). This method offers good features for the analysis of polar pesticides. In this method pesticides are extracted with acetonitrile, water is removed by salting out and the acetonitrile extract is cleaned up by mixing with an SPE sorbent rather than passing it through an SPE column.

The main feature of this technique consists of extracting a homogenized sample by hand-shaking or vortex rotary with the same amount of acetonitrile to give a final extract adequately concentrated due to the lack of need for solvent evaporation. A mixture of 4 g anhydrous mangnesium sulfate ($MgSO_4$) and 2 g sodium chloride (NaCl), which provides well-defined phase separation without dilution with dangerous non-polar organic solvent, so are added to the sample by mixing to facilitate partitioning of the analytes between the aqueous residue and the solvent. After shaking and centrifugation, clean up and elimination of residual water is carried out simultaneously using a rapid technique, called dispersive solid phase extraction (d-SPE), in which a primary-secondary amine (PSA) adsorbent, a weak anion-exchanger which removes fatty acids, sugars and other matrix co-extractives that form hydrogen bonds and extra anhydrous $MgSO_4$ are blended with the sample extract. D-SPE is based on SPE method, but the adsorbent is added directly to the extract and the clean-up is simply carried out by shaking and centrifugation. This method takes shorter time than the traditional SPE and simultaneously enables the removal residual water and a lot of polar matrix components such as organic acids, polar pigments and sugar. MeCN is the selected solvent to successfully extract all kinds of pesticides from various food matrices by using QuEChERS (Lehotay *et al*., 2005a, b; Leandro *et al*., 2005; Ferrer *et al*., 2005;

Diez *et al*., 2006; Banerjee *et al*., 2007; Kmellar *et al*., 2008; Dong *et al*., 2008; Garrido *et al*., 2008; Hernandez *et al*., 2009; Mezcua *et al*., 2009).

Leandro and co-workers have evaluated the use of two mixed adsorbents such as C_{18} and PSA in the extraction of OPPs and transformation products from baby food using QuEChERS and d-SPE clean up step. The obtained recoveries were slightly close to 100%, when 50 mg of PSA was used. Observed results were undesirable when mixed adsorbents (50 mg PSA+100 mg C_{18}) or only C_{18} were used, depending on the matrix and the class of the pesticide analyzed. Therfore, PSA was chosen as adsorbent in the analyses of these classes of pesticides by HPLC-MS/MS and UPLC-MS/MS because it achieved a clean extract and peak shape with improved signal-to-noise ratio (S/N) in comparison with crude extracts (Leandro *et al*., 2006). In their earlier study, this research group investigated the effects of different amounts of C_{18} (100-300 mg) with a constant 50 mg of PSA to the quantity of co-extractives remained after evaporation of solvent. The lack of reproducibility among the diverse range of matrices was observed when 100 mg C_{18} was used. On the other hand, non-linear calibration plots and low recoveries resulted with 300 mg C_{18}. However, cleaner extracts, improved S/N, and satisfactory calibration plots were obtained when C_{18} in the range of 100-200 mg was used as adsorbent (Leandro *et al*., 2005).

Recently Mezcua and co-workers described two methods based on GC-MS (SIM) and GC-IT-MS-MS (SRM) for identification, confirmation and quantification of two insecticides in pepper samples by using QuEChERS technique. Clean up step was performed by d-SPE using PSA as sorbent material and MeCN as extracting solvent. Average recoveries were in the range of 85-98%. In brief, no significant differences on the performance of both methods were observed in terms of sensitivity and limit of

detection, although the unambiguous confirmation capabilities provided by MS-MS could not be achieved with a single quadrupole analyzer. The potential of the proposed methods was demonstrated by analyzing real samples with excellent selectivity and sensitivity, thus enabling the unambiguous identification of trace levels of these insecticides in pepper samples (Mezcua *et al*., 2009).

Different versions of QuEChERS methods were evaluated for pesticide residues determination in fruits by use of GC and LC coupled to mass spectrometry. The three compared methods was based on the original unbuffered method, which was first published in 2003 (Anastasiades *et al*., 2003), citrate-buffered and acetate-buffered respectively. The results were excellent (overall average of 98% recoveries with 10% RSD) using all 3 versions, except the unbuffered method gave somewhat lower recoveries for the few pH-dependent pesticides. The acetate-buffered version gave higher and more consistent recoveries for pymetrozine than the other versions in all matrices and for thiabendazole in limes. None of the versions consistently worked well for chlorothalonil, folpet or tolylfluanid in peas, but the acetate-buffered method gave better results for screening of those pesticides. Also, due to the recent shortage in acetonitrile (MeCN), ethyl acetate (EtAc) was evaluated as a substitute solvent in the acetate-buffered QuEChERS version, but it generally led to less clean extracts and lower recoveries of pymetrozine, thiabendazole, acephate, methamidophos, omethoate and dimethoate. In summary, the acetate-buffered version of QuEChERS using MeCN exhibited advantages compared to the other tested methods in the study (Tadeo *et al*., 2000).

The advantages of QuEChERS method are high recovery, high sample yield, accurate results, low solvent and glassware consumption, lower labour and bench space,

less reagent costs and ruggedness. The main drawback of this method is that for 1 g sample per milliliter of final extract the concentration of obtained extracts using this method is lower than concentrated extracts achieved by the use of most conventional procedures. Hence, the final extract must be concentrated more extensively in order to provide high sensitivity and to obtain the limits of quantification (LOQ) desired. Table 2.1 reviews the applications of this method for the determination of pesticide residues in variety of food samples.

Table 2.1: Review of QuEChERS applications in the analysis of pesticides in food samples

Sample	Analyte	Extraction Solvent/ clean-up	Analytical method	Recovery (%)	RSD (%)	Ref
Grape, lemon, anion, tomatoes	105 Pesticides	MeCN/ PSA	GC-SQ-MS	70-110 & > 110	< 20	(Lesueur et al. 2008)
Grape,lemon, anion, Tomatoes	46 Pesticides	MeCN/ PSA	LC-IT-MS	70-110	< 20	(Lesueur et al. 2008)
Bananas harvested	11 pesticides	MeCN/ PSA	GC-NPD	67-118	< 16	(Hernandez et al. 2009)
Pepper	Isocarbophos, isofenphos-methyl	MeCN/ PSA	GC-MS/MS	85-98	< 8	(Mezcua et al., 2009)
Vegetables & fruits	160 Multi-class pesticides	MeCN/ PSA	LC-ESI-MS/MS	70-120	n.r*	(Kmellar et al., 2008)
Cabage & radish	107 pesticides	MeCN (HAc0.5%)/ PSA	GC-MS (SIM)	80-115	< 15	(Dong et al., 2008)
Olive oil	Multi-class pesticides	MeCN/ PSA,C_{18},GCB**	LC-QIT-MS/MS (MRM)	n.r	< 15	(Hernando et al., 2007)
Milk, eggs, avocado	32Multi-class pesticides	MeCN/ PSA	GC-QEI-MS (SIM)	> 95	< 10	(Lehotay et al., 2005a)
Milk, eggs, avocado	32 Multi-class pesticides	MeCN/ PSA	GC-QEI-MS (SIM)	> 27	n.r	(Lehotay et al., 2005a)
Baby food	12 priority pesticides	MeCN/ PSA, C_{18}	GC-QEI-MS/MS (MRM)	60-113	< 28	(Leandro et al., 2005)
Barley samples	43 Herbicides	MeCN/ PSA	GC-ESI-MS	62-78	1.1-9.3	(Diez et al., 2006)
Barley samples	43 Herbicides	MeCN/ PSA	LC-TQ-ESI-MS/MS(SRM)	37.4-135	1.0-19.5	(Diez et al., 2006)
Fruits & vegetables	15 Multi-class pesticide	MeCN/ PSA	LC-TOF-MS (SCAN)	n.r	0.8-11	Ferrer et al., 2005)
Baby food	16 OPPs	MeCN/ PSA	LC-ESI-MS/MS	85-113	2-10	(Leandro et al., 2006)
Baby food	16 OPPs	MeCN/ PSA+C_{18}	LC-ESI-MS (MRM)	92-119	1-17	(Leandro et al., 2006)
Egg, cucumber	OCs, OPPs, pyrethroids	MeCN,1% HAc***/ PSA	GC-EI-IT-MS/MS	n.r	2-16	(Garrido et al., 2008)
Egg, cucumber	OCs, OPPs, pyrethroids	MeCN,1% HAc/ PSA	GC-EI-TOF-MS/MS	n.r	1-14	(Ferrer et al., 2007)
Grapes	82 Multiclass pesticides	EtAc/PSA	LC-ESI-MS/MS (MRM)	70-120	< 20	(Banerjee et al., 2007)

*; Not Reported
**; Graphitized Carbon Black
***; Acetic acid

2.1.2 Instrumental solvent extraction methods

2.1.2.1 Supercritical fluid extractions (SFE)

Enhanced extraction methods are usually instrumental techniques, and the enhanced efficiency of these methods is because of the elevated solvent temperatures used. This temperature elevation increases the speed of extraction of analytes from solid matrices, as a result of increased solubility, better desorption, and enhanced diffusion. The new generation of enhanced extraction techniques is based on use of temperatures above the atmospheric boiling point of the extracting solvent. One such emerging technique is SFE, which resembles soxhlet extraction in which the solvent used is a supercritical fluid (SF), i.e. a substance above its critical temperature and pressure, which results in an unusual combination of properties. SFE diffused through solids like gases, but dissolved analytes like liquids, so the rate of extraction is enhanced and less thermal degradation occurs (Hawthorne *et al*., 1990; Lehotay *et al*., 1997; Matthew *et al*., 2006).

SFE in food analysis is usually performed with carbon dioxide (CO_2) as extracting solvent (Ericsson *et al*., 2000; Gomez *et al*., 2002; Sanchez *et al*., 2002; Jensen *et al*., 2003; Andreu *et al*., 2004; Pena *et al*., 2006; Hovander *et al*., 2006). Carbon dioxide is the most common super critical fluid used as a potential alternative solvent. In comparison, nitrous oxide proved dangerous because of its oxidizing power and more exotic solvents like xenon were ruled out by their cost. In many ways CO_2 is an ideal solvent as it combines low viscosity and a high diffusion rate with a high volatility. The salvation strength increases with temperature and hence the extraction can be carried out at relatively low temperatures. The high volatility simply means that the pressure is reduced and thus allowing the super

critical fluid to evaporate readily and concentrates the sample. The use of CO_2 reduces organic solvent consumption. It is also inexpensive and nonflammable. The incorporation of various solid-phase sorbents like alumina and octadecilsily-bonded silica at the extraction procedure for purification purpose is one of the advantages of SFE procedure (Shim *et al*., 2003). The major problem is while the relatively low polarity of CO_2 which is ideal for polycyclic aromatic hydrocarbons (PAHs) and halogenated pesticides or lipids and fats, it is unsuitable for most pharmaceuticals and drug samples. It has been popular for solid matrices including powdered plant material, herbal medicines, some foods, but there are some problems associated with liquids like biological fluids, which require immobilizing on a solid support material.

The addition of modifiers, such as methanol to the CO_2 enables more polar analytes to be extracted and increase the scope of the method. Aguilera *et al*. (2003) evaluated the effects of different factors like supercritical fluid volume, pressure, temperature and static modifier additions on SFE recoveries from spiked wild rice with 6% olive oil samples using 15 mL of CO_2 at 300 atm, 50 °C and 200 µL MeOH as the static modifier and alumina as a cartridge. Their studies indicated that in all cases the recoveries without modifier were less than those achieved using methanol as modifier, except for the less polar pesticides. Very polar pesticides gave poor recoveries when the modifier was less than 30%. On the other hand, when EtAc was used as modifier, the recoveries were slightly higher than those achieved with methanol, except again for polar pesticides that only showed high recovery with MeOH. In another report, this author and co-workers evaluated the retention of fat from wild rice by various sorbent materials (Celite, Extrelut, Hydromatrix, Florisil, and Aminopropyl) employing on-line SFE clean-up procedure under conditions of

15 mL of CO_2 at 200 atm and 50 °C. The results showed that the first three sorbents were unsuitable materials (the amounts of fat extracted per 100 g wild rice using Celite, Extrelut, or Hydromatrix were 1.84, 1.80, and 1.62 g, respectively). By using florisil the amount of fat extracted per 100 g wild rice was reduced up to 0.36 g, and fat-free SFE extracts were obtained only when "in-line" clean-up was performed with a 1-g layer of aminopropyl. From these results, it can be seen that "in-line" clean-up with aminopropyl is an effective method for obtaining fat free SFE extracts of rice samples (Aguilera *et al*., 2005).

Rodil *et al*. (2007) employed a useful tool based on a single step extraction and clean up to determine 15 organohalogenated pollutants in aquaculture samples using aluminum oxide and acidic silica gel in the supercritical extraction cell followed by GC-MS. Critical factors such as extraction temperature (60 ºC), pressure, static extraction time (5 min), dynamic extraction time, and CO_2 flow rate (2 mL min^{-1}) were optimized. The Doehlert design, followed by a multi-criteria decision-making strategy was then carried out in order to determine the optimum conditions for the two most important factors namely; pressure (165 bar) and dynamic extraction time (27 min). After analysis with GC-MS/MS, LODs were found to be in the range of 0.01-0.2 ng g^{-1}, with excellent linearity. However, existing information about the ability of on-line clean-up method to remove fat from matrices using SFE is not comprehensive and more studies are needed with sufficient number of analytes and sorbent materials in order to confirm the applicability of this technique to different group of pesticides. For example, sorbents such as alumina, florisil and silica can be placed in the extraction cell, or used for clean-up 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 *et al*., 1998).

2.1.2.2 Pressurized-liquid extraction (PLE)

Pressurized-liquid extraction, also known as an accelerated solvent extraction (ASE), is one of the most recent solid and semisolid sample-extraction techniques. At high temperature the rate of extraction increases because the viscosity and the surface tension of the solvent decrease whereas its solvent strength and rate of diffusion into the sample increase. Pressure keeps the solvent below its boiling point and forces its penetration into the pores of the sample. The combination of high temperature and pressure results in better extraction efficiency, thus minimizing solvent use and expediting the extraction process. The time required for extraction is almost independent of sample mass and the efficiency of extraction is mainly dependent on temperature (Richter *et al*., 1996; Smith *et al*., 2002).

PLE method can be carried out in static or dynamic modes, or the combination of both. In the static mode, the sample is placed in a stainless steel vessel containing the extracting solvent. During extraction the solvent is washed out with N_2 gas into a collection vial. In the second system, extracting solvent is pumped through the sample but with high solvent consumption and hence diluting the extract. Anhydrous sodium sulfate, diatomaceous earth, cellulose or sorbent material can be used in clean-up step. In order to optimize the conditions, statistical experimental design procedures are used in order to reduce the number of experiments (Von *et al*., 2005).

Modifiers can be added to the extracting solvent. For instance water modified with a surfactant (sodium dodecyl sulphate) was used to extract PAHs from fish tissues (Morales *et al*., 2002). Tanaka *et al*. (2007) used a simple one step extraction and clean-up by PLE under optimized conditions (extraction tempreture: 100 °C, extraction pressire: 11 MPa, static extraction time: 10 min, extraction cycle time: once, solvent flush volume: 6.6 mL) for the determination of six insecticides, one fungicide and one herbicide in vegetables via GC-MS. Among the several drying materials used such as anhydrous sodium sulfate, alumina, silica gel, florisil and graphitized carbon, the last one was reported to be desirable. Therfore, graphitized carbon was used to remove the co-extracted water because it produced a transparent and colourless solution with the mean recovery of 95%. The extracts had a dark green color when alumina (mean recovery: 92%) was used as the clean-up agent and the results were near the obtained results by florisil (mean recovery: 76%) and silica gel (mean recovery: 82%). By reducing the amount of graphitized carbon from 12 to 6 g, the colour of the extracts changed from colourless to dark green. The overall recovery obtained for the analysis of 8 pesticides ranged from 71-103%. By contrast, Blasco *et al.* (2005) used PLE extraction after the dispersion of fruit samples with acidic alumina. They reported that anhydrous sodium sulfate instead of alumina produced an extract with a cloudy and strong colour. However, they did not evaluate the cleanup effects of the extraction procedure because they used the materials as a solid support material or a drying material.

Pressurized liquid extraction-based was performed for simultaneous extraction and in situ clean-up of polychlorinated biphenyls (PCBs), hydroxylated (OH)-PCBs, methylsulfonyl ($MeSO_2$)-PCBs and their metabolites from small tissue samples. A

mix of fat retainer and diatomaceous earth pre-extracted in a short PLE cell of the combined extraction and in situ clean-up to prevent cell memory effects. After tissue extractions, the pre-extracted diatomaceous earth was eliminated from the PLE cell by mixing the liver from non-PCB exposed rats. After spiking, the PLE cells were extracted twice under the same conditions as pre-extraction (Kania-Korwel *et al*., 2008). Separation of different fractions was carried out using different physicochemical properties of selected pesticides based on the procedure proposed by Hovander et al. (Hovander *et al*., 2006). The length of static cycle (1-9 min) was investigated using extracting solvent mixture that consists of hexane-dichloromethane-methanol (50:45:5 v/v). Validation of PLE-based method under optimized conditions (hexane-dichloromethane-methanol; 48:43:9 v/v, temperature of 100 °C, pressure of 1500 psi, 6 min heating time, 1 static cycle of 5 min and a 60% cell volume flush) was performed by comparing with the extraction method described by Jensen *et al*. (2003) as well as by verifying its linearity and repeatability. This method required more labour-intensive extraction with different solvent combinations by increasing polarity and clean-up steps with additional column clean-up steps for both PCBs and $MeSO_2$-PCBs in comparison with the PLE method described by Kania *et al*. (2008) that required only a single, automated PLE extraction step. In this study, the extraction of a laboratory reference material with hexane–dichloromethane–methanol (48:43:9 v/v) and florisil as the fat retainer allowed an efficient recoveries of 78–112% with RSD: 13–37% for PCBs, 46 ± 2%; RSD: 4% for OH-PCBs and 89 ± 21%; RSD: 24% for MeSO2-PCBs respectively. Comparable results were obtained with an established analysis method for PCBs, OH-PCBs and $MeSO_2$-PCBs.

For lipid containing samples, further clean-up is usually required. Gomez *et al.* (2002) investigated the use of several sorbents and concluded that florisil produced the cleanest extracts for their samples. An alternative approach is to perform a preliminary PLE with a non-polar solvent to eliminate the hydrophobic compounds prior to extraction of the analytes of interest. The use of pressurized fluids in comparison with soxhlet extraction have the advantages of reducing solvent consumption and extraction time although they require expensive specialized equipment and clean up procedure is necessary after extraction.

Soler and co-workers examined the use of PLE to extract carbosulfan and seven of its metabolites from oranges by use of 40 mL DCM as an extraction solvent at 100 °C and 2000 psi with 100% flush volume, 2 min heating time, and two cycles of static extraction for 5 min each. LC–MS^3 was used for identification and confirmatory analysis. The authors concluded that the matrix of the samples affects the quantitative analysis of the target compounds by substantially enhancing the response to early-eluting metabolites. The magnitude of the effect was only slightly dependent on the particular orange extract analyzed, however % RSDs were never higher than 14%. They suggested an analyte-added control orange extract could be used as a standard to improve the accuracy of the analysis. Because of its simplicity and sensitivity (limit of quantification (LOQ) <0.07 mg kg^{-1}), the method enabled efficient determination of carbosulfan and its metabolites in oranges (Soler *et al.*, 2006).

In summary, the advantage of commercially available PLE systems is the capability to be easily automated for sequential unattended extraction of up to 24 samples. The amount of time spent on method development can therefore be

substantially reduced compared with other techniques. Relatively matrix-independent methods can, furthermore, be developed by using high temperature and suitable solvents. Compared with Soxhlet extraction, the use of pressurized fluids has the advantages of reducing solvent consumption and extraction time with the disadvantage of using expensive specialized equipment. The main disadvantage is that a sample cleanup is still required after extraction.

2.1.2.3 Microwave assisted extraction (MAE)

In order to achieve easier sample pre-treatment, new extraction procedures based on instrumental techniques such as MAE with smaller amounts of matrix and solvent's consumption and rapid extraction has been investigated since 1986 (Andreu *et al*., 2004). The key advantages of this method are the low temperature necessity, high extraction efficiency, automation, the chance of simultaneous extraction of different samples without interference, smaller amounts of matrix and solvent and rapid extraction. In contrast to other heating methods, the extraction vessel does not need direct heating and no additional clean up step is required so the extraction time is reduced. In conventional extraction techniques, a higher solvent volume to solid matrix mass ratio would increase recovery. However, in MAE a higher ratio of solvent to solid matrix mass may lead to lower recoveries, probably because of inadequate stirring of the solvent by the microwaves. MAE is only employed for the extraction of compounds that are thermally stable due to the increase of temperature during extraction may lead to degradation, so in such circumstances the power selected during MAE must be set correctly to avoid excessive temperatures. Antioxidants and preservatives can be extracted with this

technique if the matrix is low in fat. This technique can be used for extraction of herbicides from soil and PAHs from sediments. Alternatively, sonication can be used to enhance extraction and this has been applied for the extraction of organophosphorus pesticides (Sanchez *et al*., 2002). MAE technique, like SFE and PLE, provides simple conditions for working at high temperatures and pressures, which intensely enhance the speed of extraction. On the contrary, traditional techniques for solid matrices, such as the well-known Soxhlet extraction, together with shaking and sonication (Pena *et al*., 2006), which can also provide efficient extractions with low investment, seem to get decreasing attention due to their drawbacks: long extraction times (especially for Soxhlet), relative high solvent consumption, occasional need of a clean-up step and possible repeated extractions in the case of sonication.

Cheng *et al*. (2007) reported a multi-residue method developed for the determination of triazine herbicides (simazine, atrazine, propazine and prometryn) in sheep liver by MAE using MeOH as the extracting solvent. This group optimized the MAE operation parameters such as type and volume of solvent, time and temperature of extraction in order to increase the extraction yield. First, 10 mL MeOH was added to the extraction vessels placed in the microwave sample preparation system and the extraction was performed at 70 °C for 6 min. After cooling at room temperature and filtration, the residue was washed three times with 5 mL of MeOH each time. The methanol extract was then extracted three times with 10 mL of petroleum ether each time. After discarding the ether layer, the extraction was repeated three more times with 1 mL of 0.1% NaCl, 9 mL of water and 10 mL of chloroform each time. After distilling the chloroform extract to dryness and

reconstitution of the residue with 5 mL of MeOH, the solution was transferred to a column containing anhydrous sodium sulfate and aluminum oxide. Using chloroform as the extracting solvent resulted in emulsification and a decreased in recoveries. The results were desirable (upper than 90%) when the extraction time was increased from 2 min to 6 min. MeOH has high dielectric constant to adsorb microwave energy efficiently; however non-polar solvents such as hexane and toluene, are not potential solvents for MAE, but their extracting selectivity and efficiency can be modulated by using mixtures of solvents for example, hexane-acetone (Ericsson *et al*., 2000). Different clean-up procedures has been carried out after MAE extraction such as SPME (Sanusi *et al*., 2004) and disposable SPE cartridge packed with $C_{18,}$ silica and ion exchange material (Smalling *et al*., 2008; Pateiro *et al*., 2008). Among many studies that was done on different extraction method coupled with chromatographic techniques and MS to analyze and determine of pesticide residues in foods, only a few studies has been reported on using MAE coupled with both chromatographic systems and MS. The reason might be the requirement of sample filtration and clean-up steps after extraction, something that is impossible to circumvent, in comparison with SFE and PLE method, in which on line clean-up and filtration are possible. Sanusi and co-workers employed focused microwave-assisted extraction (FMAE) coupled with SPME and GC-MS for the extraction and analysis of pyrethroid residues in strawberry. In order to improve the conditions of FMAE-SPME, they added co-solvent (MeOH, MeCN or EtOH) to the extraction solution instead of pure water, enabling the increase in the transfer of the analytes into the solution analyzed by SPME. The results illustrated that the observed signal was better when co-solvent was used instead of pure water. Among the three co-solvents, MeCN was the most

sensitive. The obtained LODs were in accordance with MRLs (Sanusi *et al*., 2004). In a second study, Iglesias et al. developed an analytical method based on MAE with hexane-acetone (50:50 v/v) as the extracting solvent, SPE clean up with three different sorbents (alumina/ ENVI^{TM}-Florisil, ENVI^{TM}-Carb and ENVI^{TM}-Carb II/PSA) and hexane-EtAc (80:20 v/v) as the eluent for the determination of organochlorine pesticides in animal feed. The analytes were determined by GC-ECD and quantified via GC-MS. According to the obtained results, both ENVI^{TM}-Carb and ENVI^{TM}-Carb II/ PSA furnished colourless eluates but with lower interfering peaks in ENVI^{TM}-Carb II/ PSA chromatograms, so the latter system was employed for the purification of the extracts. The recoveries (100%) were similar to those achieved with soxhlet extraction. Validation method was performed with the analysis of certified reference material (CRM-115 BCR) and the results were in accordance with certified values. The range of LODs were between 2 and 19 µg kg^{-1} and LOQs ranged from 5 to 37 µg kg^{-1} corresponding to the MRLs and below (Iglesias *et al*., 2008).

You *et al*. (2007) described a method using pressurized microwave-assisted extraction (PMAE) without clean-up step for the determination of triazine herbicides in infant nutrient cereal-based foods coupled with HPLC-ESI/MS. After improving the key factors of PMAE such as extracting solvent, extraction temperature and extraction time, the method was validated with atmospheric pressure microwave assisted extraction (AMAE), ultrasonic extraction (UE) and soxhlet extraction (SE). The recoveries obtained (66.2-88.6%) from the proposed method were better with more efficiency, faster and without clean-up procedure. Among the different solvents used such as MeOH, MeCN, acetone, acetone-n-hexane (1:1 v/v) and MeOH-DCM

(1:1 v/v), the required microwave radiation time to reach a temperature of 85 °C was less by using MeOH (241s). The ability and specific heat of solvent for absorbing microwave energy seemed to affect the temperature.

Fuentes *et al*. (2007, 2008) used MAE in combination with GC-FPD for the determination of selected organochlorine pesticides in agricultural soil. In this study, they used water-MeOH as a modifier for desorption and simultaneous partitioning with n-hexane (MAEP). In second study, they employed MAE coupled to SPE and GC-MS for the determination of organoclorine pesticides in olive oil. In this study, MeCN-DCM was used as the solvent for LLE, while for clean-up step ENVI-Carb and DCM were used as SPE cartridge and elution solvent respectively. Confirmation was performed by GC-MS/MS. First, olive oil was used as a matrix mimic in order to optimize GC-FPD signals and improve the extraction method. By adding $KHPO_4$ to the mixture of water-MeOH, the recoveries were increased (up to73%) compared to using the mixture of water-MeOH alone where recoveries were 54-77%. However, in latter study, when MeCN was used alone as the solvent for partitioning of LLE, the recoveries were ranged 62-99% and by adding DCM to the extracting solvent caused the increase of the recoveries (Fuentes *et al*., 2008). Most MAE applications to date have been for the extraction of environmental samples. As for the MAE coupled with GC-MS or LC-MS, more investigations are needed in the next few years.

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2.1.3 Sorptive extraction methods

2.1.3.1 Solid-phase extraction (SPE)

Solid phase extraction is the extensively accepted alternative extraction-clean up method including a preliminary LLP step, although it can be used without this step, which involves the use of disposable cartridges to trap analyte and separate them from the bulk of the matrix. The adsorbent materials in SPE procedure play a significant role in achieving greater enrichment efficiency and lower cost of organic solvents. In SPE the sample is passed through a cartridge or a packed column filled with a solid adsorbent on the surface of which the analytes are adsorbed while the other sample components pass through the bed. When the analytes have been retained on the SPE adsorbent they are then eluted with an organic solvent. Advantages of SPE are that the analytical procedure is much simpler, small volumes of solvents are used, and much cleaner extracts and greater recoveries are usually obtained. SPE also enables avoidance of the emulsion formation often encountered in LLE, and automation is also possible (Morales *et al*., 2006). Method development in SPE is usually accomplished by optimizing pH, type and solvent strength of the sample matrix, polarity and flow rate of the eluting solvent, and physicochemical characteristics of the adsorbent bed. For example, sample pH can be crucial to obtaining high pesticide retention on the adsorbent. Occasionally, therefore, sample pH modification can be necessary to stabilize the pesticides and increase their absorption by the solid phase. Recently, use of high flow-rates through extraction cartridges has been claimed to give improved extraction (Blasco *et al*., 2004).

Many types of adsorbents such as Florisil (Barrek *et al*., 2003), alumina, magnesium silicate, graphitized carbon (GCB) and oasis HLB (Debayle *et al*., 2008;

Gervais *et al*., 2008), adsorbents with weak anion-exchange and polar capabilities (NH_2) (Melo *et al*., 2005), polystyrene-divinylbenzene supports (Perreau *et al*., 2007), C_{18} (Fernandez *et al*., 2002; Ferrer *et al*., 2007; Martinez *et al*., 2007) SWCNTs and MWCNTs (Zhou *et al*., 2006a, b; Min *et al*., 2008; Ravelo *et al*., 2008) mixed mode phases and bamboo charcoal (Zhao *et al*., 2008), have been shown to be valuable adsorbents for sample enrichment and clean-up of a variety of pesticides in food matrices and environmental samples. The most commonly used material, however is the reversed-phase octadecyl silica (RP-C_{18}) (Fernandez *et al*., 2002; Blasco *et al*., 2003; Karazafiris *et al*., 2008; Lourenecetti *et al*., 2008) because it is sufficiently reactive to enable its surface to be modified by chemical reaction and yet sufficiently stable to enable its use with a wide range of solutions. The introduction of the disposable pre-packed SPE cartridges had major effects on methods for the examination of the analysis in solution. The SPE cartridge introduced two critical factors namely, standardization and hence greater reproducibility and a much wider range of phases. More importantly the use of reversed-phase and ion-exchange materials enables aqueous solutions to be treated and additional trapping mechanisms to be utilized. A wide range of phases means that polarity, hydrophobicity or ionization can be used as trapping mechanisms and the sample matrix may now be non-polar or aqueous. Once trapped, the analyte can be released into a small volume of an extraction solvent by altering the polarity or pH. Although the cartridges are single use and disposable and thus present a significant consumable cost, this has been claimed to be much lower than the cost of chemicals and manpower needed for the corresponding traditional solvent extraction methods.

After an adsorbent has been selected, on the basis of its retention efficiency for the target pesticides, the second step consists in determining the best solvent or mixture of solvents to disrupt this link and to displace the analytes from the SPE materials. An eluent is usually chosen on the basis of its high-performance, low volume, weak toxicity, non-interference with compounds, and compatibility with the chromatographic system used (GC or LC).

Niu *et al*. (2009) used single-walled carbon nanotubes disk as sorbent material and MeCN as eluent in SPE method for the extraction of sulfonylurea herbicides in water samples by HPLC-DAD. They demonstrated that when the selected analytes (sulfonylurea herbicides) are weak acids the solubility of them in water is increased drastically with increasing solution pH, indicating the high polarity of the analytes. Since adsoption of analytes on the adsorbent material (SWCNTs) is based on hydrophobic interaction, the sample pH is a significant factor in the enrichment of the analytes. As a result of the high adsorption of the all analytes in acidic solution, the maximum recovery was obtained at pH 3.0 and it dropped clearly with the increase of pH. Flow rate was another factor to increase the enrichment efficiency of analytes and control the extraction time that was adjusted by the manifold vacuum pressure to 150 mL min^{-1} with SWCNTs as adsorbent. However, when double or triple layered disk was used, the flow rate was decreased to approximately 50 mL min^{-1} similar to C_{18} and activated carbon. Zhou and co-workers used multiwalled carbon nanotubes for pre-concentration of simazine and atrazine prior to HPLC-DAD. Under the optimal SPE procedure, the recoveries of the two analytes were in the range of 86.2-103.7% (Zhou *et al*., 2006a). However, Zhao *et al*. (2008) used bamboo charcoal as SPE adsorbent in the determination of atrazine and simazine in

environmental water samples by HPLC-MS. Following optimization of enrichment conditions, among three different polarity solvents (MeOH, MeCN, and acetone), MeCN had a high elution performance. To obtain proper sample solution pH to obtain high extraction efficiency of the analytes, different pH ranging from 3 to 11 was studied and the recoveries at pH 5-9 were found to be appropriate. In investigation of the flow rate of working solution in the range of 1.0-2.5 mL min^{-1} in order to obtain enhancement efficiency, the flow rate of 2.5 mL min^{-1} was selected. Effect of sample volume on the recoveries was investigated in the range of 100-1000 mL. Quantitative recoveries were obtained for sample volume up to 500 mL. The recoveries for simazine and atrazine slightly reduced when a volume of more than 500 mL was used, so in subsequent experiments a volume of 500 mL was selected. In this study, the recoveries ranged between 75.2% and 107.1% with % RSD in the range of 8.3-8.7%.

The results of different studies have revealed that the most broadly used eluents for desorption of pesticides and their degradation products from adsorbent materials are EtAc (Min *et al*., 2008; Fernandez *et al*. 2002), MeOH (Barrek *et al*., 2003), DCM (Ravelo *et al*., 2008), MeCN (Gervais *et al*., 2008; Niu *et al*., 2009; Vega *et al*., 2005) acetone (Zhou *et al*., 2006), or mixtures of these (Seccia *et al*., 2005; Bogialli *et al*., 2006; Martinez *et al*., 2007). Most of the examples cited are multiresidue, including several groups of pesticides, and so mixtures of solvents are usually used to ensure high recoveries for all the target compounds. Different mixtures have been used, including EtAc-n-hexane (Nozal *et al*., 2005; Karazafiris *et al*., 2008), EtAc-MeOH–H_2O (Wang *et al*., 2007a, b), acetone–hexane (Albero *et al*., 2005), and EtAc–acetone (Nozal *et al*., 2005). MeOH was also used as SPE eluent

by Wang *et al*. (2005) who developed and validated a method for the identification and quantification of trace levels of thirteen pesticides in apple-based infant food by LC–ESI-MS–MS. SPE has been successfully applied for the extraction of pesticide residues in food samples. Anastasios and co-workers reported a sensitive multi-residue liquid chromataography-tandem mass spectrometry based on SPE for determination of pesticides in wine samples. In this study, SPE procedure with oasis HLB cartridges combined isolation of the pesticides and sample clean-up in a single step. The cartridges were conditioned with MeOH and pure water and then rinsed by water. Finally the retained pesticides were eluted by 10 mL MeOH. LODs and LOQs were in the range of 0.0003-0.003 mg L^{-1} and 0.001-0.01 mg L^{-1} respectively with recovery between 70 and 110% (Anastasios *et al*., 2009).

A novel application of carbon nanotubes was described by Lopez et al. for the determination of pesticides (chlortoluron, diuron, atrazine, simazine, terbuthylazin-desethyl, dimetoathe, malathion and parathion) in virgin olive oil samples. In evaluation of adsorbents, two carbon nanotubes such as multi-walled and carboxylated singlewalled, the later being the most appropriate for the aim of the work. For this purpose, SPE columns were washed with n-hexane then the pesticides were eluted with ethyl acetate. After analysis by GC-MS, LODs levels achieved between 1.5 and 3.0 µg L^{-1} (Lopez-Feria *et al*., 2009).

The selection of proper adsorbent and eluting solvent to match the physicochemical characteristics of multiresidue analysis that is, small extracted sample volume by SPE adsorbents, high blank values are the problems that must be researched and removed. Table 2.2 reviews some SPE applications for the determination of pesticides residues in food matrices and environmental samples.

Table 2.2: Examples of SPE application for determination of pesticide residues in food and environmental samples

Sample	Analyte	Conditioning solvent	Adsorbent	Eluting solvent / Flow rate	Analytical method	Recovery (%)	RSD (%)	Ref
Apple, orange, grape &pineapple fruit juices	8 OPPs	MeCN-water	MWCNTs**	20mL DCM/ Eluted by gravity	GC-NPD	> 73	< 8.5	(Ravelo et al., 2008a)
Royal jelly	9 multiclass pesticides	EtAc-n,hexane (1:1), MeCN, H_2O	RP-C_{18}	2 mLEtAc-n,hexane/ Eluted by gravity	GC-μ-ECD	70-110	< 13	(Karazafiris et al., 2008)
Honey	Coumaphos, carbendazim, Amitraz	MeCN-water	Oasis HLB (waters)	3mL MeCN-MeOH-DCM(50:25:25, V/V)/Eluted by gravity	LC-TQ-MS/MS (MRM)	n.r*	n.r	(Debayle et al., 2008)
Water	Sulfonylurea herbicides	HCl 0.1 mol L^{-1}	SWCNTs	MeCN (0.1% acetic acid)/1mLmin^{-1}	HPLC-PDA****	79-102	n.r	(Niu et al., 2009)
Tomatoes	5 triazine herbicides & a fungicide	MeOH-water	Oasis HLB	3mL DCM-MeOH (99:1)/Eluted by gravity	RP-HPLC-UV	60-130	n.r	(Melo et al., 2005)
Environmental water	Atrazine &simazine	DCM	Laboratory-made NH_2(aminopropyl)	10mL MeCN/ 2.5mLmin^{-1}	HPLC-UV	75.2-107.1	8.3, 8.7	(Zhao et al., 2008)
Bovine milk	30 herbicides& Fungicides	MeCN-water	Octadecylsiloxane (ODS)	1.5mLMeOH,6mL DCM-MeOH/5mLmin^{-1}	LC-ESI-MS/MS (MRM)	78-104	< 13	(Bogialli et al., 2006)
Drinking water	Nicotinamid insecticides	MeOH, water	Carbograph 4 ™	DI-water-3 mL EtAc/MeOH (50:50)/ 10 mL min^{-1}	LC-ESI-MS (SIM)	94-104	< 20	(Seccia et al, 2005)
Camercial juices	50 Multi-class pesticides	MeCN-water	MWCNTs	5mLHexane-EtAc (1:1, v/v)/Eluted by gravity	GC-EI-MS (SIM)	> 91	< 9	(Albero et al., 2005)
Wine	8 Azolic fungicides	MeOH, water	Teflon frit,C_{18}	3mLMeOH/ Eluted by gravity	LC-APCI-MS (SIM)	83-109	< 10	(Nozal et al., 2005)
Citrus essential oils	12 Pesticides	Pentane	Polymeric cartridge	5mLPentane,DCM/ 1mLmin^{-1}	GC-ESI-MS	65-95	< 7	(Barrek et al., 2003)
Citrus essential oils	12 Pesticides	Pentane	Florisil- C_{18}	5mLPentane,DCM/ 1mLmin^{-1}	LC-ESI-MS	50-94	< 2	(Barrek et al., 2003)
Honey	22 OPPs	MeOH	C_{18}	3mLEtAc/ 10mLmin^{-1}	LC-APCI-MS	16-102	< 17	(Fernandez t al., 2002)
Bovine milk	Nicotinoid insecticides	DCM	C_{18}	5mLMeCN,CH_2Cl_2/ 10mLmin^{-1}	HPLC-DAD	85.1-99.7	10	(Garcia et al., 2009)

*;Not Reported

**; Multiwalled Carbo Nanotube

****;Photodiode Array Detector

2.1.3.2 Matrix solid-phase dispersion (MSPD)

In order to analyze semi-solid and solid food matrices and overcome the serious restrictions of SE and SPE methods and achieve high efficiency especially in environmental analysis, Barker and co-workers described a procedure for the extraction of target analytes in solid matrices by MSPD (Barker *et al*., 1989). The main difference between MSPD and classic SPE is that, in SPE, the samples must be in liquid state before application to the column whereas MSPD can handle solid or viscous liquid samples directly. The interactions of the components of the system are greater in MSPD and different, in part, from those in SPE. This technique enables disruption and dispersion of analytes simultaneously onto a solid support, thereby isolating the extracts from the matrices. Reversed phase sorbent materials such as octyl-bonded silica (C_8) and octadecyl-bonded silica (C_{18}) are the most commonly used adsorbents because of the lipophilic characteristics of them that cause a good disruption and dispersion. In this method, the adsorbent is mixed with the sample using mortar and pestle or a related mechanical device. MSPD allows the extraction of pesticides from homogenously dispersed target samples onto a solid support such as florisil and silica. The homogenized mixture is then placed in co-columns to obtain further fractionation and to assist in extract cleanup. A co-column material (for example florisil, silica, and alumina,) can be packed at the bottom of the same cartridge, containing the dispersant/matrix blend, or used as an external column. Such columns may be literally stacked so as to collect and fractionate the sample as it emerges from the MSPD column.

Garcia *et al*. (2006) reported the use of MSPD after preliminary LLE with MeCN saturated with petroleum ether for the analysis of four herbicides in olive oil by LC-

MS. The obtained extracts from LLE step was homogenized with aminopropyl-bonded silica as dispersant sorbent by means of a glass mortar and pestle. The mixture was then transferred into the mini-column containing packed florisil that was connected to a vacuum system for clean-up procedure. After eluting with MeCN, the extracts were evaporated and dissolved in MeCN-water (1:1 v/v) prior to LC-MS. The recoveries obtained were in the range of 81-111%. Numerous adsorbents with different selectivity are available that may be selected based on the analytes of interest, type of matrix and interferences. The use of MSPD in food analysis has been reported using sorbents such as silica (Santana *et al*., 2008), florisil (Abhilash *et al*., 2007; Zhu *et al*., 2007; Hercegova *et al*., 2006) and C_{18} or C_8 bonded silica (Geovania *et al*., 2008; Wang *et al*., 2007). Inert adsorbents, for example diatomaceous earth (Radisic *et al*., 2009; Chu *et al*., 2005) and sand (Bogialli *et al*., 2004; Bogialli *et al*., 2007a), instead of reversed or normal-phase dispersant have been successfully used in the analyses of pesticides in fruit juices, fatty foods and tissues because they enable early elution of interferences that would not be retained by any adsorbent during elution of the target analytes. The combination of sand as dispersant with water as eluting solvent for polar analytes in any type of matrix enables almost quantitative recoveries. Recently, it has been proved that replacement of C_{18} by aminopropyl silica or primary and secondary amine (PSA) sorbents leads to cleaner extracts from complex fatty samples, e.g. olives (Garcia *et al*., 2006; Garcia *et al*., 2007a, b; Cunha *et al*., 2007). It seems that the weak anion-exchange character of amino materials is responsible for this better selectivity due to more effective retention of the fatty acids present in biological samples. Reversed-phase octadecyl silica (RP-C_{18}) (Fernandez *et al*., 2002; Blasco

et al., 2003) is another commonly used dispersant because of its high reactivity that leads to the modification of its surface by chemical reaction. Another important parameter in MSPD procedure is the selection of extraction solvents that depends on the analyte polarity. Non-polar solvents such as hexane, DCM, or mixture of both can be used in extraction of non-polar compounds, whereas medium or high polar substances can be recovered using polar solvents such as acetone, MeCN, EtAc, water-ethanol or methanol.

Geovania *et al*. (2008) determined pesticides in coconut based on MSPD under optimized conditions such as type and amount of solid-phase (C_{18}, alumina, silica-gel and florisil), selection of eluent (DCM, MeCN, EtAc, acetone, n-hexane and n-hexane-water (1:1 v/v)) by GC-MS (SIM). The best result was obtained when C_{18}, florisil and acetonitrile saturated with n-hexane were used as dispersant sorbent, clean-up sorbent and eluting solvent respectively. The mean recoveries were between 70.1 and 98.7% with RSD from 2.7 to 14.7% except for two pesticides.

Radisic *et al*. (2009) developed a rapid and sensitive LC-MS-MS method for the analysis of selected pesticides in fruit juices based on MSPD extraction process by using diatomaceous earth as dispersant and DCM as eluent. In order to avoid the use of chlorinated solvents such as DCM, other extraction solvents such as EtAc and MeOH were tested for all selected pesticides. The results of recoveries were undesirable when EtAc was used. However, the use of MeOH as the extracting solvent required an additional clean-up step, so DCM was used as extracting solvent. The effects of pH were studied over the range of pH 2-8 and the highest recoveries were obtained at pH 6. The recoveries obtained were between 71 and 118% with RSD in the range of 5-15%.

One paper devoted an effective MSPD method for determination of cypermethrin and deltamethrin in porcine tissues with a neutral alumina-based MSPD column and HPLC-UV using reversed-phase C_{18} column. In this study, in order to obtain high elution efficiency, dissimilar solvents polarities (n-hexane, n-hexane-EtAc (1:1), n-hexane-DCM (1:1), EtAc and n-hexane-acetone) were tested. When EtAc, acetone and DCM were used as the extracting solvent, a greater number of interferences were extracted into the eluate. N-hexane is a nonpolar solvent thus, a mixture of it with other solvents has intermediate polarity and so n-hexane was used as eluent solvent. The ratio of mobile phase was optimized and acetonitrile-water (85-15 v/v) was selected. The recoveries were between 83.5 and 109%. When the traditional method is compared with the MSPD-SSEC method, the MSPD–SSEC method reduces sample contamination during the procedure, and decreases the amount of organic solvent used (Cheng *et al*., 2008).

Santana *et al*. (2008) compared and evaluated a variety of dispersant materials such as C_{18}, alumina, silica and florisil with regards to the amount of solid-phase and eluent such as n-hexane, DCM, n-hexane-DCM (8:2 and 1:1 v/v), DCM-EtAc (9:1, 8:2 and 7:3 v/v) in analysis of buprofezin, tetradifon, vinclozolin, and bifenthrin from propolis by GC-MS. The results were excellent when 1.0 g silica and 1.0 g Florisil and DCM-EtAc (9:1 v/v) were used as dispersant, clean-up sorbent and elution solvent respectively. The main factors in the selection of elution solvent are, its capability to selectively and quantitatively recover target analytes and its compatibility with the subsequent determination technique, harmlessness, low cost, low consumption solvent, and environmental friendliness are also desirable attributes. Hot water has been used for extracting polar to moderately polar

contaminants from solid matrices because of the drop of its polarity with increase the temperature (Morales *et al*., 2006).

Extraction with high temperature water is carried out at atmospheric or elevated pressures. MSPD was developed for the determination of pentachloronitrobenzene, pentachloroaniline, methyl-pentachloro-phenylsulfide and procymidone in ginseng extract using gas chromatography. The optimal conditions selected for MSPD extraction were as follows: after blending 5 mL of aqueous ginseng extract (10%,w/v) with florisil (10 g), the mixture was passed into a small chromatographic column and extracted twice with 10 mL of ethyl acetate-hexane solvent mixture (70:30 v/v) for 15 min in an ultrasonic bath at room temperature. The mean recoveries were found in the range of 83-97% with RSD below 10% (Xiangyang *et al*., 2010).

From the papers reviewed the main conclusion that can be drawn is that MSPD has become a well-established sample-preparation technique in food analysis (Garcia-lopez *et al*., 2008). Low toxic solvent consumption, reducing cost and analysis time, simplifying and speeding up the sample treatment procedure, increasing reliability and in most cases integrating of extraction and clean-up in a single step are the advantages of this technique. Solvent evaporation remains a problem, however, and literature reports of on-line coupling of MSPD to LC or GC instruments are scarce.

MSPD method for the analysis of pesticide residues in food samples are listed in Table 2.3.

Table 2.3: Review of MSPD applications in the analyses of pesticides in food samples

Sample	Analyte	Dispersant/Clean up	Eluting solvent	Analytical method	Recovery (%)	RSD (%)	Ref
Plant matrices	Hexachlorocyclohexane isomers	Florisil/Neutral alumina	n-Hexane-EtAc (70:30, v/v)	GC-ECD	91-98	5.40-9.85	(Zhu et al., 2007)
Fruit juices	Selected pesticides	Diatomaceous earth/Teflon frit	DCM	GC-MS/MS	71-118	5-15	(Chu et al., 2005)
Porcine tessues(liver, muscle, heart,kidney)	Cypermethrin,deltamethrin)	Neutral alumina/ Diatimaceous earth	n-Hexane	HPLC-UV	87.8-105.3	< 7	(Cheng et al., 2008)
Bovine samples	Five OPPs	Octadecylsilyl (C_{18})/ silica gel	MeCN	HPLC-DAD-UV	> 94%	≤ 15	(Garcia et al., 2009)
Coconut	8 Pesticides	C_{18}/ Florisil	MeCN saturated with n-hexane	GC-MS (SIM)	70.1-98.7	2.7-14.7	(Geovania et al., 2008)
Wine	Fungisides & their metabolits	Florisil	EtAc-hexane (70:30, v/v)	GC-ECD	82.4-93.7	< 8	(Zhu et val., 2007)
Propolis	4 pesticide residues	Silica/ Florisil	DCM-EtAc (9:1, v/v)	GC-MS (SIM)	67-175	5.6-12.1	(Santana et al., 2008)
Animal fats	DDT	Activated carbin fiber (KF)	Acetic acid, ethanol, Heptanes (10:20:80,v/v)	HPLC-PDA	58-93	< 7	(Furusawa et al., 2005)
Fruits & vegetables	Fungicides	C_{18}-bonded silica/ glass filter paper underlay	EtAc	LC-QIT-MS	71-102	< 13	(Wang et al., 2007b)
Meat	Sulphonamide	C_{18}/Teflon frit	MeOH	LC-API-MS/MS	87-101	n.r*	(Sergi et al., 2007)
Olive & olive oil	Triazines,OPPs,OCs,prethroids	Aminopropyl/ Florisil	MeCN saturated with Petroleum ether	GC-Q-MS (SIM)	73-130	n.r	(Garcia et al., 2007a)
Olive oil	4 s-Triazines	Aminopropyl/ Florisil	MeCN saturated with Petroleum ether	LC-ESI-TOF-MS	81-111	< 4	(Garcia et al., 2006)
Vegetables & fruits	8 Carbamate insecticides	Sand/ no	Water (50ºC)	LC-ESI-MS(SIM)	88-110	< 9	(Bogialli et al., 2004)
Baby food	20 Multiclass pesticides	Florisil	EtAc	GC-EI-MS (SIM)	70-110	n.r	(Hercegova et al., 2006)
Apple juices	266 Multiclass pesticides	Diatomaceous earth	Hexane-DCM	GC-EI-MS (SIM)	64-117	2-23	(Chu et al., 2005)
Animal fats	Aldrin, dieldrin, DDTs	Acidic alumina oxide/filter disc	Heptane	HPLC-PDA	84-98	n.r	(Furusawa et al., 2004)
Tomato juices	Endosulfane isomers, endosulfan sulfate	Florisil	EtAc	GC-EI-MS (SIM)	81-101	< 6	(Albero et al., 2003)

*;Not Report

2.1.3.3 Solid-phase microextraction (SPME)

SPME is an easily automated, simple, one-step, rapid, solvent-free method of extraction. The technique is based on establishment of equilibrium between analyte in a sample and analyte adsorbed by a fused-silica fiber coated with a stationary phase, which can be a liquid polymer, a solid adsorbent, or a combination of both. SPME is increasingly being used instead of classical and time consuming extraction and leaching processes.

A number of different fiber coatings are available, which offer a range of analyte solubilities and porosities, including the non-polar polydimethylsiloxane (PDMS), semi-polar PDMS-divinylbenzene (PDMS-DVB) and polar polyacrylate (PA) and Carbowax-divinylbenzene and the coated porous particle phase PDMS-Carboxen. Among these, PDMS fiber has been widely used in head-space (HS) extraction methods because it is the highest capacity fiber for non-polar compounds and enables successfully the collection of the different compounds from the sample. Recently SPME has been interfaced with HPLC and LC-MS for the analysis of compounds that are nonvolatile and thermally unstable. In this system, a SPME-HPLC interface equipped with a specific desorption chamber is used before LC separation instead of thermal desorption in the injection port of the GC. Two types of SPME techniques can be used to extract the analytes: head-space (HS-SPME), and direct immersion (DI-SPME). In HS-SPME, the fiber is exposed in the vapour phase above a gaseous, liquid or solid sample. In DI-SPME, the fiber is directly immersed in clean liquid samples. Agitation of the sample is often performed with a small stirring bar to increase the rate of equilibration. In SPME, the amount of analytes extracted depends on the partition coefficient between the sample solution and the

fiber. The main advantages of SPME are good analytical performance, simplicity, and low cost. SPME produces relatively clean and concentrated extracts, and is ideal for MS applications. This technique does not suffer from the plugging or channeling problems encountered with SPE. It also completely eliminates use of organic solvents. A relatively long equilibration time (up to 1 h) is needed, and methods such as sample stirring, sample sonication, fiber vibration, and fiber rotation have been used to reduce this absorption time (Kataoka *et al*., 2000; Beltran *et al*., 2000). An inherent disadvantage of SPME is that quantitative work is still rather laborious because carry-over between samples can be severe.

Zeng *et al*. (2008) used polymethylphenylsiloxane-coated fiber for SPME-GC-ECD for the determination of OCs and pyrethroid pesticides (non-polar pesticides) in vegetables with recoveries of 42.9 to 105.3%. In this study, extraction efficiency of the synthesized hydroxyl-terminated polymethylphenylsiloxane (PMPS-OH) coated fiber (70 µm) was compared with commercial fibers such as PDMS (100 µm), PA (85 µm) and PDMS/DVB (65 µm). Since the mentioned pesticides are non-polar and have low solubility in water, they contain one or more phenyl groups and so PMPS is one of the most common non-polar silicon oils with a chain structure. In comparison with PDMS, PMPS furnishes better thermal stability and its polarized phenyl has stronger л-л interaction with the phenyl group in aromatic compounds. The results showed that the extraction efficiency of the PMPS coated fiber for selected pesticides with HS-SPME was higher than commercial fibers and DI-SPME mode. Among the three commercial SPME fibers tested, PDMS (100 µm) resulted in the highest overall extraction efficiency and PA (85 µm) showed the lowest

extraction efficiency, while the efficiency of the PMPS-coated fiber was much better than of PDMS (100 µm).

HS-SPME is often used as a routine technique for the extraction of pesticides from liquid and solid samples. OPs and OCs are the most widely investigated compounds by this method because of their thermal stability and volatility. The extraction process of SPME method can be relatively slow because it relies on the sufficient stirring or diffusion to bring the analytes in to the location of the fiber. In addition, good reproducibility requires that equilibrium is established.

A kind of SPME extraction is the single-drop microextraction (SDME) that is a solvent-minimized sample pretreatment procedure and also has been used to analyze carbamates and organophosphorus pesticides in water samples (Xiao *et al*., 2006; Basheera *et al*., 2007). However, the disadvantages of SDME are as follows: fast stirring often break up the organic solvent drop, easy formation of air bubble (Shen *et al*., 2002) and the extraction procedure is time-consuming where in most cases equilibrium is not easily attained even after a long time (Chen *et al*., 2007). In order to eliminate these restrictions, hollow fiber membrane-protected extraction LPME (HFM-LPME) (Zhang *et al*., 2006; Xiong *et al*., 2008) has been reported as an alternative. In this technique, the solvent is held and protected by a hollow fiber membrane (HFM). However, some drawbacks, such as memory effects caused by the on line configuration, and poor reproducibility because of manual cutting or/and sealing of the membrane in the laboratory have been reported (Rasmussen *et al*., 2004).

Fua *et al*. (2009) applied dispersive liquid-liquid microextraction (DLLME) as sample pretreatment method coupled with HPLC-FID for the analysis of triazophos

and carbaryl pesticides in water and fruit juice samples. The extraction was performed under optimized conditions including, extracting solvent; tetrachloroethane (15 µL), dispersive solvent: MeCN (10 mL), without addition of salt and the extraction was less than 5 s. The enrichment factors obtained for carbaryl and triazophos were 87.3 and 275.6 respectively. In this technique, a proper mixture of extraction and dispersive solvents was rapidly injected into an aqueous sample by a syringe, resulting in the formation of a cloudy solution. This technique (DLLME) has been employed in the analysis of trace organic contaminants and metal ions in liquid environmental samples (Rezaee *et al*., 2006).

Ravelo and co-workers used a combination of DI-SPME with sample stacking micellar electrokinetic chromatography (MEKC) for the analysis of 11 multiclass pesticide residues in red wine. SPME was performed by using PDMS/DVB fibers and the large sample volumes were injected into the capillary by reversed-electrode polarity stacking mode (REPSM). Apparent recovery values with REPSM-MEKC-DAD ranged between 90 and 107% (Ravelo *et al*., 2008_b). The application of SPME-MS has not been considerably established in pesticide analysis. This, along with GC-MS and LC-MS should be expected in the near future.

2.1.3.4 Stir-bar sorptive extraction (SBSE)

Another very elegant enrichment extraction technique based on the same principle as SPME is the recently developed SBSE. This technique was developed to extract organic analytes from liquid samples and is based on adsorption of analytes on to a thick film of PDMS coated on to an iron stir bar. The stir bar is placed in a liquid sample and analytes are adsorbed on this as the samples are stirred for a given time. The stir-bar is then either thermally desorbed on-line for capillary GC–MS or extracted with organic solvent.

The sample is typically stirred for 30-240 min and the extraction time is controlled and determined by means of sample volume, stir bar dimensions and stirring speed. In order to optimize the extraction time, the analyte recovery must be measured as a function of the extraction time. When the extraction time increases, no additional recovery is observed. However, in SPME technique, selected sampling times are often shorter than the time needed to reach full equilibrium. The non-equilibrium conditions are actually preferable in getting good sensitivity and repeatability since the extraction time is not too long. Application of sorptive extraction with PDMS for sample preparation furnishes considerable enrichment, no displacement effects, rapid thermal desorption at mild temperatures. This technique enables the absolute amount of an analyte in a sample to be determined.

Bicchi *et al*. (2003) studied the analysis of nine pesticide residues in heterogeneous matrices and determined the experimental recovery of these pesticides from pear pulp on the basis of their absolute amounts in the sample. In this study the amount of analyte present was evaluated in matrix and the extraction of diluted samples was performed by the stir bar technique. The main difficulty of this method

is that it is hard to automate the rinsing and the extraction processes as well as the removal of the stirring-bar from the sample matrix.

Liu *et al*. (2004, 2005) used sol-gel technology in order to achieve thin layers of PDMS on stirring road. In another study Bicchi and co-workers reported the use of a dual phase stir bar both in DI-SBSE mode and in HD-SBSE mode with PDMS coating and a carbon adsorbent material inside. This system caused the combination of both sorption and adsorption with high recovery of volatile compounds emitted from plant material. In liquid desorption technique the stir-bar is placed in a small vial and is desorbed by using non-polar solvent for GC analysis or with polar solvents for LC analysis. It should be noted that stir bar can be reused for 20-50 extractions (Bicchi *et al*., 2005).

Leon *et al*. (2006) described a multi-residue method for the analysis of PCBs and PAHs and pesticides combined with GC-MS based on ISO/EN 17025 method. In this study, thermal desorption procedure was carried out during14 h by using 2 cm stir-bar coated with a 0.5 mm thick PDMS film followed by GC-MS in scan mode. LODs were 0.1-1.0 ng L^{-1} and the results obtained were very close to the results obtained by classical method.

Stir-bar sorptive extractions followed by liquid desorption and large-volume injection capillary gas chromatography (SBSE-LD-LVI-GC-MS) was developed by Serodio and co-workers for the analysis of pyrethroid pesticides in water samples. The extraction was performed using of stirring bar coated with 47 µL PDMS under conditions of an equilibrium time of 60 min, 5% MeOH as an organic modifier and MeCN as a back-extraction solvent. Good accuracy (81.8-105%) and remarkable

reproducibility (< 11.7%) with excellent recovery were obtained (Serodio *et al*., 2005).

SBSE is more sensitive and accurate than SPME but the main drawback of SBSE is in the desorption step because loaded analyte on coated stir-bars cannot be desorbed directly in the injection port of a GC and so the analyte must be back extracted into a appropriate solvent which causes an additional desorption step.

SPME and SBSE in combination with LC-MS were compared by Blasco and coworkers for the analysis of pesticide residues from honey. According to the results obtained both techniques are simple, cheap and can be done with low consumption of solvents without any preliminary sample preparation step. Linearity and precision obtained by the two methods were similar, while the results obtained by SBSE were more accurate and sensitive than SPME (Blasco *et al*., 2004).

SBSE method is applied for the analysis of halogenated solvents, volatile aromatics, PAHs, PCBs pesticides, odor compounds and organic compounds. Due to the apolar character of PDMS, it is not successful for the extraction of polar compounds except when they have been previously derivatised hence SBSE has been applied commonly for the extraction of non-polar and weakly polar compounds. Even after derivatisation of strong polar analytes to produce more hydrophobic species, this method is not suitable and extraction of them is difficult by PDMS-coated stir bars.

A comprehensive of all early applications of SBSE has been presented by Sanchez-Rojas *et al* (2009). SBSE is nevertheless, regarded as superior to SPME in terms of sensitivity and accuracy. Despite these advantages, its disadvantages have restricted its widespread application in food analysis. The most important of these is

the desorption step, because analyte loaded on coated stir bars cannot be desorbed directly in the injection port of a gas chromatograph. The analyte must therefore be backextracted into a suitable solvent, which adds an additional step to the overall analytical method, or a specially designed thermal desorption unit must be used. This desorption unit is usually a relatively sophisticated instrument, because of problems with high dead volume. Another disadvantage is that the stir bar must be transferred manually to the desorption unit. This may cause partial loss of the sensitivity gained by use of an extended adsorbent surface (Dimitra *et al*., 2007). Table 2.4 reviews some recent applications of SPME and SBSE methods in the analysis of pesticide residues in different samples.

Table 2.4: Examples of SPME and SBSE applications for the analysis of pesticides in food matrices.

Sample	Analyte	Extraction Method	Adsorbent	Eluting solvent	Analytical method	Recovery (%)	RSD (%)	Ref
Radish	12 OCPs & their metabolites	HS-SPME	C[4]/OH-TSO**	-	GC-ECD	78-119	n.r*	(Dong et al., 2005)
Water	Organochlorine pesticides	HS-SPME	PDMS/DVB	Acetone-n-hexane	GC-EI-TOF-MS	n.r	< 20	(Mmualefe et al., 2009)
Strawberries greenhouse	Pesticides	SPME	PDMS		GC-IT-MS/MS	98-124	n.r	(Wang et al., 2009)
Vegetables	OCPs, pyrethroide pesticides	SPME	PMPS-OH	-	GC-ECD	42.9-105.3	< 16.2	(Zeng et al., 2008)
Black rice & ormosia	Triazins & OPPs	SPME	MAA/TRIM*** co-polymers	- -	GC-FTD	79.5-102.2 & 79.8-98.7	5.1-9.0	(Zeng et al., 2008)
Different fruit juices	54 pesticides	SPME	PDMS-DVB		GC-EI-MS/MS	71-108	< 16	(Cortes et al., 2008)
Cucumber & potato	OPPs	SBSE	PDMS	-	GC-TSD	n.r	< 20	(Kirchner et al., 2005)
Water	8 Pyrethroid pesticides	SBSE	PDMS	-	GC-MS(SIM)	67-100	< 11	(Serodio et al., 2005)
Olive oil	9 OPPs	HS-SPME	PDMS	MeCN	GC-FPD	80-106	< 10	(Tsoutsi et al., 2006)
Water	Pyrethroid pesticides	SPME	PDMS	-	GC-μECD	n.r	< 16	(Casas et al., 2006)
Fruits	OPPs	SPME	PA	-	GC-NPD	n.r	2.5-8	(Fyttianos et al., 2006)
Biological samples	Four OPPs	HS-SPME	PA	-	GC-NPD	n.r	< 9	(Tsoukali et al., 2005)
Fruit juices	Carbamate & phenylureas	SPME	PDMS-DVB	- -	LC-ESI-MS (SIM)	25-82	1-17	(Sagratini et al., 2007)
Herbal & tea infusions	OCPs, OPPs, pyrethrin	SPME	PDMS		GC-NPD	73.5-108.3	n.r	(Campillo et al., 2007)
Water	Chloroacetanilide herbicides	SPME	PDMS	-	GC-MS (SIM)	79-102	n.r	(Xu et al., 2007)
Water	46 Multi-class pesticides	SPME	PDMS/DVB		GC-MS (SIM)	n.r	< 20	(Gonzalez et al., 2007)
Honey	6 OPPs	SPME	PDMS	-	LC-APCI-MS (SIM)	52-75	3-10	(Blasco et al., 2005)
Honey	6OPPs	SBSE	PDMS	- -	LC-APCI-MS (SIM)	75-115	5-9	(Blasco et al., 2004)
Grapes	6 pesticides	SBSE	PDMS		LC-APCI-MS	15-100	10-19	(Juan et al., 2004)
Vegetables	Phenylurea herbicides	SPME	PA	-	GC-EI-MS	76-95	< 10	(Berrada et al., 2004)
Wine & strawberries	4 Triazoles	SPME	PA	-	GC-EI-MS	n.r	7-28	(Asperger et al., 2002)

*;Not Reported
**; Sol–gel Calix[4]arene/Hydroxy-Terminated Silicone Oil
***; Methacrylic Acid-Trimethylolpropanetrimethacrylate

2.2 Overview of the instrumentation analysis of pesticide residues in food matrices

The variety of fields of application described in this section show that, because of their versatility, chromatographic MS techniques have been proved successful in virtually any analytical challenge; this makes them robust and effectively applicable options for analysis of pesticides in food. Many pesticides in different chemical groups have been anlalyzed by GC-MS, LC-MS, or MS-MS. The analytical MS methods used in the pesticide food publications on which this part of review is based are listed in Tables 2.5 and 2.6.

Toress *et al*. (1996) made an extensive review on the non-chromatographic techniques employed to determine pesticide residues which were namely immunoassay, biosensors, spectrophotometry, and electrochemistry. In the early days of pesticide residue analysis, colorimetric methods were used, whereby pesticides were analyzed in vegetables employing derivatization to yield the certain colour with subsequent colorimetric determination. The drawbacks of these methods are the impossibility to analyze more than one pesticide simultaneously.

2.2.1 Gas chromatography mass spectrometry (GC-MS)

Although GC-MS, especially with EI ionization, furnishes fingerprint spectra, qualitative and quantitative GC-MS analysis of pesticides can be complicated by interference from matrix components co-eluting with the analytes of interest. Analytes with low and, hence, unspecific m/z value ions in their mass spectra are especially troublesome. Conventional GC-MS methods may therefore, fail to

identify and quantify these analytes at sufficiently low concentrations. This problem becomes critical if a MRL is set to improve the GC separation. A new approach to chromatographic separation, known as comprehensive two dimensional gas chromatography (GC×GC) has recently been introduced as alternative to conventional GC separation because of its outstanding separation potential and capability of solving demanding analytical tasks (Adahchour *et al*., 2006a). In this approach second column, coated with a stationary phase different from that of the primary column, is used for rapid chromatography with TOF-MS detection. This technique uses only TOF-MS as the detector because it has the most sensitivity for fast-eluting peaks (Adahchour *et al*., 2006a, b).

Zrostlíková *et al*. (2003) described the use of GC×GC coupled with TOF-MS for the determination of residues of 20 modern pesticides in apple and peach baby food. Good separation was achieved on a DB-XLB×DB-17 column set and most of the analytes tested could be identified reliably in fruit at levels below 0.01 mg kg^{-1}. Despite its potential, little attention has been devoted to trace-level determination of pesticides in food and very few studied have been reported in the literature. This is probably at least partly because there are many detailed procedures for their precise and accurate analysis by ID-GC-MS (Adahchour *et al*., 2006a, b).

Mezcua et al. described two methods based on GC-MS in selected ion monitoring (SIM) mode and GC-MS-MS using an ion trap operating in the multiple reaction monitoring (MRM) mode for identification, confirmation and quantitation of two EU-banned insecticides in pepper samples. From the obtained results, no significant differences on the performance of both methods were noticed in terms of sensitivity and limit of detection, although the unambiguous confirmation

capabilities provided by MS-MS cannot be achieved with a single quadrupole analyzer. Therefore the information was provided by the ion trap MS-MS method that is the second generation full scan mass spectrum, exceeds that provided by a SIM single quadrupole method (Mezcua *et al*., 2009).

From the literature published, it is clear that GC-MS has proved itself successful for analysis of non-polar, semi-polar, volatile, and semi-volatile pesticides in food. Nevertheless, for polar, non-volatile, and thermally unstable pesticides, for example phenylureas, carbamates, pyrimidines, triazines, phenoxylkanoic acids, and most pesticides transformation products use of GC is impossible and LC coupled to MS is the technique of choice (Reemtsma *et al*., 2003).

Table 2.5 shows some application of GC-MS for analysis of pesticides in foods.

Table 2.5: GC-MS method for determination of pesticides in foods

Pesticide	Matrix	Extraction	Adsorbent	Ionization mode	Detection	LOD ($ng\ g^{-1}$)	Recovery (%)	RSD%	Ref
7 OPPs	Strawberies, Cherries	HS-SPME	PDMS 100m	EI	Q-MS (SIM)	6.3-12.7	74-91	7-15	(Vega et al., 2005)
7 Pyrethroids	Strawberries	MAE-SPME	PDMS 100m	EI	Q-MS (SIM)	0.9-13.8	n.r	1.2-14	(Wang et al., 2007)
6 Phenylurea herbicides	Vegetables	SPME	PA 85m	EI	Q-MS (SIM)	0.1-0.7	76-95	<10	(Seccia et al., 2005)
4 Triazoles	Wine, strawberries	SPME	PA 85m	EI	Q-MS (SIM)	0.03-0.1	n.r	7-28	(Furusawa et al., 2004)
9 OCls	Honey	SPE	C_{18}	EI	Q-MS (SIM)	n.r	79-98	3-18	(Barrek et al., 2003)
Non-authorized insecticides	Peper	QuEChERS	PSA	EI	Q-MS (SIM)	0.1-0.3	85-98	<8	(Milagros et al., 2009)
150 multi-residue	Fruits, vegetables	QueChERS	PSA,C_{18}, GCB	EI	Q-TOF-MS	n.r	70-120	<20	(Tadeo et al., 2000)
Multi-residues	Fish	SPE	NH_2	EI	Q-MS (SIM)	0.5-20	81-113	≤13.5	(Shubing et al., 2009)

2.2.2 Liquid chromatography

High performance Liquid chromatography (HPLC) or simply liquid chromatography (LC) is a less harmful separation procedure in separating thermally labile chemicals and non-volatile components. Nowaday, LC process applies for analysis of both pesticides and their degradation products due to their high polarity and thermolibility or low volatility.

The diverse methods used to determine pesticides in food samples by HPLC have been well documented by Bushway *et al.* (1992). Motohashi *et al.* (1996) presented a review article on the multiresidual methods using GC, thin layer chromatography (TLC), and high performance liquid chromatography (HPLC) in analysis of pesticides in vegetable, fruits and soil.

Extracted compounds of complex sample can be recognized by ultra violet (UV) absorbance detection with diode array (DAD) (Otero *et al*., 2003; Huck *et al*., 2001) but with low specificity, or fluorescence (Bernal *et al*., 1997; Li *et al*., 2004) with more specificity than UV detection, but the results obtained by use of this technique are not desirable. Cheng *et al*. (2008) optimized the operation conditions of LC with UV to obtain the excellent selectivity and sensitivity for the quantitative analysis of pyrethroids in different porcine tissue samples. In this study, the highest peak area of selected pesticides was achieved at wavelength of 210 nm. In evaluation of ratio of mobile phase, all peaks could be separated well when acetonitrile: water at ratio of 85:15 v/v was used as the mobile phase.

In order to increase of sensitivity and specificity, HPLC systems can be combined with the mass analysis capabilities of mass spectrometry. This technique

usually tends towards special detection and potential identification of chemicals in a complex mixture.

Seccia *et al*. (2008) described a rapid and simple sample preparation for the determination of insecticide residues in bovine milk using solid-phase extraction and HPLC-DAD. All analytes were extracted in a single step with dichloromethane, using chem Elut cartridge containing diatomaceous earth materials. Determination and quantification were performed by HPLC with diode-array detection (DAD). LOQ% ranged from 0.01-0.04 mg kg^{-1} with RSD% below 10%.

2.2.3 Liquid chromatography mass spectrometry (LC-MS)

Numerous mass analyzers can be employed in LC-MS, such as quadrupole, triple quadrupole, ion trap, time of flight (TOF) and quadrupole-time of flight (Q-TOF). At present, the techniques that used routinely for introduction and analysis of liquid samples by mass spectrometry can be classified in two groups: those that introduce the sample into the ionic source of the spectrometer, namely the particle beam (PB), and those that allow soft ionization of the sample namely, Thermospray (TSP) or interfaces of atmospheric pressure ionization (API). Slobodnik *et al*. (1995) presented a review covering the field of environmental applications of LC-MS. In their review development and advances on thermospray, particle beam and atmospheric pressure ionization interfaces were discussed.These interfaces were restricted because of low sensitivity, the narrow mass and polarity range of analytes, and common preservation requirements. The arrival of electrospray (ES) ionization resolved the problem of the interface between a liquid phase methods and a gas phase method performed in a vacuum.

More recently, three atmospheric pressure ionization (API) interfaces: electrospray ionization (ESI), ionspray (ISP), and atmospheric pressure chemical ionization (APCI), have replaced almost completely thermospray and particle beam techniques. These interfaces have a broad range of analyte molecular weights and polarities, high sensitivity, improved usability, and reduced maintenance needs. Selection of the appropriate LC-MS interface for an application depends on factors such as the polarity, molecular weight, and thermal lability of the analyte.

Under optimized conditions, all above mentioned interfaces, operating in negative-ion (NI) or positive-ion (PI) modes have worked well and been found complement each other with regard to polarity, molecular mass of analytes, and chromatographic conditions for determination of pesticide residues in food. In most studies positive ion mode has been the ionization mode of choice for all interfaces. MS conditions promoting limited fragmentation and a single predominant ion have been selected as optimum to furnish maximum sensitivity under SIM conditions. These predominant ions correspond either to the protonated molecular ion ($[M+H]^+$) or to adducts of the analyte molecule with one sodium atom ($[M+Na]^+$). Occasionally, however, an additional single for m/z corresponding to K^+ or NH_4^+ adducts appear as the base peak in the spectra and can be selected as the predominant ion. For example, in LC-APCI-MS analysis of carbamates and other polar pesticides in fruit and vegetables, ammonium adduct ions are observed as base peaks. In this particular study some of the pesticides were detected as protonated molecules and other adducts ion (Granby *et al*., 2004).

2.2.3.1 Atmospheric pressure ionization (API)

These interfaces include a wide range of analyte molecular weights and polarities, high sensitivity and decreased preservation requirements. As can be seen in Figure 2.1., in electrospray, effluent is directed through a nebulizing needle into a high-voltage field where charged droplets are formed, then the charged droplets are dried and, as they shrink, analyte ions are desorbed. The ions are transported to the mass analyzer through the series of vacuum stages and ion-focusing elements. The ion source region is separated from the high vacuum mass analyzer region by small ion sampling orifice. The LC column effluent is sprayed in the vicinity of the orifice, so that free jet expansion and concomitants adiabatic cooling can occur. The formation of a spray is affected by applying heat, a coaxial nebulizer gas stream, an electrostatic potential with ultrasonic vibration of the capillary, or a combination of these can be effective on the formation of the spray. The free jet contributes to the formation of large clusters of analyte and solvent molecules bounded by van der waals forces.

At first, electrospray has been applied successfully to small polar molecules with high molecular weight. Many multiresidue methods using LC-API-MS to determine of pesticides in foods have been reported (Volmer *et al*., 1998; Zang *et al*., 1998). API contains a group of interfaces, normally called electrospray (ESI), ionspray (ISP) and atmospheric pressure chemical ionization (APCI).

The introduction of MS^2 instrumentation with atmospheric pressure ionization (API) sources in the past decade has revolutionized trace analyses of chemicals in food and the environment (Pico *et al*., 2004). This coupling combines the advantages

of LC and MS for the separation and the unequivocal identification of pesticides at low-mg kg^{-1} levels in complex matrices (Pico *et al.*, 2004; Pico *et al.*, 2006). LC-MS^2 methods greatly reduce the need for dedicated clean-up steps, resulting in optimized analysis time and costs, with little chance of false-positive findings. Pesticide analysis by $LCMS^2$ is already used in the regulatory area due to its optimum capability in performing multi-residue analyses (Amadeo *et al.*, 2008).

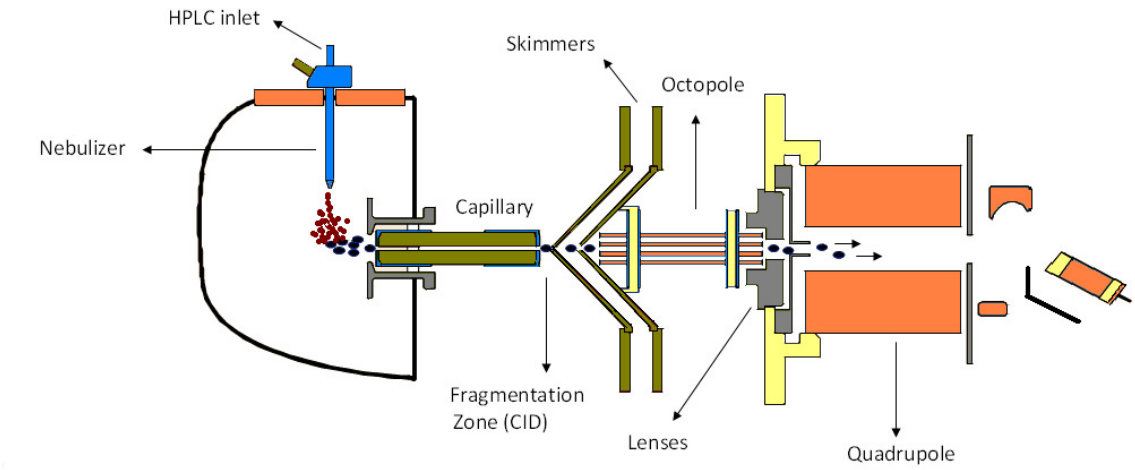


Figure 2.1: API-electrospray LC-MS interface

2.2.3.1.1 Electrospray ionization (ESI)

ESI is the softest ionization technique available for LC-MS and has permitted large labile molecules to be studied intact. In ESI, sample molecules are simultaneously nebulised and ionized at atmospheric pressure. The sample solution is passed through a steel capillary tube. A potential difference of several thousand volts is maintained between the capillary and the counter electrode so that the solvent emerging from the capillary forms an electrostatic spray towards the counter electrode. Gas phase is formed by ion evaporation at atmospheric pressure and then sampled through a two stage momentum separator into the high vacuum of the mass analyzer. The major drawback of this technique is that the maximum allowable flow rate is in the order of 10 L min^{-1}, with lower flow rates giving better performance.

Molina *et al*. (1994) used the first commercially available LC-ESI-MS devices for the determination of organophosphorus pesticides. In comparison between TSP and ESI, they observed that triclorfon degraded to dichlorvos under TSP ionization conditions, whereas this compound could be simply analyzed under ESI conditions. LODs were ranged 10-200 pg, 100 times better than using a TSP- interfacing system.

Hogenboom *et al*. (2000) successfully described the use of LC-ESI-MS for the quantitative analysis of a wide polarity range of pesticides in carrots and potatoes.

In general, ESI is the ionization technique recommended for polar, ionized, and high-molecular-weight compounds, and so is frequently used for analysis of pesticides containing sulfonic acid or carboxyl groups in the chemical structure. Selection of appropriate mobile phase is crucial for the ionization process in ESI; it should always contain at least a small amount of a volatile buffer, acid, or base. For

some compounds better results are obtained by use of ESI and APCI as are summarized in Table 2.6, suggesting that these API sources should be considered when establishing new methods for analysis of foods.

2.2.3.1.2 Ionspray (ISP)

The ISP interfaces, developed by Bruins et al. was originally introduced to enhance the ion evaporation of the ESI. In the ISP interface, the electrospraying process is assisted by coaxial pneumatic nebulisation of the LC column effluent (Bruins *et al*., 1987). The main advantage of this interface in comparison with ESI interface is the tolerance of higher flow rates. Flow rates of 40-50 μL min^{-1}, which are compatible with 1 mm inner diameter LC column, can be accommodated. ISP also shows improved performance over TSP with thermolabile ionic compounds as ISP operates at room temperature, while heat has to be applied with TSP.

Corcia *et al*. (1996) studied the feasibility of using LC-ISP-MS for measuring traces of N-methylcarbamate insecticides in ten different types of fruits and vegetables. Analysis of a tomato extract spiked with carbamates at the individual level of 5 ng g^{-1} of vegetable performed by SIM showed that the detection limits of these analytes could be set at a few hundred pictogram per gram of fruit or vegetable.

2.2.3.1.3 Atmospheric pressure chemical ionization (APCI)

APCI is a gas phase ion-molecule process, which leads to the ionization of the analyte molecules under atmospheric pressure conditions. Because ionization is

CI, however, this is a soft ionization technique and no informative fragmentation occurs. As can be seen in Figure 2.2., APCI interface uses a nebulizer and a make-up gas flow (N_2) to ionize the LC effluent. The spray travels through a large diameter quartz tube, which is heated sufficiently to dry the vapour. A corona discharge needle initiates ionization of solvent molecules. The solvent ions which are formed produce the analyte ions by atmospheric pressure chemical ionization of the analyte. The ions are focused and declusterd through the dry nitrogen curtain gas and are then pass through the orifice into the high vacuum analyzer region of the mass spectrometer where they are mass analyzed. APCI enables very sensitive analysis of weakly basic compounds, and pesticides such as triazines and phenylureas can be easily protonated by gas-phase or mobile-phase ions, depending on their proton affinity.

Blasco *et al*. (2004) compared ESI and APCI interfaces in both ionization modes (NI and PI) for determination of dithiocarbamates and their metabolites in plants. At the concentrations studied, the analytes (thiram, disulfiram, dazomate, ETU, and PTU) were detected in PI mode but not in NI mode. Comparison of APCI and ESI revealed sensitivity differences. When ESI was used sensitivity for ETU and PTU was a factor of 5-10 less than when APCI was used; ESI was much more sensitive than APCI for thiram and disulfiram, however. The APCI interface was eventually selected by the authors because of the better sensitivity for ETU and PTU and its greater robustness resulted in reproducible spectra of the compounds without adduct formation.

Another interface, called atmospheric pressure photo-ionization (APPI), has recently been proposed for complex sample analysis, because it can overcome the

suppression problems encountered with APCI and ESI sources. The APPI was recently used for LC-MS analysis of carbamate pesticides in fruits and vegetables (Yoshioka *et al*., 2004). But no applications of APPI-MS-MS have been reported in the literature.

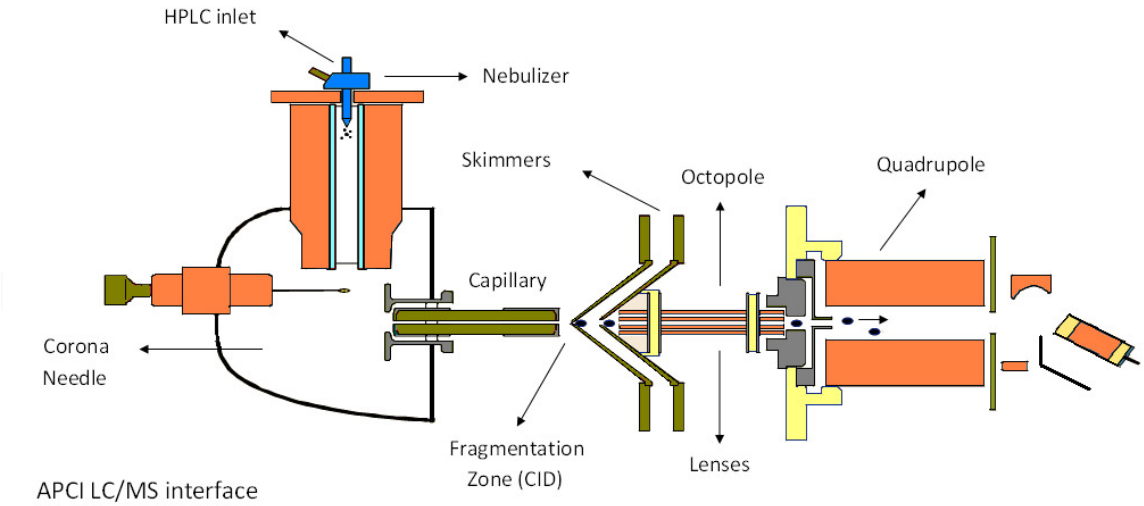


Figure 2.2: APCI LC-MS interface

Recently, among mass spectrometers enabling MS or MS-MS experiments, most of research work was performed with quadrupole (single or triple) and ion-trap instruments. This is principally because of their greater ease of operation, their greater robustness for rutine analysis, and their relatively low cost compared with time-of flight (TOF) instruments. Tandem MS (MS-MS) or in-source collision-induced dissociation (CID) is required to obtain structural information, to improve selectivity and sensitivity, and to confirm the identity of pesticides. In most instances, among the analyzers capable of MS-MS, triple-quadrupoles operating in MRM mode for improving sensitivity have been most frequently used, proving they

were most suitable for achieving the strict MRLs introduced for pesticides in foods. The sensitivity of ion-trap instruments is usually similar to or less than that of triple-quadrupole analyzers. Because of the possibility of obtaining product-ion scans (PIS) without loss of sensitivity, and ability to perform multiple-stage fragmentation (MS), ion trap have been selected for screening purposes. More recent approaches to MS-MS analysis, including linear trap, new generation triple quadrupoles, and hybrid instruments, for example Q-TOF and Q-linear traps can be good alternatives, because of their high scan speeds, accurate mass measurement (QqTOF), and higher sensitivity. The use of TOF instruments increased the accuracy m/z measurements and resolution of mass, which are usually within a few parts-per-million (ppm) of the extract m/z values calculated from the nuclide masses and the ionic charge z. Besides, as the number of target compounds in a single run increases, identification will be a problem. So the main advantage of TOF-MS analysis for large-scale screening is its ability to test a data file for a theoretically unlimited number of pesticides.

Garcia *et al*. (2007b) described a comprehensive method for the analysis of 100 pesticides in food based on the combined use of LC-TOF-MS and LC-MS-MS using QqLIT and compared three stages including: automated pesticide screening by LC-TOF-MS; identification by LC-TOF-MS accurate-mass measurements; and confirmation and quantitation by LC-MS-MS. In the first stage a set of data were obtained, including m/z accurate-mass windows (within 20 mDa width) and retention time in order to build the automated screening procedure, which was created automatically by assigning retention time and the m/z mass window for each target pesticide. After analysis and identification by LC-TOF-MS and confirmation

using two MRM transition, quantitation was carried out by LC-MS/MS using a QqLIT instrument. The results obtained were satisfactory.

Soler *et al*. (2006) compared LC-TQ-MS and LC-QIT-MS and discussed the advantages and disadvantages of both for analysis of pesticides in orange. The results indicated that precision, linearity, and robustness were better for the TQ, which was better than the QIT for quantitative analysis, although both mass spectrometers could be used for both qualitative and quantitative analysis of conventionally targeted oranges.

LC of target pesticides in extracts obtained from food samples has been performed with different columns. Pesticides have usually been separated by reversed-phase chromatography on C_{18} columns (4.6 mm i.d.). Column type is, nevertheless, always critical and other types of column have been proposed for more specific separations. Hernando *et al*. (2007) evaluated different LC-QLIT-MS (MRM) conditions to obtain sufficient sensitivity for the detection of pesticides in olive oil by using turboionspray source in positive mode. In this work, two different chromatographic columns (C_{18}, 100×2.1 mm i.d., 1.8 µm and C_{18}, 150×4.6 mm, 5 µm), different working flows (200 and 600 µL min^{-1}) and different injection volumes (5 and 10 µl) were studied. The mobile phases used in both columns were HPLC water, 0.1% formic acid as mobile phase B, and MeCN as mobile phase A. One approach applied to improve the sensitivity was by using small particle size (e.g., 1.8 µm) columns, which can provide increased column efficiency with better baseline separation and narrower peaks than standard particle size columns (e.g., 3.5–5 µm). On the other hand, the sensitivity achieved in small particle size columns is limited by the volume of sample that can be injected. In high-demand conditions,

small particle size columns such as 2.1×100 mm, could even support the injection of higher volumes (e.g., 10 µL) than the maximum volume recommended (5 µL) without significant changes in the column pressure. But the disadvantage is a worsening of the peak shape. Another option to optimize sensitivity is the flow rate. Upon exploring two flow rates of 200 and 600 µL min^{-1} in term of sensitivity, a superior response was observed at 200 µL min^{-1}, and so this was judged to be more suited to the trace determination of pesticides. The benefit of using higher flow rates is the reduction in analysis time, which is ideal for routine laboratory analysis. However, reduced sensitivity was observed at the higher flow rate explored, which could be associated with a dilution effect or a less stable spray. Therefore, the use of C_{18} (4.6×150 mm 5 µm), at flow rate of 200 and injected sample of 10 µL in MRM mode gave the best sensitivity with LODs ≤1 µg kg^{-1} for 84 pesticides, ≤5 µg kg^{-1} for 12 and ≤10 µg kg^{-1} for 4 pesticides.

Table 2.6 shows some application of LC-MS for analysis of pesticides in foods.

Table 2.6: LC-MS methods for determination of pesticides in foods

Pesticide	Matrix	Extraction	Adsorbent	Ionization mode	Detection	Mobile phase	LOD (ng g^{-1})	Recovery (%)	RSD (%)	Ref
6 Pesticides	Grapes	SPE	C_{18}	APCI+	MS-Q (SIM)	H_2O:MeOH	n.r	60-100	7-17	(Shim et al., 2003)
160 multi-residiue	Vegetables	QuEChERS	PSA,C_{18}, GCB	ESI	MS-Q (SIM)	H_2O:MeCN	<5	70-120	<20	(Kmellar et al., 2008)
80 multi-residues	Oranges, grapes, Wheat flour, wine	QuEChERS	PSA,C_{18}, GCB	ESI	MS-$Q_{3(LIT)}$ (MRM)		≤10	n.r	<10	Payá (2007)
22 Opps	Honey	SPE	C_{18}	APCI+-	MS-Q (SIM)	H_2O:MeOH	n.r	16-102	<7	(Debayle et al., 2008)
6 Pesticides	Grapes	SBSE	PDMS 1mm	APCI+	MS-Q (SIM)	H_2O:MeOH	n.r	15-100	10-19	(Shim et al., 2003)
Multi-residues	Fruits	MSPD	Diatomaceous	ESI+	MS-Q (SIM)	H_2O:MeCN	n.r	71-118	5-15	(Radišic´ et al., 2009)

CHAPTER

III

CHAPTER III: METHODOLOGHY

3.1 Experimental procedures

3.1.1 Glassware

All glassware was cleaned thoroughly using diluted Decone with water and a bottle brush and rinsed with tap water. Then the glassware were soaked overnight in a chromic acid bath which was prepared by adding potassium dichromate ($K_2Cr_2O_7$) to concentrated sulphuric acide (H_2SO_4) until saturation was reached. After that, the glassware was rinsed with abundant tap water and distilled water and then dried in a drying oven at 105 °C. The glassware was then wrapped in aluminium foil to protect from dust and vapour. All glassware was rinsed with acetone prior to use throughout the work.

3.1.2 Reagents and samples

The use of high purity reagents and solvents help to minimize interference problems. HPLC grade acetonitrile (MeCN), methanol (MeOH), acetone and n-hexane were purchased from Merck (Darmstadt, Germany). Reagent grade anhydrous magnesium sulphate, sodium chloride and primary secondary amine (PSA) sorbent (SPE Bulk packing, 50 µm) were purchased from Sigma-Aldrich (Steinheim Loius, MO, USA). C_{18} sorbent (50 µm) and GCB cartridges (SPE Bulk packing, 120–400 mesh) were obtained from Supelco (Bellefonte, PA, USA). Florisil cartridges (50 µm, 12 mL) were purchased from Agilent Technologies and neutral alumina from Merck (Darmstadt, Germany). A Milli-Q-Plus ultrapure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC-grade water used during the analyses. As pre-treatment prior to

LC-QTOF-MS analysis, the extract samples were merely filtered through a 0.45 μm filter (Millex FG, Millipore, Milford, MA, USA).

In this study, several samples of virgin olive oil and palm oil both from two different brands which were purchased from local supermarkets in Kuala Lumpur, Malaysia were sampled and analyzed following the purposed sample preparation methods for the determination of seven multiclass pesticide residues.

3.1.3 Pesticides and standard stock solutions

In this study, seven analytes namely dimethoate, carbaryl, simazine, atrazine, terbuhylazine, diuron, and malathion were selected among different classes of compounds (organophosphates, carbamates, triazines and phenylureas) and based two chemical uses which are insecticides and herbicides. Analytical pesticide standards simazine, terbuthylazine, atrazin, diuron, dimethoate, malathion and carbaryl were obtained from Fluka (Buchs, Switzerland, HPLC grade 99.9%). Individual pesticide stock solution of the analytes were prepared at 1.0 mg mL^{-1} in pure methanol and kept in an amber-colored bottle at 4 °C. These solutions were kept for 2 h at ambient temperature prior to their use. The mixed standard stock solution containing all of the studied pesticides was prepared by pooling aliquots of the individual pure pesticide standard solutions and then diluting with methanol. Table 3.1 shows the concentration of each pesticide used in the mixed standard stock solution. Working standard solutions (5 and 10 μg mL^{-1}) of a mixture of pesticides were prepared by appropriate dilutions in methanol and stored at -20 ºC. The calibration standard solutions were prepared by serial dilution of the mixed standard stock solution with methanol. The working standard mixture solutions were freshly

prepared every day in order to avoid the influence on the results from the possible degradation of pesticides.

Table 3.1: Concentration of selected pesticides used in the mixed standard stock solution

Pesticides	Concentration (μg mL^{-1})
Dimethoate	10
Simazine	5
Carbaryl	10
Atrazine	5
Diuron	5
Terbuthylazine	5
Malathion	10

3.2 Apparatus

3.2.1 Centrifuge

A centrifuge instrument (Kubota 2420, Japan) was used for separation of solid and liquid phases.

3.2.2 Ultrasonic system

An ultrasonic water bath (Model: Power Sonic 405, Hwashin Technology, Korea) was used in the extraction procedure equipped by a generator with an output of 350 W and input of AC 230 V and 50 KHz.

3.2.3 Liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS)

The separation of the selected herbicides was carried out using an HPLC system (consisting of a vacuum degasser, an autosampler, and a binary pump-SL; Agilent Technologies 1200 Series) equipped with a reversed phase C_{18} analytical column of 50 mm × 2.1 mm and 1.8 µm particle size (Zorbax SB-C_{18}). Column temperature was maintained at 40 °C. The injected sample volume was 5 µL. Mobile phases A and B were acetonitrile and water with 0.1% formic acid, respectively. In the optimized chromatographic method, the initial mobile phase composition (10% A) was held constant for 5 min, followed by a linear gradient to 100% A after 30 min. The flow-rate was optimized at 0.25 mL min^{-1}. A 10- min post-run time was used after each analysis. This HPLC system was connected to an Agilent MSD QTOF (Agilent Technologies, 6530 Accurate Mass QTOF), equipped with an electrospray interface operating in positive ion, using the following operation parameters: capillary voltage 4000 V; nebulizer pressure 40 psig; drying gas 9 $Lmin^{-1}$; gas temperature 300 °C; fragmentor voltage 190 V; skimmer voltage 65 V; octopole RF 750 V. LC/MS accurate mass spectra were recorded across the range 50-1000 *m/z*.

3.3 Identification and confirmation by LC-QTOF-MS

Although first introduced commercially only 6 years ago, quadrupole–time-of-flight (TOF) mass spectrometers have rapidly been embraced by the analytical community as powerful and robust instruments with unique capabilities. In this study we used the relatively generic term QTOF to refer to both the technique and to the

instrument, where Q refers to a mass-resolving quadrupole and TOF refers to a time-of-flight mass spectrometer. This technique uses the principle of orthogonal injection from a high-pressure ion source, and its history (and principles) is well described in several recent reviews (Chernushevich *et al.,* 1999). The configuration can be regarded either as the addition of a mass-resolving quadrupole and collision cell to an ESI-TOF, or as the replacement of the third quadrupole (Q3) in a triple quadrupole by a TOF mass spectrometer. This configuration gives excellent selectivity from high mass resolution (typically around 10000) and high mass accuracy with full spectra. Particular advantage for full-scan sensitivity (over a wide mass range) is provided in both modes by the parallel detection feature available in TOF-MS. As a result of this, interfering peaks of ions having the same nominal mass can be resolved partially or completely, the charge state of multiply charged ions can be determined from their isotopic spacing in many cases, and signal-to-noise ratio is improved owing to grouping of ions into narrower peaks (increasing the peak height). The high mass accuracy of the TOF can be achieved in a very practical way is due to two main factors: the high mass resolution and the simplicity (and hence predictability) of the mass calibration scale. The high mass resolution is important because it minimizes the possibility of overlap of two mass peaks, since the centroid of a peak can be significantly affected by even a minor underlying component of a slightly different *m*/*z* value. So the instrument is able to provide high mass accuracy and sensitivity in a product ion mode with electrospray ionization (Chernushevich *et al.,* 2001).

The identification of the targeted species was performed basically by retention time matching combined with accurate mass measurements of the targeted

protonated molecules and, when available their main fragment ions and or isotope signature (i.e. ^{37}Cl). In this sense, the combination of in-source colision induced dissociation (CID) and the comparison and evaluation of the theoretical and experimental isotope patterns (from the elemental composition of the species) are powerful tools for identification purposes in most of the targeted species. The accurate mass of characteristic isotopic signals, and the distance in the *m/z* axis between them can be combined by the software to provide a user-created weighted coefficient estimating how similar the experimental mass spectrum is when compared to that obtained with standards.

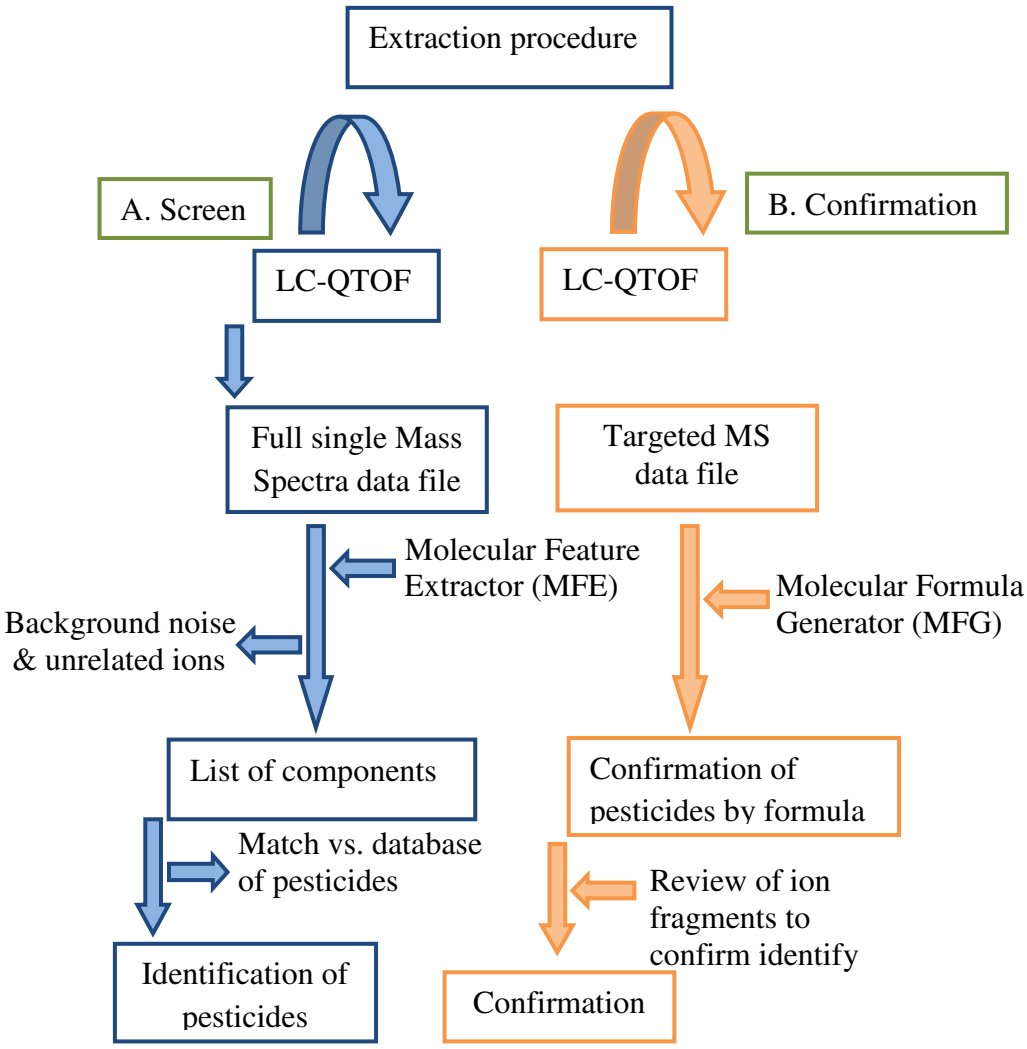


Figure 3.1: Screen and confirm-LC-QTOF analysis and software workflow

3.4 Optimization approaches

3.4.1 Optimization of chromatographic conditions

Oil is one of the difficult food matrices due to the presence of numerous interferences that show up in full-scan mode. For this reason, LC-TOF-MS parameters affecting the performance of LC-MS such as nebulizer and drying nitrogen flow rates, drying temperature and the effect of the fragmentor voltage were studied and optimized. For optimization of these parameters, the analytical standard of pesticides was injected individually and then these parameters were adjusted to maximize signal-to-noise (S/N) ratio of the compound. The study of the influence of variations in each parameter setting on the S/N ratio of individual compound was performed in the flow injection analysis (FIA) mode and determined by repeated injections of the solution, keeping the other parameters constant. For each compound, the parameter settings which gave the highest S/N ratio were selected for the analysis.

3.4.2 Optimization of extraction and clean-up procedures

In this study, a comprehensive evaluation of the potential of two widespread sample treatment procedures based on (a) low temperature precipitation (LTP) followed by dispersive solid-phase extraction (d-SPE) clean-up and (b) liquid-liquid extraction (LLE/LTP) followed by matrix solid-phase dispersion-sonication (MSPD) used for pesticide residues analysis in food were carried out using LC-QTOF-MS as a detection method. Concerning MSPD method, this technique has appropriate application as an analytical process for the preparation, extraction and

fractionation from solid, semi-solid and biological matrices (Carvallho *et al*., 2009, Garcia-Chao *et al.,* 2010, Sobhanzadeh *et al*., 2011, Rodrigues *et al*., 2010). MSPD technique involves dispersion of sample in adsorbent, followed by preliminary purification and elution of analytes with a relatively small solvent volume (Yang *et al*., 2008, Garcia *et al*., 2008).

High fat food samples such as oil represent a particular analytical challenge for pesticide residues analysis due to the inherent complexity of the matrix that may have an adverse affect on the results of analysis. Methods applied to determine pesticide residues in fatty food may require many steps and analytical time. Therefore, to reduce the co-extracted fat and obtain the extract with minimal interferences as well as to reduce the analysis time, optimization approach of sample treatment methodologies are performed in order to solve these challenges and to provide the best clean-up of total matrix components prior to multi-residue pesticids determination in oil samples.

For MSPD extraction, type and quantity of sorbents, nature and volume of the eluting solvent were studied. The extraction conditions in terms of with and without sonication were also evaluated in this work.

3.5 Spiking procedures

For recovery studies, the samples were spiked with the studied pesticides before the corresponding extraction procedure. A representative 200 g portion of oil sample was weighed and fortified homogeneously with different volumes of working standard solution to obtain 25, 50 and 100 ng g^{-1} of the studied pesticides in the spiked sample. The sample was incubated at room temperature for 6 h, to make sure the solvent was completely evaporated.

3.6 Matrix matched calibration curves

The use of matrix-matched standards provides reliable quantitation capabilities for food analyses (Ferrer *et al*., 2005). Therefore, matrix-matched standards of the studied pesticides were applied using proposed sample preparation methods in order to avoid quantitative errors. For this purpose, oil matrix-matched standards were prepared by adding known amount of working solution to the extracts in order to obtain the desired concentration in the range of 5-1000 ng g^{-1}. Blank extracts of oil matrix were also measured in this study.

3.7 Analytical methods

3.7.1 Liquid-Liquid extraction (LLE) followed by low temperature precipitation (LTP)

5.00± 0.01 g homogenous oil sample was weighted in a 50 mL screw capped centrifuge tube. The sample was fortified when required, with 250 µL of pesticide standard mixture in MeOH to obtain concentration of 50 ng g^{-1}, while 250 µL of MeOH was added in non-fortified samples. The use of MeOH was to improve the sample distribution throughout the column. LLE was performed using 15 mL different organic solvents to optimize the efficiency of the pesticides extraction from oil for LLE and freezing process (see section 4.2.1). The resulting mixture was then shaken for 10 min using a vortex mixer. After centrifugation at 4000 rpm for 3 min, the centrifuge tube was kept horizontally in a freezer at -20 °C for 2 h. The organic phase containing the organic solvent and extracted pesticides remained as a liquid and rose to the top whereas the oil were frozen and precipitated at the bottom of the tube.

3.7.2 Matrix solid-phase dispersion sonication (MSPD-sonication)

3 mL (1/5 extract volume, about 1/5 initial weight) of the extract obtained from LLE step was transferred into a 10-mL glass test tube, to be carefully evaporated under nitrogen stream, up to a final volume of about 1.5 mL. This remaining extract was gently blended with 750 mg PSA as dispersing phase in a glass mortar using a glass pestle until a homogenous mixture was obtained. The mixture was then transferred into a 100 mm × 20 mm I.D. glass column containing Whatman No. 1 and 250 mg of florisil as a clean-up adsorbent which was placed at the bottom end of the column. The column was then set in a tube rack and closed with one-way stopcock and extracted with 15 mL of acetonitrile for 15 min at room temperature in an ultrasonic bath. The water level in the bath was adjusted to be at level with the solvent inside the column. After extraction, the columns were set on a vacuum manifold and the analytes were eluted and collected in graduated conical tubes. Elution step was carried out by gravity flow. The extracts were concentrated to slightly dryness with gentle stream of nitrogen, then reconstituted with acetonitrile/water (1:1 v/v), reaching a final volume of 1 mL. The extract finally contained the equivalent of 1 g of sample per mL and then was filtered through a 0.45 μm PTFE membrane filter (Millexm FG, Millipore, Milford, MA) prior to LC-TOF-MS analysis. In order to obtain cleaner samples, the extracts were diluted in a ratio of 1:2 before they were injected in the LC-MS instrument. In this case, 500 μL of the extract was taken and diluted with 500 μL of solvent (MeOH 20 %), so all samples contained 80% of water.

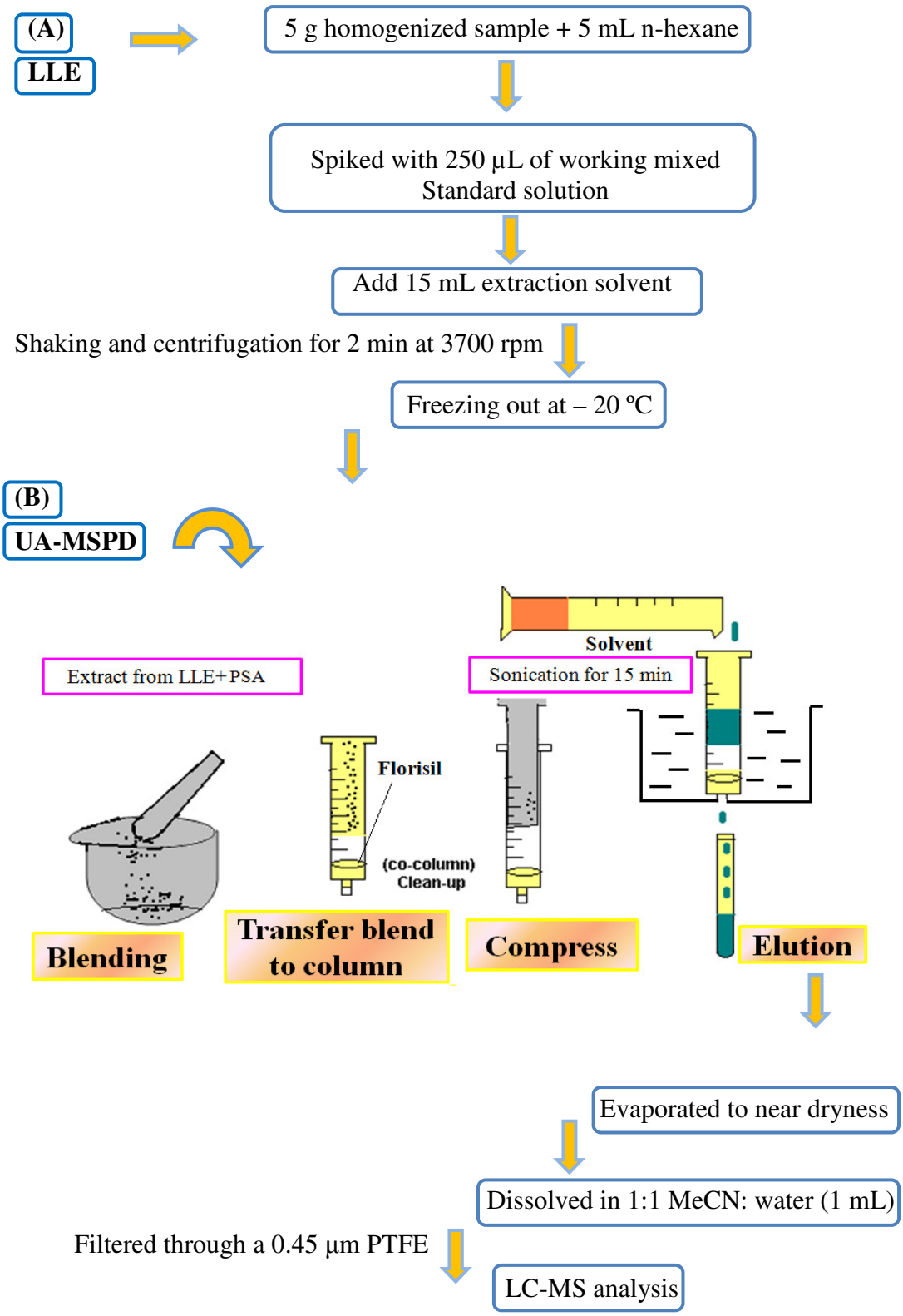


Figure 3.2: Flow chart of multiresidue analysis of pesticides in oil sample using LLE/LTP; (A) followed by MSPD sonication procedure: (B).

3.7.3 Dispersive solid-phase extraction (d-SPE) procedure

3.00± 0.01 g of a homogenous oil sample was weighed in a 50-mL screw capped centrifuge tube with 7 g of ultra-pure water. The sample was fortified, when required with 150 µL of pesticide standard mixture in MeOH, while 150 µL of MeOH was added to non-fortified sample. 10 mL of MeCN was added along with 4 g of anhydrous magnesium sulphate ($MgSO_4$) and 1 g of sodium chloride (NaCl). After shaking for 3 min and centrifugation at 3700 rpm for 2 min, all samples were kept horizontally in a freezer at - 20 °C for 2 h. Aliquots of extract obtained from LTP were subjected to further clean-up by d-SPE procedure. Therefore, 5 mL of the extract was transferred into a 15-mL micro-centrifuge vial containing 150 mg PSA and 50 mg GCB and 150 mg $MgSO_4$. After shaking for 1 min, the mixture in the tube was centrifuged at 3700 rpm for 2 min. 3 mL of the supernatant was then evaporated to slightly dryness and reconstituted with 1 mL to a final composition of 20 % MeOH in water. Then the extract was filtered through a 0.45 µm PTFE filter prior to LC/MS analysis. Now the extract contained the equivalent of 1 g of sample per mL. In order to obtain cleaner sample, the extract was diluted 1:2 prior to injection into LC–MS instrument. This step was carried out by taking up 500 µL of the extract and adding 500 µL of solvent (20% MeOH). Finally, all samples contained 80% of water. Figure 3.3 shows the flow chart of sample treatment method based on LLE/LTP and modified d-SPE procedure for oil samples.

Figure 3.3: Flow chart of multiresidue analysis of pesticides in oil sample using LLE/LTP; (A) and d-SPE clean-up procedure (B).

3.8 Quantification

3.8.1 Multiresidue analysis of pesticides in oil samples

The analytical performance of the proposed method was studied in order to evaluate its usefulness for quantitative determination of pesticide residues in oil extract. For quantitative analysis in pesticide formulations, the response of peak area against concentration of matrix matched standard solutions was tabulated. The calibration was carried out using spiked matrix-matched standards prepared by the proposed extraction method as described in section 3.7. Linearity was estimated by analyzing these matrix-matched standard solutions prepared at different concentration levels in the range of 5-1000 ng g^{-1}. The slope and intercept values, together with relative standard deviations were estimated using regression analyses.

The instrumental limit of detection (LOD) and limits of quantification (LOQ) of the prepared in overall method, were estimated using matrix-matched standard at low concentration levels giving signal-to-noise ratio of three (S/N=3) and ten (S/N=10), respectively, measured by peak-to-peak method at the lowest calibration level. Recovery studies of the developed analytical method to obtain precision were performed by spiking untreated oil samples with the appropriate volumes of composite working standard solution at three different concentration levels: 25, 50, 100 ng g^{-1}. Intra-day and inter-day repeatability were carried out by running six extractions of oil samples spiked at three concentration levels (50, 500, 2500 ng g^{-1}) using both sample treatment methodologies. The relative standard deviation (RSD) (n=6) for run-to-run studies (running in single day) and inter-day (running within six days) were also calculated.

3.8.2 Matrix effects

Matrix components can provide variation in the detector response to pesticides. Matrix effect can reduce or enhance the response of the detector and it can be evaluated by comparing the detector response for pesticide standards prepared in solvent with that for standards prepared in the sample extract. A value <1 indicates signal suppression due to the matrix, while values >1 involves enhancing effect of the matrix on analyte signal. In this study, these possible effects were evaluated by comparing the slopes obtained in the calibration with matrix-matched standards and those obtained with solvent-based standards in order to calculate matrix/solvent slope ratio for each pesticide. %ME is the %difference in the best-fit slope of the matrix-matched calibration standards vs. the best-fit slope from reagent-only standards. it was considered to be a mild signal suppression or enhancement effect between−20% and 0% and between 0% and +20%; it was considered to be of medium effect when the slope values were between −50% and −20% or +20% and +50%; and it was considered to be a strong effect of signal suppression or enhancement below −50% or above +50%.

The percentages of signal suppression or enhancement (calculated by formula: matrix/solvent slope ratio × 100 − 100) were also estimated. Depending on the decrease/increase in the percentage of the slope, different matrix effect could be observed. Negative values indicate signal suppression of the matrix, while positive results show enhancement due to the matrix.

CHAPTER

IV

CHAPTER IV: RESULTS AND DISCUSSION

4.1 Optimization parameters for LC-QTOF-MS

Standard electrospray ionization conditions were selected to achieve the best possible sensitivity and selectivity for the selected compounds. Standard values were set for nitrogen flow rates, capillary voltage and vaporizer and drying gas temperatures. Besides the typical electrospray parameters, the parameter associated with in-source collision induced dissociation (CID) fragmentation (Fragmentor voltage) which had a strong influence on the sensitivity and relative abundance of protonated molecules were carefully studied.

4.1.1 Nitrogen flow rate

In this study, the signal to noise (S/N) ratio was calculated by subtracting the minimum value of the background signal from the maximum background signal (Graham, 1993). This difference was then compared to the signal obtained from the presence of the chemical phenomenon. The signal was then divided by the difference of the background signal.

$$\frac{S}{N} = \frac{\text{Signal}}{\text{Max}_{\text{background}} - \text{Min}_{\text{background}}}$$

In order to optimize the flow rate of drying nitrogen gas, different flow rates (0.05-1.0 mL min^{-1}) were delivered to the LC system. Our results revealed that although the signal response remained unchanged the use of the lower flow rates for most of the pesticides improved the baseline stability, thereby increasing the signal-

to-noise ratio. This is because the difference between the maximum and minimum background signal remains more stable at lower flow rates. Figure 4.1 shows the intensities of the selected pesticides obtained at different gas flow rates. The optimum flow rates were between 0.2 and 0.3 mL min^{-1}, so the elution at the flow rate of 0.25 mL $min^{-1}$was selected for this study which gave rise to high signal-to-noise of the compounds as well as good chromatographic separation of the pesticides.

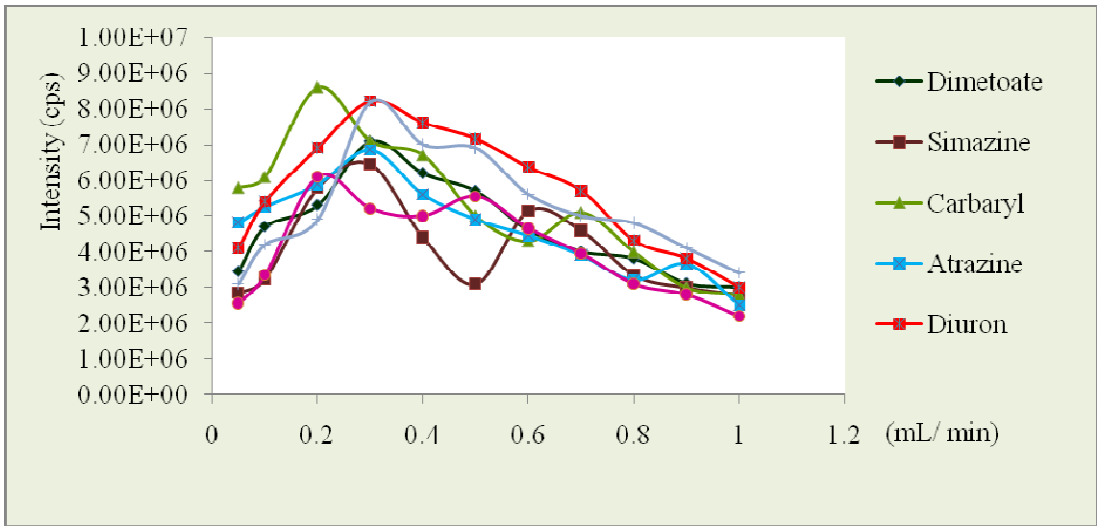


Figure 4.1: Effect on intensity of fragment ion of each analytes using different nitrogen gas flow rates for LC-QTOF-MS.

4.1.2 Capillary voltage

In general, the higher capillary voltage and gas temperature, the greater energy imparted to the ions entering the analyzing region of the mass spectrometer. The energy helps to decluster the ions and to reduce the chemical noise in the spectrum resulting in an increase in signal-to-noise ratio or sensitivity. On the other hand, in some instances, this fragmentation phenomenon can become a valuable tool providing additional structural information for identification purposes. For achievement of the best sensitivity, the results showed that the capillary voltage should be kept at 4000 V to minimize further fragmentation in the pre-analyzer zone. Figure 4.2 shows the graphic presentation of capillary voltage affecting the signal-to-noise ratio.

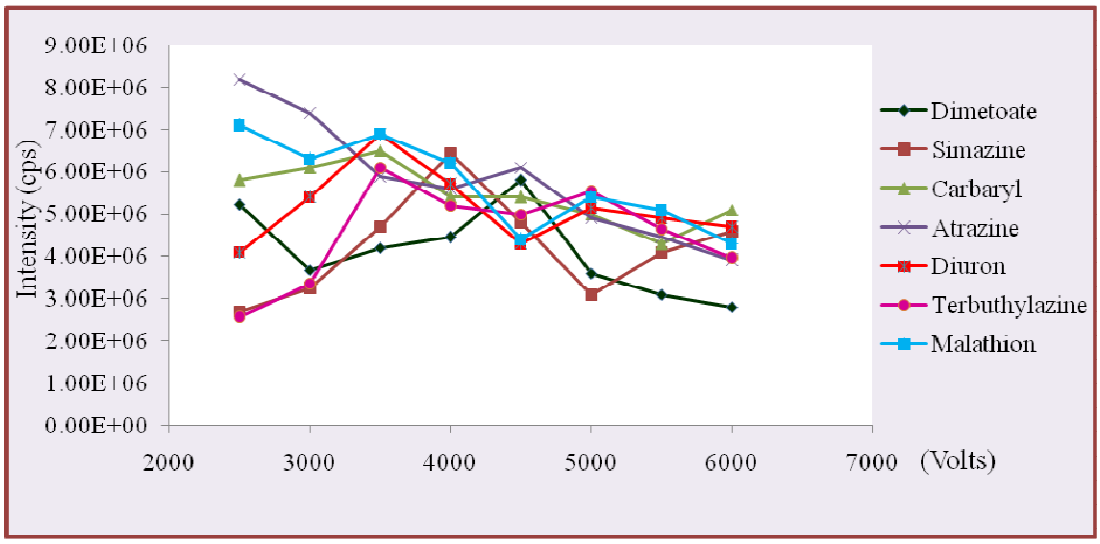


Figure 4.2: Effect on intensity using various capillary voltages for LC-QTOF-MS

4.1.3 Drying gas temperature

In LC-QTOF-MS, heat is used to vaporize the sample and solvent sprayed into the ion source. If the temperature is set too low, the vaporization is incomplete, whereas setting the temperature too high induced thermal degradation of the sample. The optimum temperature is the lowest setting which ensures complete vaporization of the sample. Therefore, the optimum gas temperature was set at 300 ℃. A graphical presentation of the results from the optimization of temperature is shown in Figure 4.3.

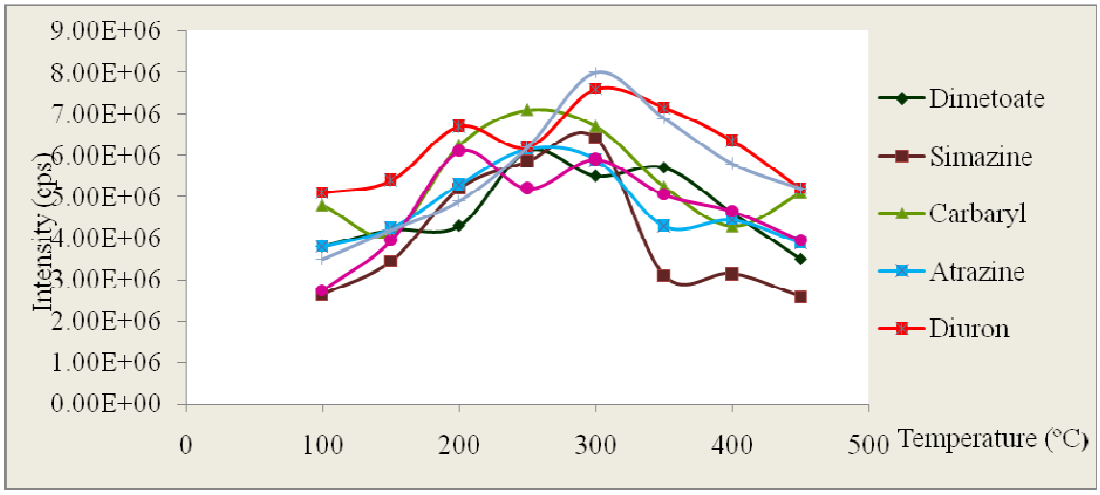


Figure 4.3: Effect on intensity using different gas temperatures for LC-QTOF-MS.

4.2 Identification and confirmation of pesticides

The identification of the targeted species was performed basically by retention time matching combined with accurate mass spectrum features of each compound and, when available, their main fragment ions and or isotope signature (i.e. ^{37}Cl). For this purpose, narrow mass window extracted ion chromatograms were used. To achieve the high selectivity, better signal-to-noise ratio and to reduce the possibilities of finding false positives, the extracted ion chromatograms (XICs) of each analyte was obtained using a mass window of ±5 mDa. For quantitation purposes, peak areas of the XICs of the protonated molecules ($[M+H]^+$) were used for all of the species except when the relative intensity of sodium adducts ($[M+Na]^+$) was higher than that of the protonated molecule in the selected conditions such as malathion, or relative intensity of characteristic fragment ion was higer than the protonated molecule such in the case of dimethoate and carbaryl. The high intensity of protonated pseudomolecular ions made them possible to achieve high specificity. Therefore, high specific analysis could be performed by monitoring these ions for quantitative purposes.

4.2.1 Collision Induced Dissociation (CID)

The in-source collisionally dissociation fragmentation is greatly enhanced at high fragmentor voltage. This provids highly valuable structural information since the accurate mass of the characteristic fragment ion could be used along with that of the protonated molecule for confirmation purposes.

The effect of the fragmentor voltage was studied in order to obtain additional informations from characteristics fragments of the studied compounds. For this purpose, three different voltages consisting; 160 V (mild), 190 V (moderate) and 230 V (high) were studied under the optimized instrumental parameters such as capillary voltage of 4000 V; nitrogen flow rate of 0.25 mL min^{-1}; drying gas at 9 L min^{-1} and gas temperature of 300 °C. The relative abundances for both the protonated molecules and the main fragments (*m/z*) of the pesticides at three fragmentor voltages (160 V, 190 V, 230 V) are shown in Table 4.1. As can be seen in the Table, the extent of the fragmentation is primarily compound-dependent. For instance, organophosphorus compounds such as dimethoate and malathion or carbamate insecticides such as carbaryl yield several fragment ions even under mild and moderate conditions, while other compounds such as s-triazines and diuron are difficult to cleave unless a moderate or high fragmentor voltage was applied. Simazine and atrazine showed the higher characteristic fragment ions (at *m/z* 174 and *m/z* 132 for simazine and at *m/z* 174 for atrazine) at moderate level voltages. Terbuthylazine required medium voltage in order to obtain significative abundance for the fragmentats. Diuran showed a good fragmentation at a medium voltage and also presented a minor sodium adduct ($[M+Na]^+$ at *m/z* 255) and a characteristic fragment ion at *m/z* 72.

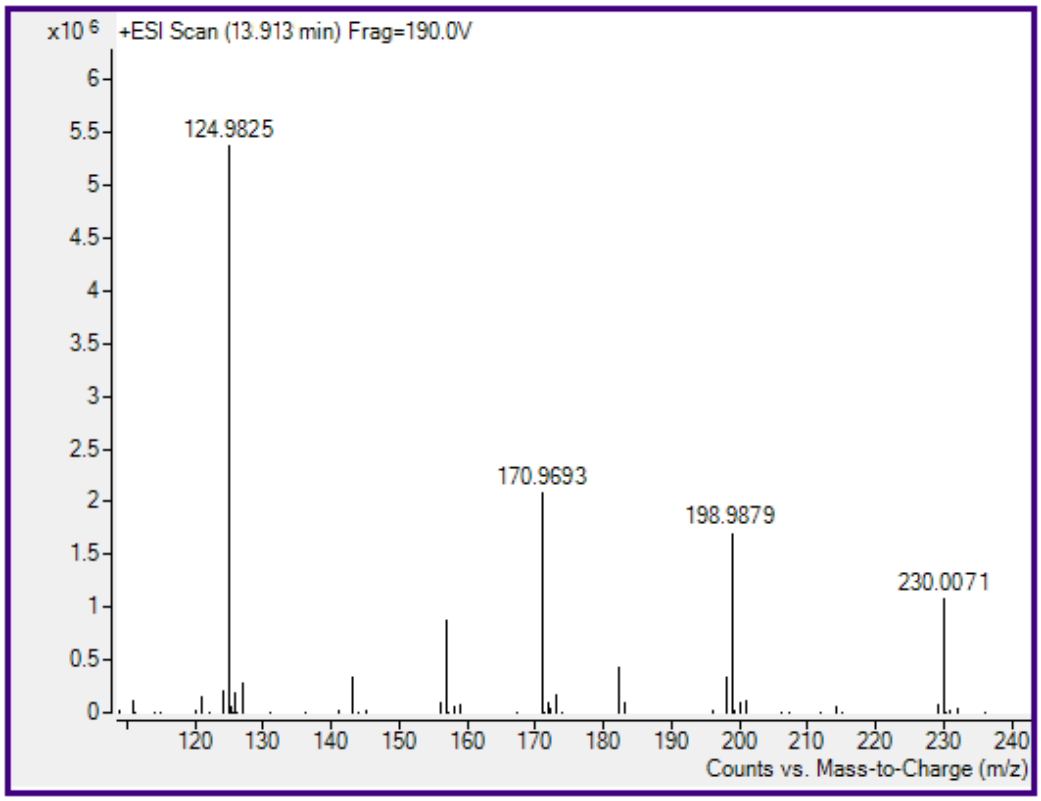
In carbaryl and dimethoate, the loss of methylisocyanate (CH_3NCO) and ethylisocyanate (C_2H_5NCO) respectively, from the protonated pseudomolecular ions give rise to the exhibited base peaks of fragment ion (*m/z* 145, and *m/z* 124 respectivly) at fragmentor voltage of 190 V. This is most likely due to the thermal degradation of these compounds. Therefore for further study, *m/z* 145 and *m/z* 124

fragment ions were used for quantitative analysis. Nevertheless, malathion showed a good fragmentation and presented a sodium adduct ($[M+Na]^+$) at *m/z* 353 as a main peak at fragmentor voltage of 190V. As a result, the fragmentor voltage was set at 190V as a balance between sensitivity for quantitation and additional mass spectrum information for structural/confirmation purposes.

The mass spectrum of each compound and the proposed fragmentation scheme of the selected pesticides in positive ionization mode are shown in Figures 4.4-4.10.

Table 4.1: Effect of different fragmentor voltages on CID Fragmentation for LC-QTOF-MS

		Relative abundance		
Analyte	**Fragment ion**	**160 V**	**190 V**	**230**
Dimethoate	230 $[M + H]^+$	100	10	75
	199 $[M + H – CH_3O]^+$	76	26	100
	171$[M + H – CH_3NCO]^+$	33	42	< 5
	124 $[M + H– C_2 H_6NCOS]^+$	37	100	38
	251 $[M + Na]^+$	nd	nd	61
Simazine	202 $[M + H]^+$	100	100	100
	174 $[M + H – C_2H_4]^+$	< 5	10	47
	132 $[M + H – C_2H_6N_2]$	nd	14	23
Carbaryl	202 $[M + H]^+$	100	7	5
	145 $[M + H – CH_3NCO]^+$	34	100	100
Atrazine	216 $[M + H]^+$	100	100	100
	174 $[M + H – C_3H_6]^+$	nd	30	42
Diuron	233 $[M + H]^+$	100	100	53
	255 $[M + Na]^+$	nd	12	7
	72 $[M + H – C_6H_5NCl_2]^+$	nd	7	< 5
Terbuthylazine	230 $[M + H]^+$	90	100	23
	174 $[M + H – C_4H_8]^+$	100	87	100
Malathion	331 $[M + H]^+$	100	10	100
	285 $[M +H – OC_2 H_5]^+$	5	30	7
	127 $[M + H– C_8H_{12}O_4S]^+$	50	43	52
	353 $[M + Na]^+$	48	100	60



Dimethoate ($C_5H_{12}NO_3PS_2$)

MW: 229.29

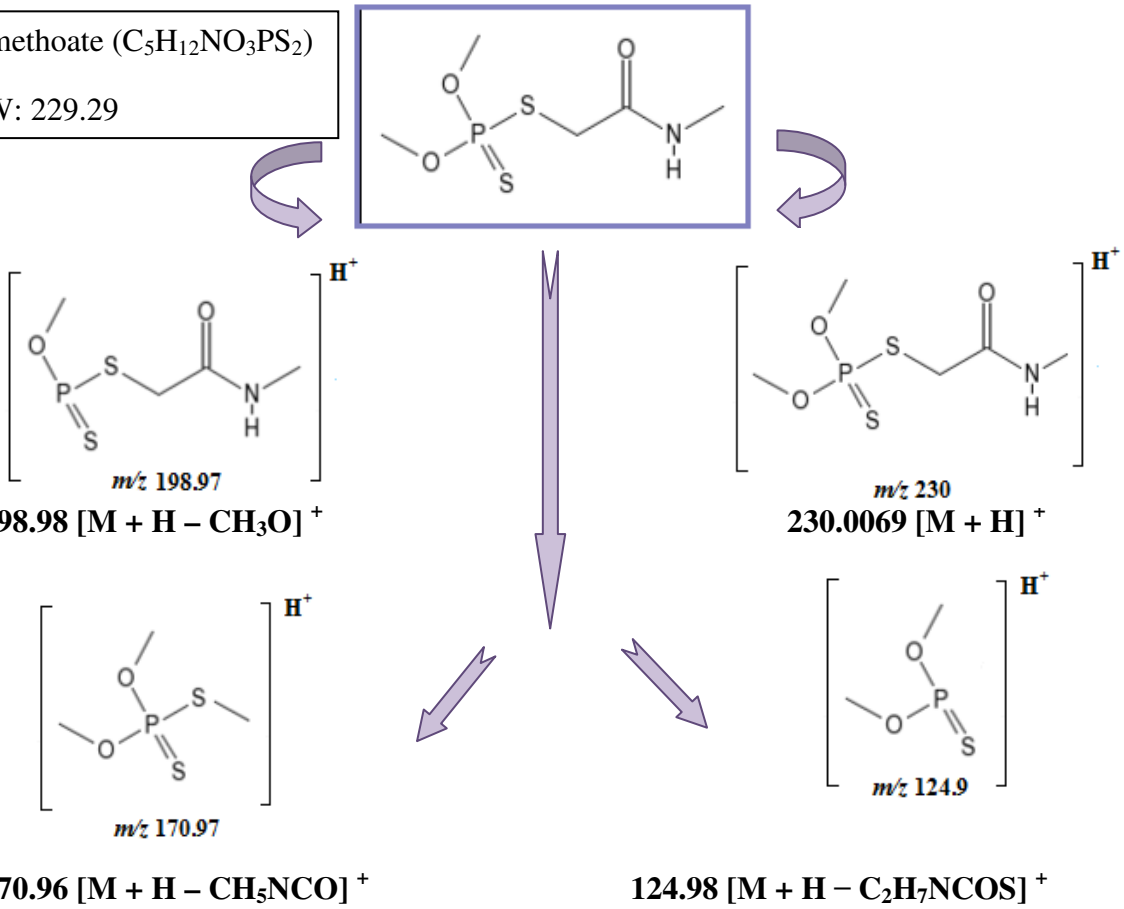


Figure 4.4: The mass spectra and the proposed fragmentation scheme of dimethoate

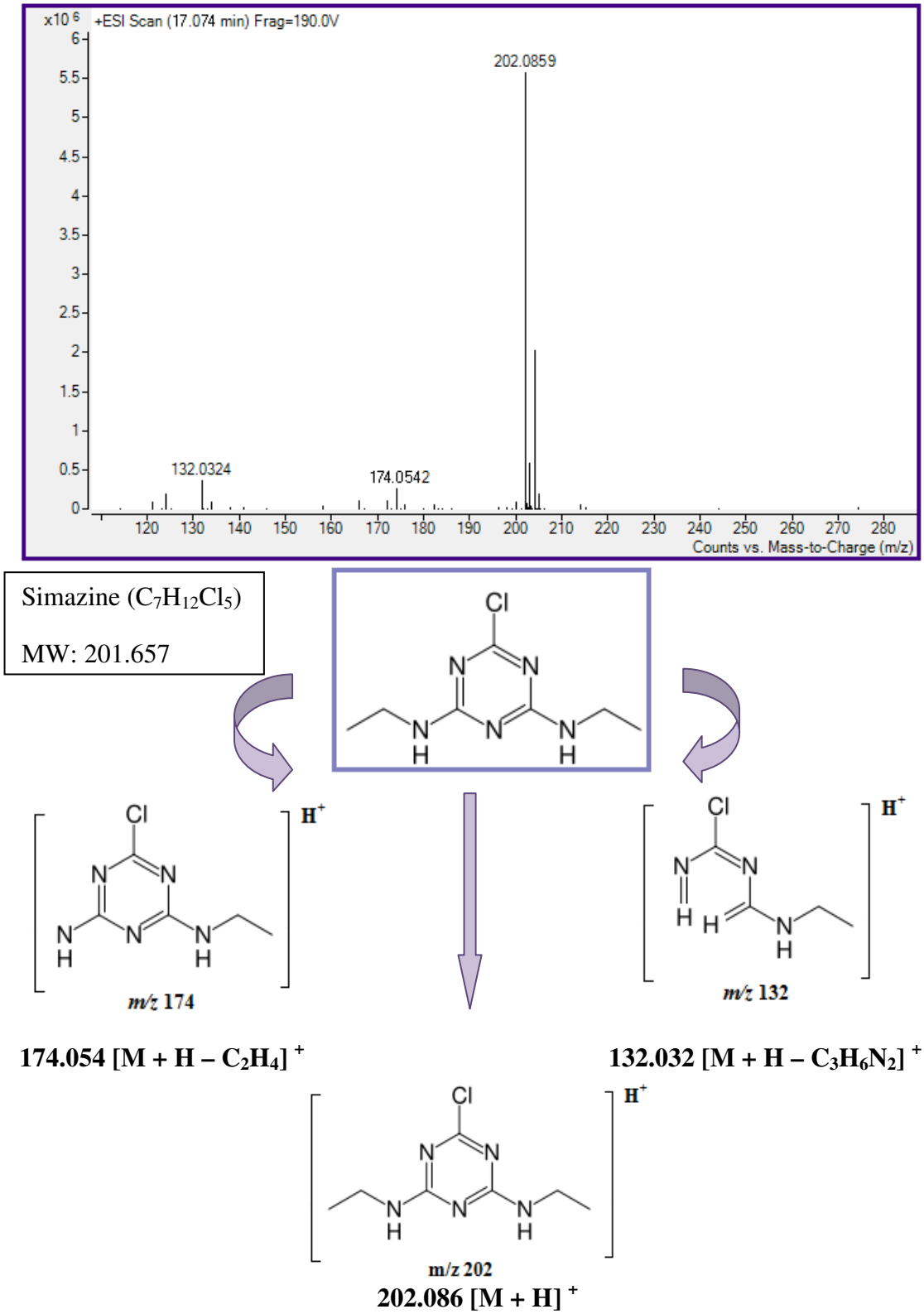


Figure 4.5: The mass spectra and the proposed fragmentation scheme of simazine

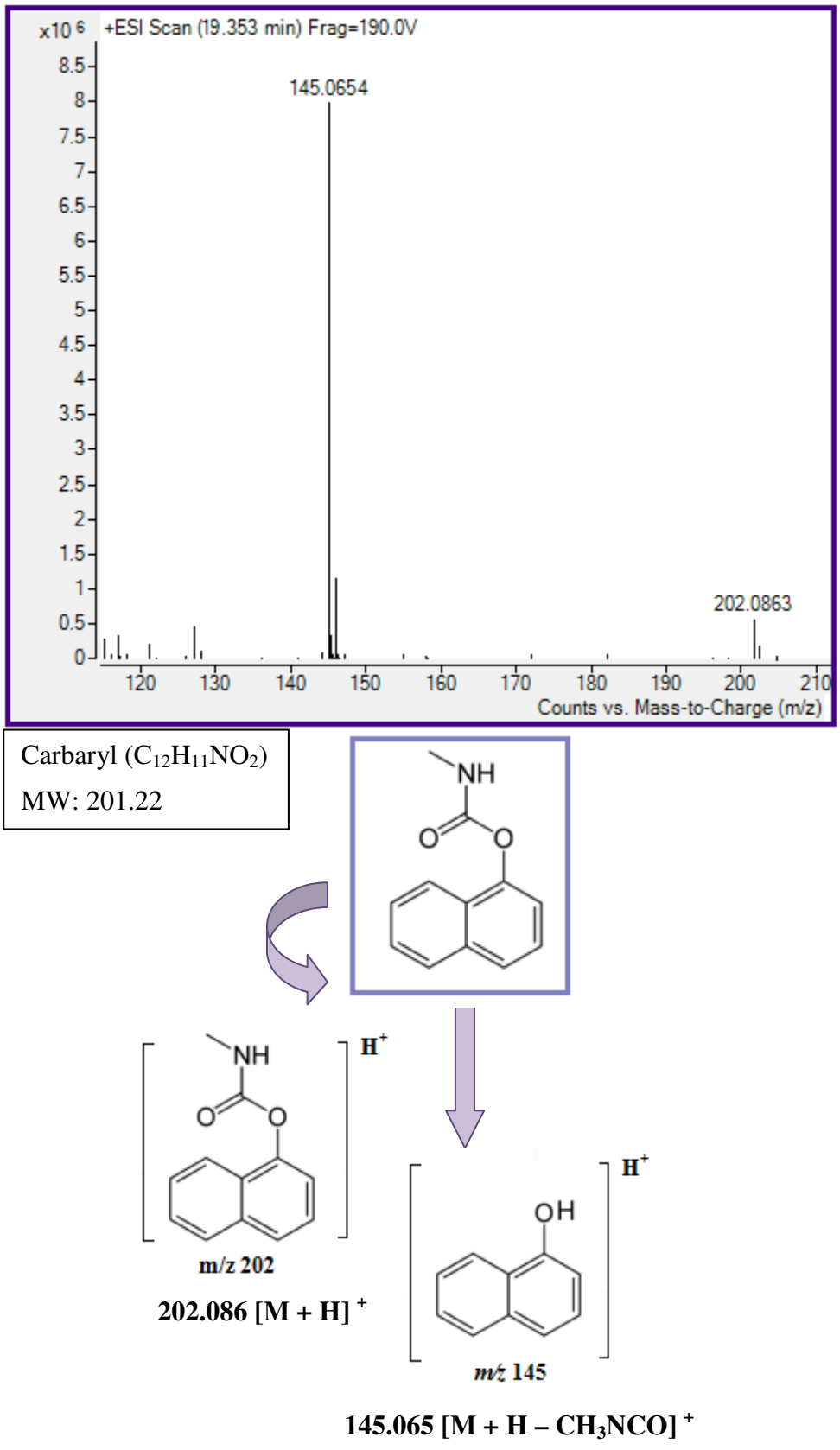


Figure 4.6: The mass spectra and the proposed fragmentation scheme of carbaryl

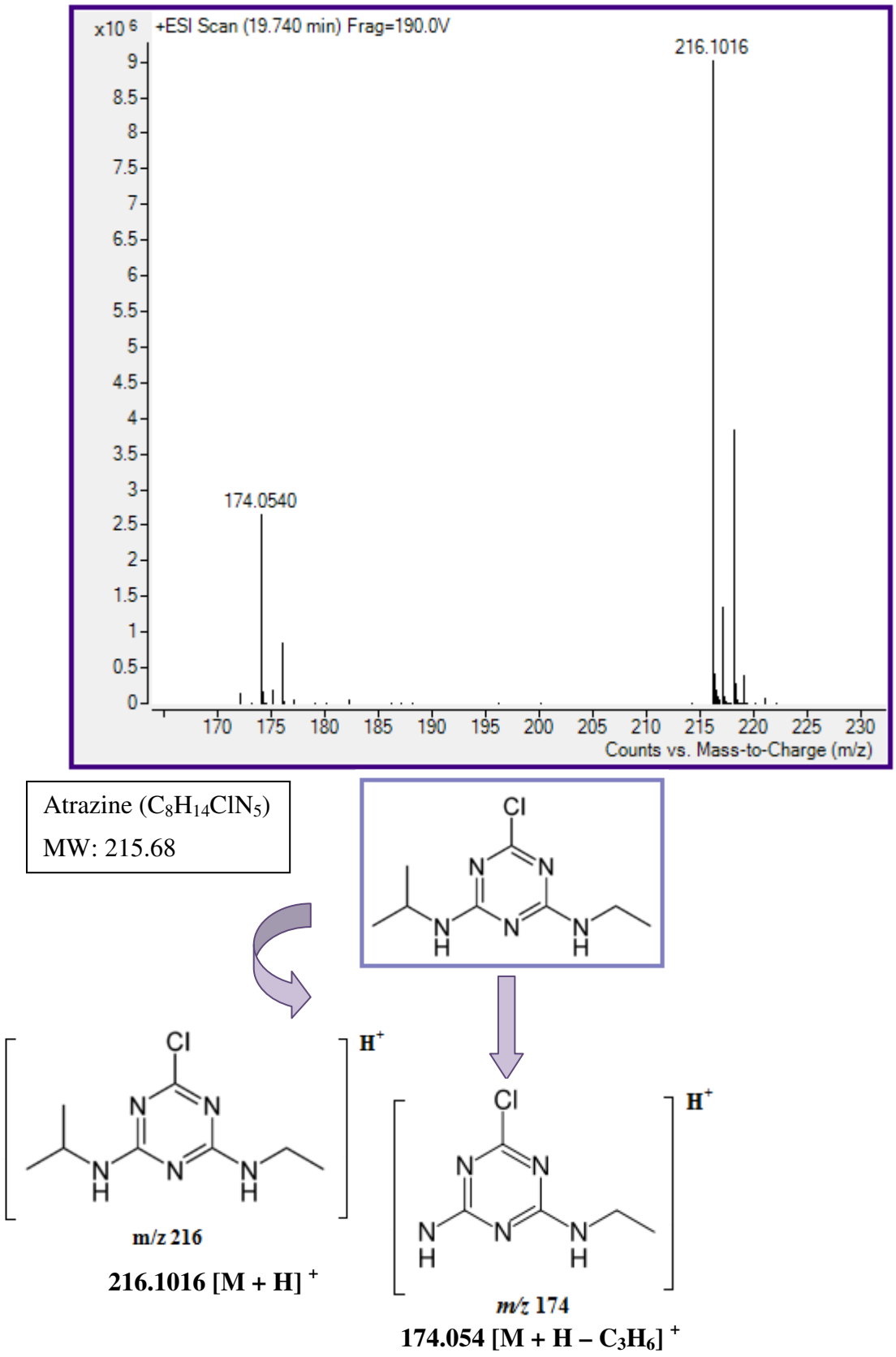


Figure 4.7: The mass spectra and the proposed fragmentation scheme of atrazine

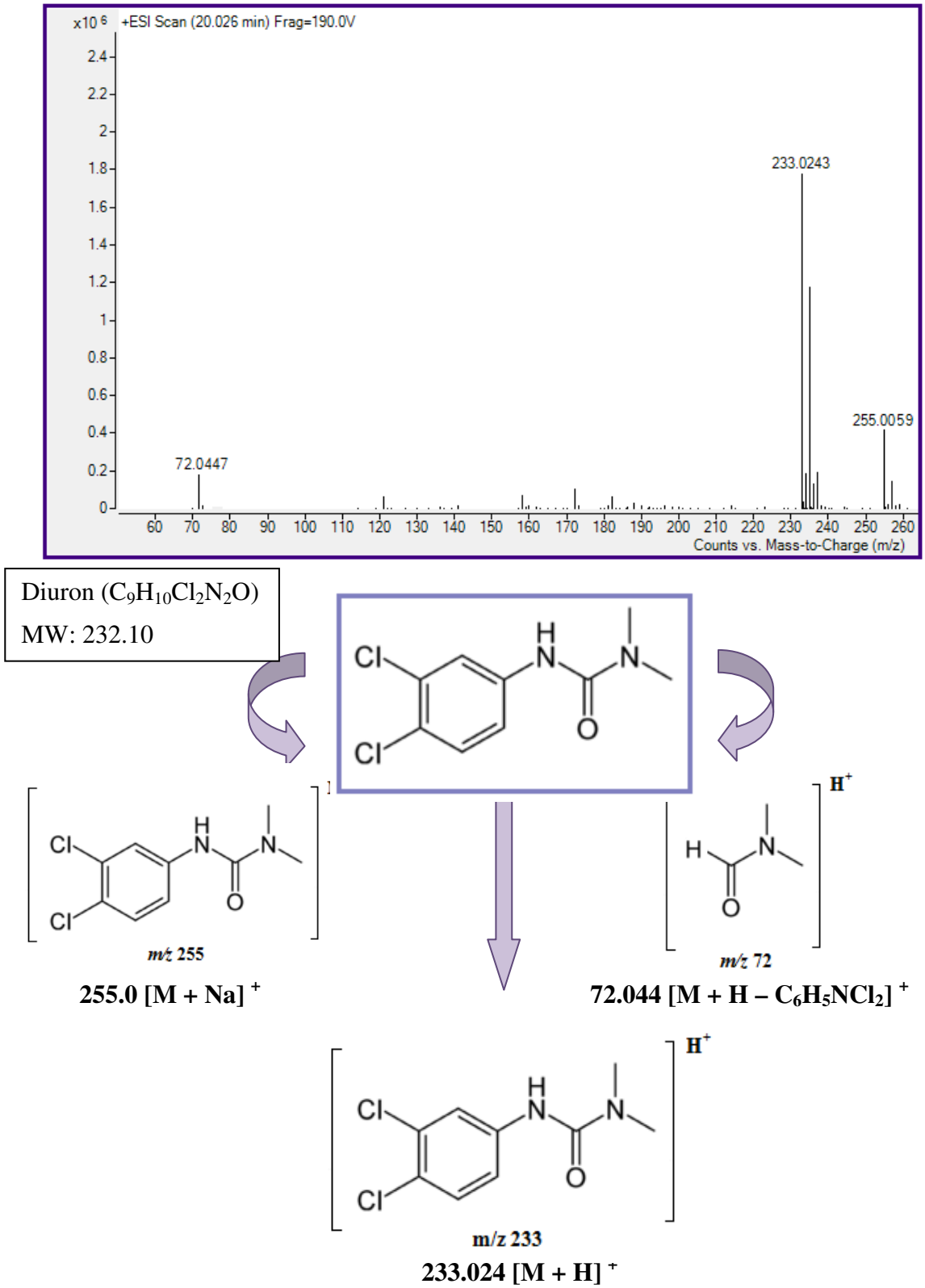


Figure 4.8: The mass spectra and the proposed fragmentation scheme of diuron

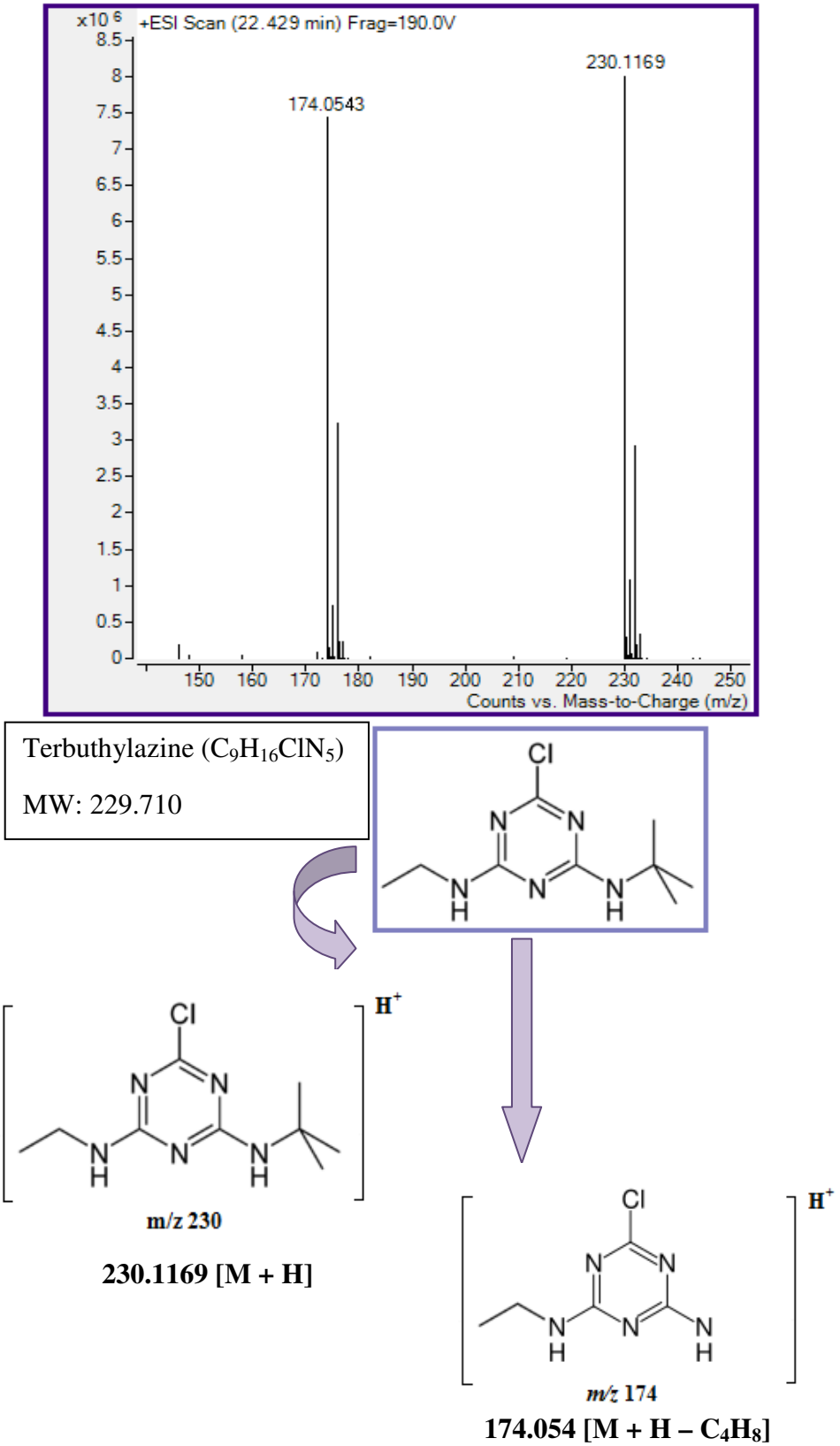
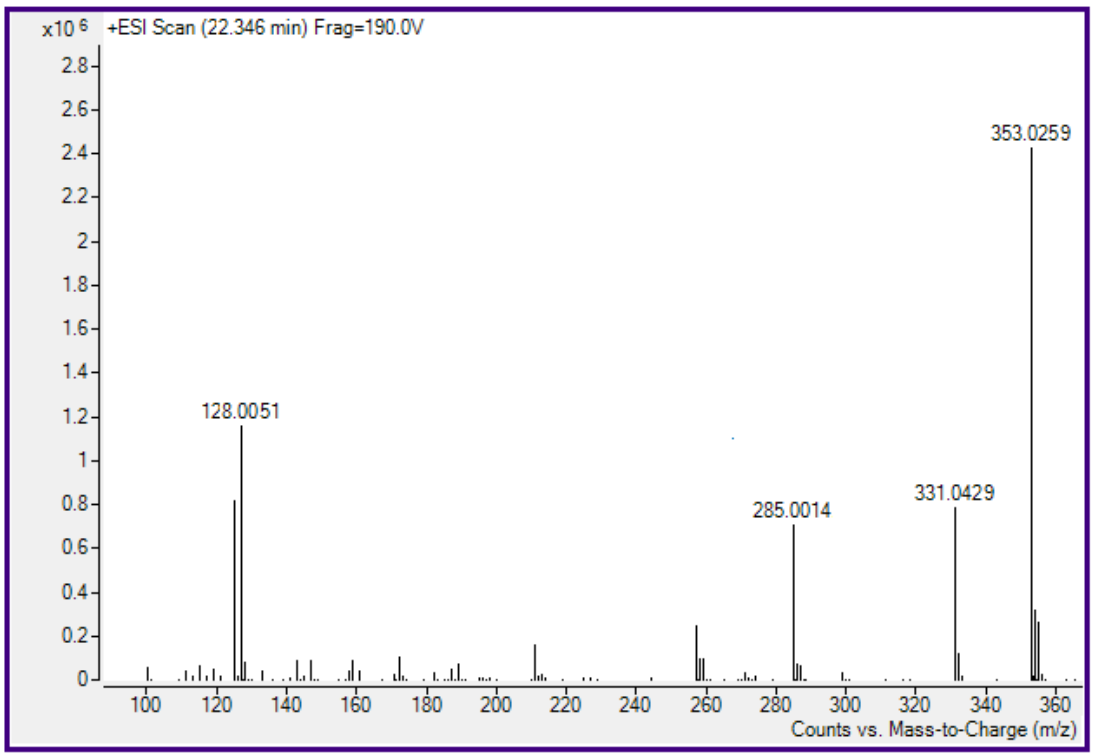


Figure 4.9: The mass spectra and the proposed fragmentation scheme of terbuthylazine.



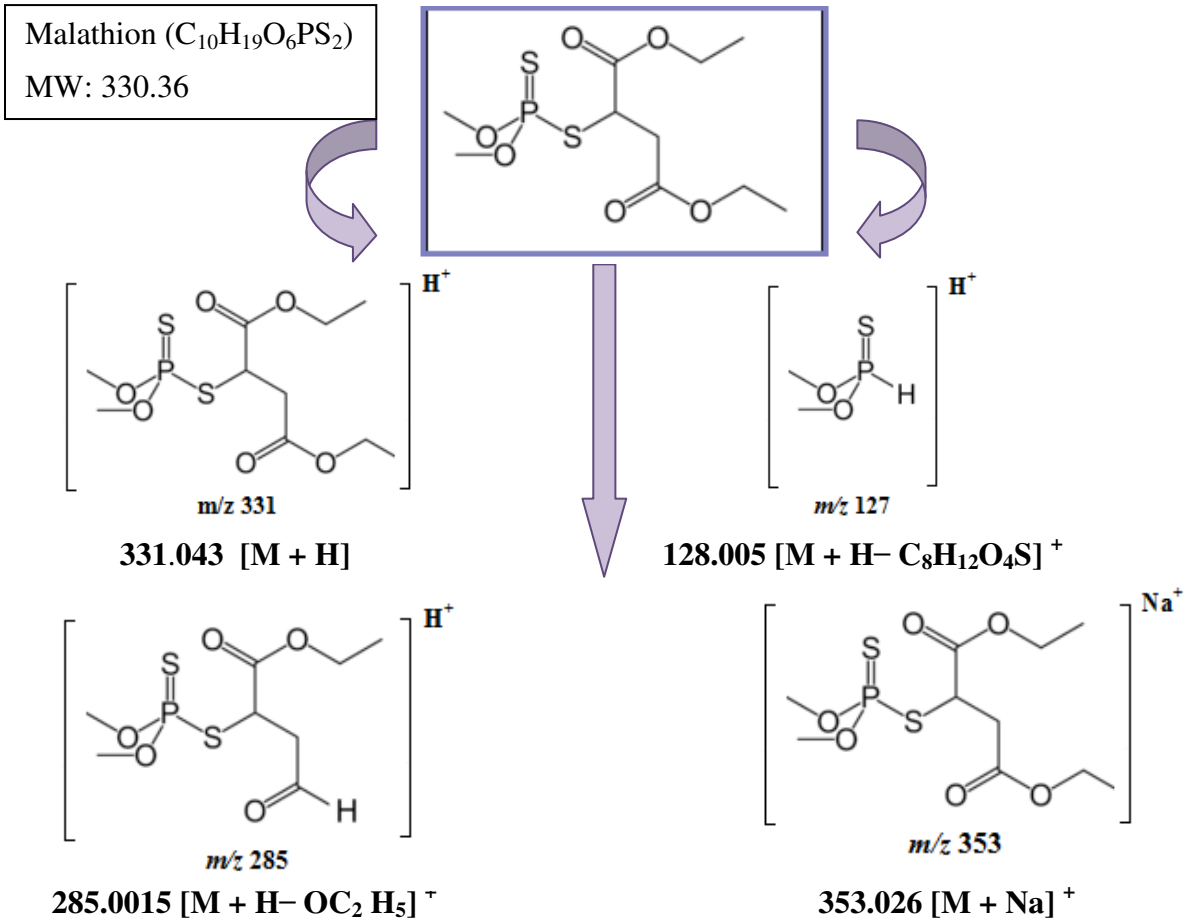


Figure 4.10: The mass spectra and the proposed fragmentation scheme of malathion

In addition, the difference between the accurate mass of the protonated molecule and the fragment ion of dimethoate, carbaryl and malathion at two different fragmentor voltages (160 and 190 V) were shown in Figures 4.11-4.13 respectively.

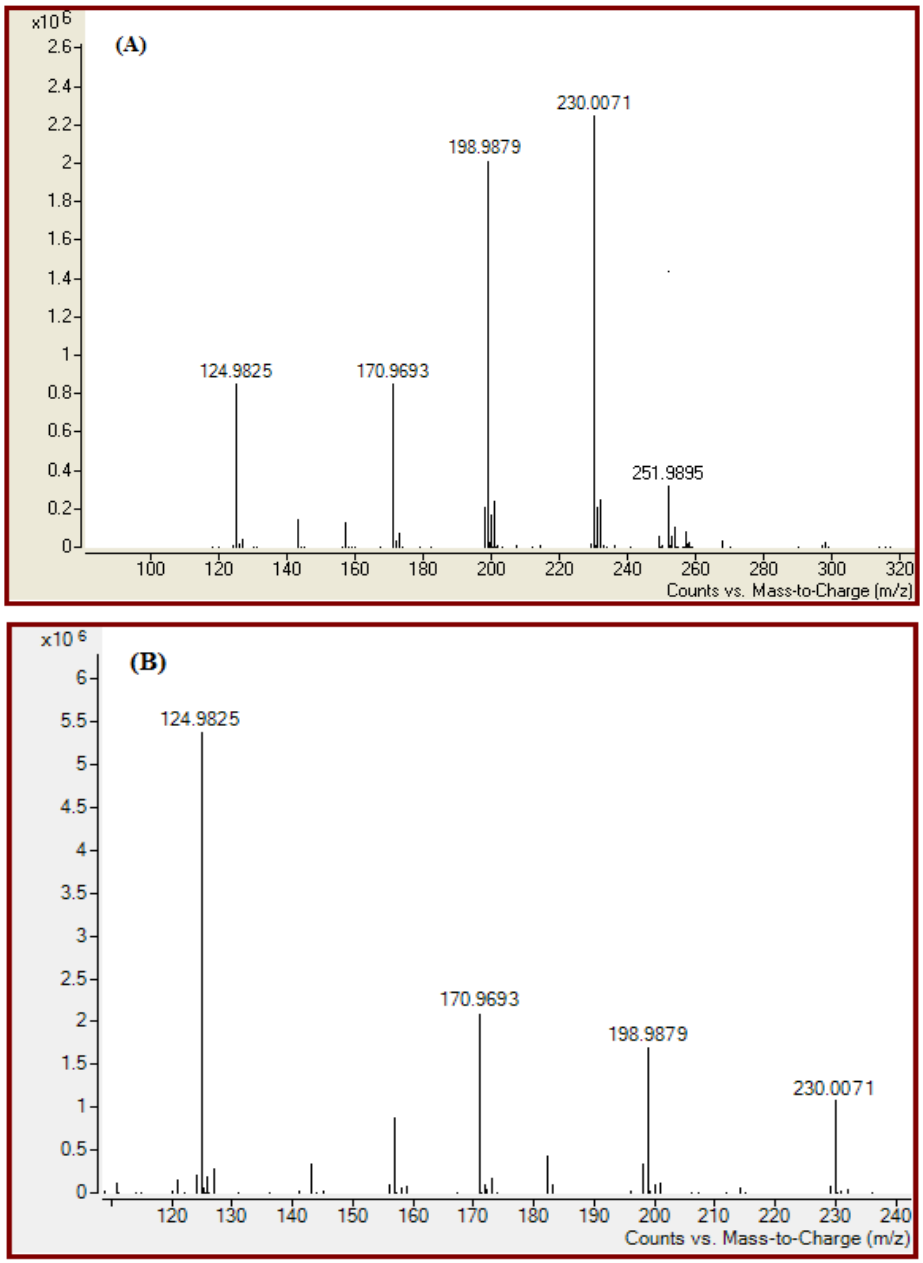


Figure 4.11: LC-TOF-MS accurate mass spectrum of the protonated molecule and fragment ion for dimethoate at fragmentor voltage of: (A) 160 V and (B) 190 V.

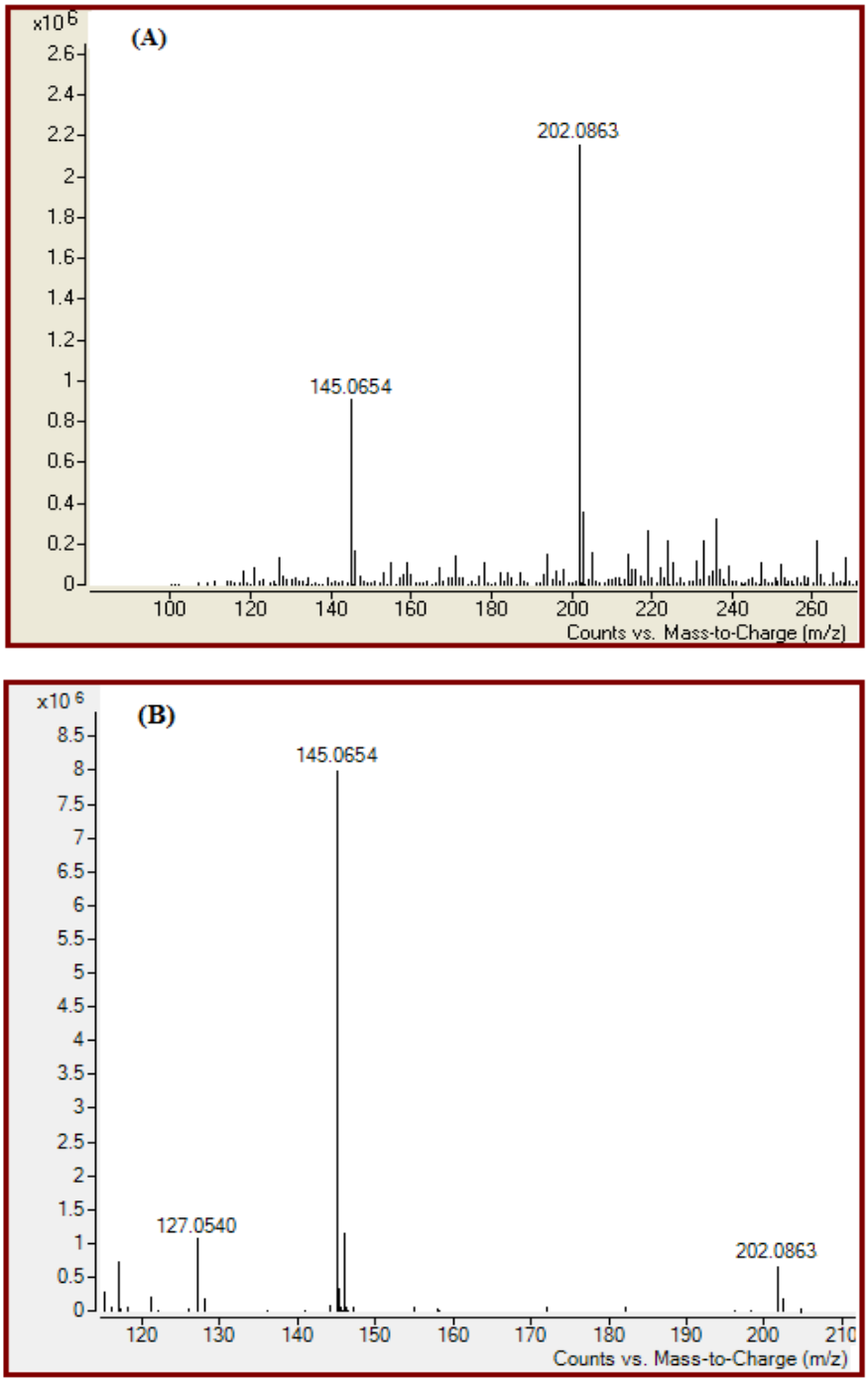


Figure 4.12: LC-QTOF-MS accurate mass spectrum of the protonated molecule and fragment ion for carbaryl at fragmentor voltage of: (A) 160 V and (B) 190 V.

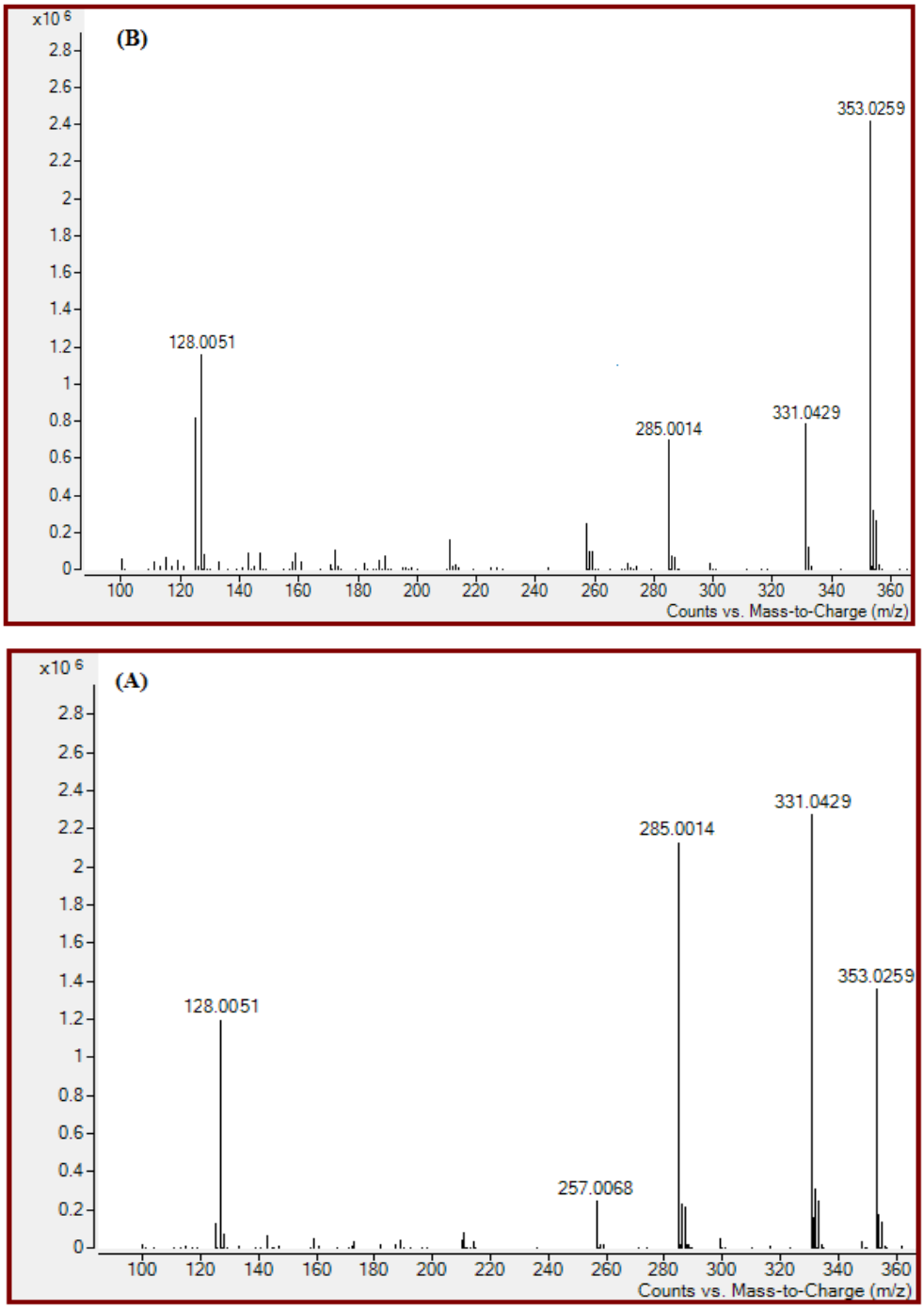
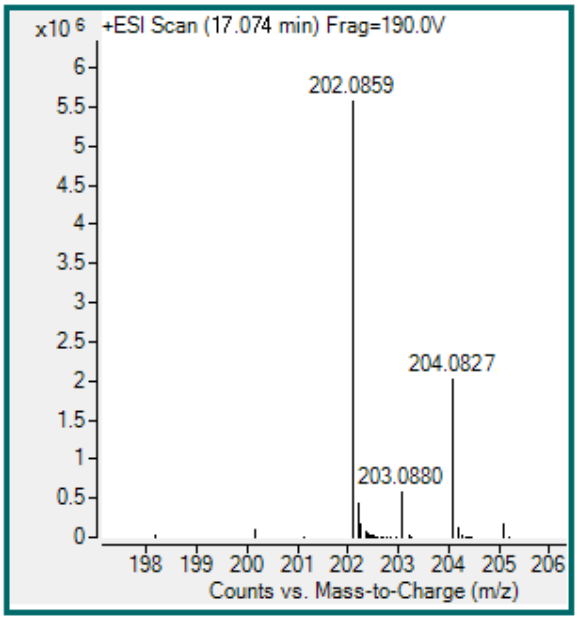


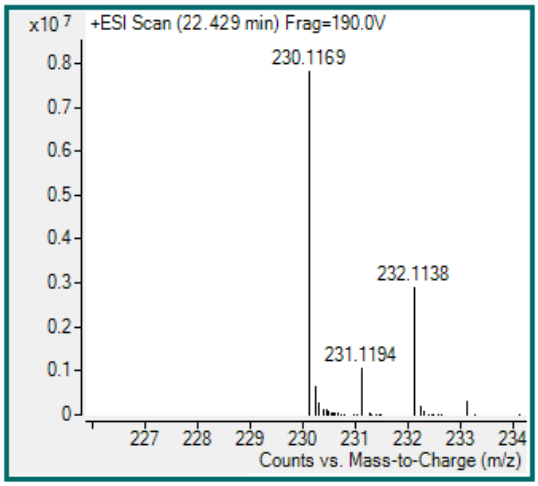
Figure 4.13: LC-QTOF-MS accurate mass spectrum of the protonated molecule and fragment ion for malathion at fragmentor voltage of: (A) 160 V and (B) 190 V.

Among the selected pesticides studied in this work, simazine, terbuthylazine, atrazine and diuron have the special feature which enables the unequivocal identification/confirmation of these chemical species. This is due to the presence of at least one chlorine atom in chemical structures. The signal intensity pattern of the ^{37}Cl isotope signal evidences that the peak contains chlorine atom unequivocally. In addition, the relative abundance of the isotopic signal for ^{37}Cl will suggest whether the compound contains a unique chlorine atom such in the case of simazine, terbuthylazine and atrazine or two atoms as in the case of diuron. Besides the usefulness of the chlorine isotopic profiles in this sense, the accurate mass obtained for the ^{37}Cl isotope, which is one of the characteristic features of time-of-flight when applied to halogen containing pesticides, is useful. Therefore, the accurate mass of each protonated molecule along with the characteristic fragment ion, the corresponding generated elemental compositions, the presence of the chlorine signature, and the characteristic retention time represent enough information to unequivocally identify and confirm members of this class of pesticides in such complicated matrices. In this way, the method based on accurate mass measurements meets the European Commission (EC) criteria for the spectrometric identification and confirmation of organic residues and contaminants based on the use of identification points (IPs) (Thurman *et al*., 2004, Herna´ndez *et al*., 2004, Commision Decision 2002/657/EC 2002).

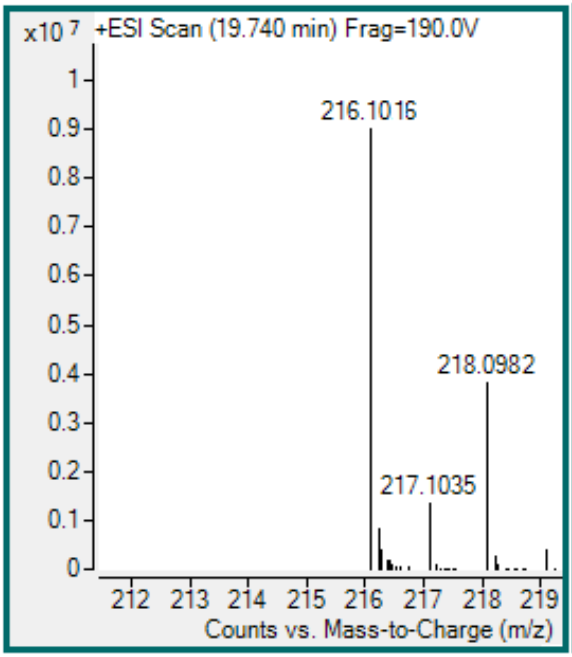
The chlorine isotopic profile and the LC-TOF-MS accurate mass spectrum of the four herbicides (simazine, terbuthylazine, atrazine and diuron) are shown in Figure 4-14.



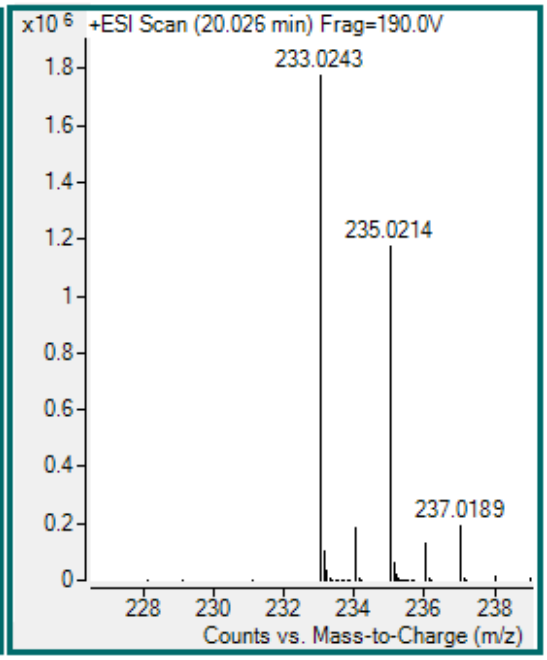
(a) Simazine



(b) Terbuthylazine



(c) Atrazine



(d) Diuron

Figure 4.14: LC-QTOF-MS accurate mass spectra of the protonated molecules for (a) simazine, (b) terbuthylazine, (c) atrazine and (d) diuron.

4.2.2 Accurate mass measurements

In order to achieve accurate mass information of protonated molecules and their characteristic fragment ions with fragmentor voltage of 190 V, matrix-matched standard spiked at 50 ng g^{-1} was used. As a result, more accurate mass information was obtained for both protonated molecules, which consisted of chlorine ^{35}Cl and chlorine ^{37}Cl isotope. Simazine, terbuthylazine and atrazine have one chlorine atom however diuron contains two chlorine atoms, so we can get up three ions and their respective accurate masses in this study, which is much wider information than that obtained from single quad and selected ion monitoring techniques. The results obtained for mass accuracy of the protonated molecules and their fragment ions are summarised in Table 4.2. As can be seen, no significant difference was observed in the mass accuracy obtained in the matrix matched standards when compared with that obtained with standards in pure solvent. Therefore, we can deduce that the accurate mass measurements have the capability for the unequivocal confirmation of these species in oil matrices at different concentration levels.

Table 4.2: LC-QTOF-MS accurate mass measurements for the protonated molecules and the main fragment ions for the pesticides studied in matrix-matched standard.

Analyte	Empirical formula	Theoritical mass	Measured mass	Error	
				mDa	ppm
Dimethoate	$C_5H_{13}NO_3PS_2$	230.0069	230.0071	0.2	0.87
	$C_4H_{10}NO_2PS_2$	198.9885	198.9879	-0.6	-3.01
	$C_3H_7O_2PS_2$	170.9698	170.9693	-0.5	-2.92
	$C_2H_5O_2PS$	124.9821	124.9825	0.4	3.20
	$C_5H_{12}NO_3PS_2Na$	251.9888	251.9894	0.6	2.38
Simazine	$C_7H_{13}N_5{}^{35}Cl$	202.0854	202.0859	0.5	2.47
	$C_7H_{13}N_5{}^{37}Cl$	204.0824	204.0827	0.3	1.47
	$C_5H_9N_5{}^{35}Cl$	174.0541	174.0542	0.1	0.57
	$C_4H_7N_3{}^{35}Cl$	132.0323	132.0324	0.1	0.75
Carbaryl	$C_{12}H_{12}NO_2$	202.0863	202.0863	0.0	-0.1
	C10H9O	145.0648	145.0654	0.6	4.13
Atrazine	$C_8H_{15}N_5{}^{35}Cl$	216.1010	216.1016	0.5	2.32
	$C_8H_{15}N_5{}^{37}Cl$	218.0980	218.0982	0.2	0.92
	$C_5H_9N_5{}^{35}Cl$	174.0541	174.0540	-0.1	-0.57
Diuron	$C_9H_{11}N_2O^{35}Cl_2$	233.0243	233.0243	-0.1	-0.43
	$C_9H_{11}N_2O^{35}Cl^{37}Cl$	235.0213	235.0214	0.1	0.42
	$C_9H_{11}N_2O^{37}Cl_2$	237.0183	237.0189	0.6	2.53
	$C_9H_{10}N_2OCl_2Na$	255.0062	255.0059	-0.3	-1.17
	C_3H_6NO	72.0444	72.0447	0.3	4.16
Terbuthylazine	$C_9H_{17}N_5{}^{35}Cl$	230.1167	230.1169	0.2	0.87
	$C_9H_{17}N_5{}^{37}Cl$	232.1137	232.1138	0.1	0.43
	$C_5H_9N_5{}^{35}Cl$	174.0541	174.0543	0.2	1.15
Malathion	$C_{10}H_{20}O_6PS_2$	331.0433	331.0429	-0.4	-1.21
	$C_{10}H_{19}O_6PS_2Na$	353.0253	353.0259	0.6	1.70
	$C_8H_{15}O_5PS_2$	285.0015	285.0014	-0.1	0.35
	$C_2H_8O_2PS$	128.0055	128.0051	-0.3	-2.34

4.2.3 Retention time

In this study, emphasise on the combination of retention time data, molecular mass information from the $[M+H]^+$ ion and other characteristic fragment ions provide satisfactory information for the identification of target analytes with a low probability of false positive. The percentage of relative standard deviation (RSD %) values of retention times recorded within one week were typically less than 1.90%. Table 4.3 shows the mean retention times and the RSD% (n=10) and also the *m/z* ions of the pesticides studied in pesticide formulations analysis. For qualitative analysis purposes, even one point per peak with spectrum of acceptable quality is sufficient for positive compound identification employing full scan mode experiments with quadrupole mass spectrometer. The typical ion chromatogram of the investigated pesticides obtained in full scan mode is presented in Figure 4.15.

Table 4.3: The mean retention times (t_R) with RSD (%) and *m/z* ions of the selected pesticides (font bold: *m/z* ions selected for quantification and confirmation of pesticides by LC-TOF-MS).

Pesticide	Mean retention time (t_R) (min)	RSD (%)	*m/z*
Dimethoate	13.91	1.13	230.0069, 198.9885,170.9698, **124.9821**
Simazine	17.07	1.02	**202.0854**, 174.0541, 132.0323
Carbaryl	19.38	0.84	202.0863, **145.0648**
Atrazine	19.41	0.61	**216.1010**, 174.0541
Diurone	19.98	1.85	**233.0243**, 255.0062, 72.0444
Terbuthylazine	22.19	0.92	**230.1167**, 174.0541
Malathion	22.23	1.90	331.0433, 285.0015, 128.0055 **353.0253**

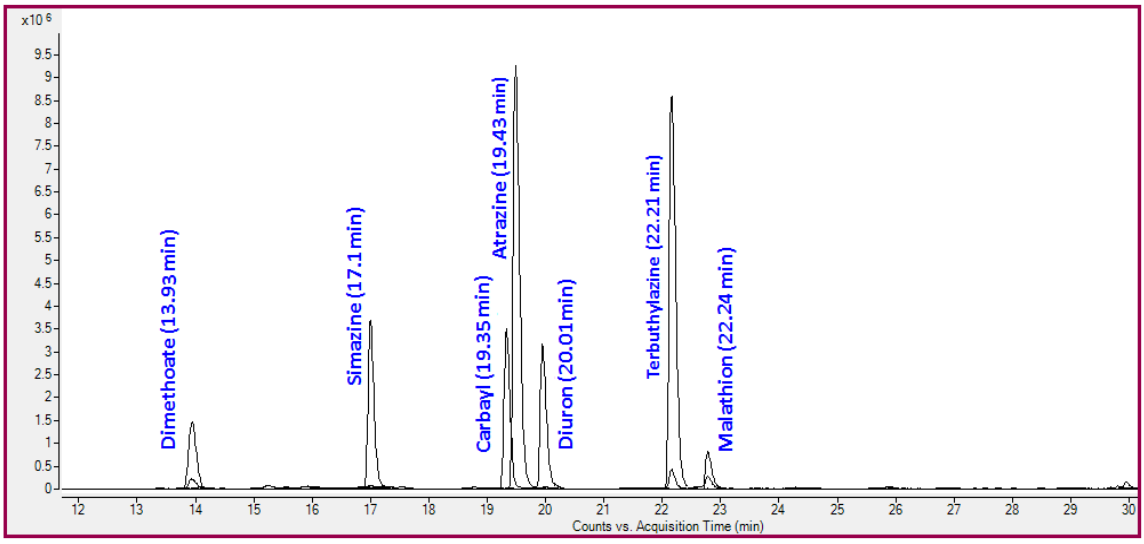


Figure 4.15: Typical chromatogram obtained from the investigated pesticides and the respective retention time (min) as shown in parentheses.

4.3 Optimization of two different sample treatment techniques

4.3.1 Optimization approach of LTP and MSPD procedure

Due to the inherent complexity of the high fatty matrix, the main challenge in developing the clean up method was the separation of co-extracted fatty matrix from the pesticides of interest. In this study, first LLE with LTP was evaluated for multiresidue pesticide analysis in palm oil. The objective of extraction procedure is to remove as much as possible of the analyte from the matrix. For this reason it is decisive to optimize the extraction parameters. First seven different LLE procedures were applied for the isolation of pesticides from palm oil matrix. Then, an efficient LLE procedure was selected and studied in part of with or without centrifugation and LTP then the co-extracted fat was weighed.

4.3.1.1 LLE, centrifugation and LTP studies

LLE_1: 5.00 g of palm oil samples was put in a 15-mL screw-cap conical bottomed glass and then extracted twice (first time with 10 mL and second time with 5 mL) with MeCN.

LLE_2: 5.00 g of palm oil samples was mixed and dissolved in 5 mL of petroleum ether saturated with acetonitrile in a 15-mL screw-cap conical bottomed glass. The mixture was extracted twice with MeCN saturated with petroleum ether.

LLE_3: 5.00 g of palm oil samples was mixed and dissolved in 5 mL of petroleum ether saturated with acetonitrile in a 15-mL screw-cap conical bottomed glass. The mixture was then extracted twice with MeCN.

LLE_4: 5.00 g of palm oil samples was mixed and dissolved in 5 mL of n-hexane saturated with acetonitrile in a 15-mL screw-cap conical bottomed glass. The mixture was then extracted twice with MeCN.

LLE5: 5.00 g of palm oil samples was mixed and dissolved in 5 mL of n-hexane in a 15-mL screw-cap conical bottomed glass. The mixture was then extracted twice (5 mL for each time) with MeCN.

LLE_6: 5.00 g of palm oil samples was put in a 15-mL screw-cap conical bottomed glass and then the mixture was extracted twice with MeCN- n-hexane (3:1).

LLE_7: 5.00 g of palm oil samples was mixed and dissolved in 5 mL of n-hexane in a 15-mL screw-cap conical bottomed glass. The mixture was then extracted twice with acetone.

4.3.1.1.1 Extraction without centrifugation

10 mL of solvent was added to 5.00 g sample in a 15-mL screw-cap conical bottomed glass and shaken with a vortex mixer for 10 min and then the mixture was allowed to stand for equilibration. The upper layer was transferred to another container and the extraction repeated with other 5 mL solvent. After combination of the extracted solvents, they were evaporated to dryness and then the co-extracted fat was weighed.

4.3.1.1.2 Extraction with centrifugation

10 mL of solvent was added to 5.00 g sample in a 15-mL screw-cap conical bottomed glass and agitated with a vortex mixer for 10 min and then centrifuged for

3 min using a Kubota-2420 apparatus at 3700 rpm. The upper layer was taken to other container and the extraction was repeated with other 5 mL solvent. After combination of the extracted solvents, they were evaporated to dryness and then the co-extracted fat was weighed.

4.3.1.1.3 Low temperature precipitation (LTP)

Low temperature precipitation method was performed to decrease the amount of fatty co-extractants with limited solubility in cold acetonitrile (Lentza-Rizos *et al*., 2001; Li *et al.,* 2007; Goulart *et al*., 2008; Chen *et al*., 2009).

The similar experiment of extraction with centrifugation was performed as explained in part 4.3.1.1.2. Then, the extracted solvent was horizontally kept in a freezer at -20 °C for 2 h. The cold extract was immediately centrifuged for 2 min at 3700 rpm. The organic phase containing the organic solvent and extracted pesticides remained as a liquid and rose to the top whereas the oil were frozen and precipitated to the bottom of the tube. The extracts were evaporated to dryness under stream of nitrogen gas for quantifying the remaining fat.

The mean values (n = 3) of the fat residues in the extract expressed as mg g^{-1} of palm oil extracted without centrifugation was found to be 13.63 ± 2.75 for LLE_1, 12.93 ± 1.83 for LLE_2, 14.85 ± 3.11 for LLE_3, 12.03 ± 2.05 for LLE_4, 11.77 ± 1.25 for LLE_5, 42.15 ± 10.64 for LLE_6 and 24.07 ± 8.14 for LLE_7.

The remaining fat after LLE with centrifugation was found to be 4.96 ± 1.02 for LLE_1, 4.89 ± 0.72 for LLE_2, 5.02 ± 0.81 for LLE_3, 4.72 ± 0.64 for LLE_4, 4.08 ± 0.37 for LLE_5, 12.21 ± 3.40 for LLE_6 and 10.54 ± 2.43 for LLE_7, respectively.

The remaining fat in the extracts after centrifugation and freezing step was found to be 0.92 ± 0.084 for LLE_1, 0.71 ± 0.062 for LLE_2, 0.85 ± 0.070 for LLE_3, 0.77 ± 0.064 for LLE_4, 0.66 ± 0.058 for LLE_5, 3.18 ± 0.56 for LLE_6 and 2.37 ± 0.32 for LLE_7, respectively.

Considering the obtained results in Figure 4.16, the mass of co-extracted fat after LLE with centrifugation was reduced to 63.6, 62.18, 66.2, 60.8, 65.33, 71.03, and 56.21% for LLE_1, LLE_2, LLE_3, LLE_4, LLE_5, LLE_6 and LLE_7 respectively. LLE with centrifugation was able to remove more than 63% of lipid from the matrix except LLE_7. On the other hands after LLE with centrifugation in combination with freezing procedure, the mass of the remaining fat in comparison with the mass of remaining fat after only LLE with centrifugation was reduced by 81.4, 85.5, 83.07, 83.6, 83.8, 73.9, 77.5% for LLE_1, LLE_2, LLE3, LLE_4, LLE_5, LLE_6 and LLE_7, respectively (see Figure 4.17). The results revealed that, freezing-out procedure was able to remove up to 94% of lipid from the matrix. Among LLE_1, LLE_2, LLE_3, LLE_4 and LLE_5, the latest was selected for further experiments due to its easier preparation and as well as good RSD. LLE_6 and LLE_7 were not considered due to its high mass of co-extracted fat.

In addition the ratio of the volume of acetonitrile to that of the sample was tested (2:1, 3:1, 4:1). The results also revealed that the best recoveries were obtained by using acetonitrile: palm oil matrix at a ratio of 3:1 (15 mL MeCN and 5 g palm oil), and any further increased of the volume of acetonitrile did not improve the recovery of the pesticides studied. In evaluation of freezing time, different times in the range of 2-24 h were tested. The minimum time for satisfactory fat removal during low temperature precipitation was found to be 2 h. There was no significant difference in

pesticides recovery with an increase in freezing time after 2 h, indicating the method is robust. However the freezing time less than 2 h was not sufficient to remove the fat completely.

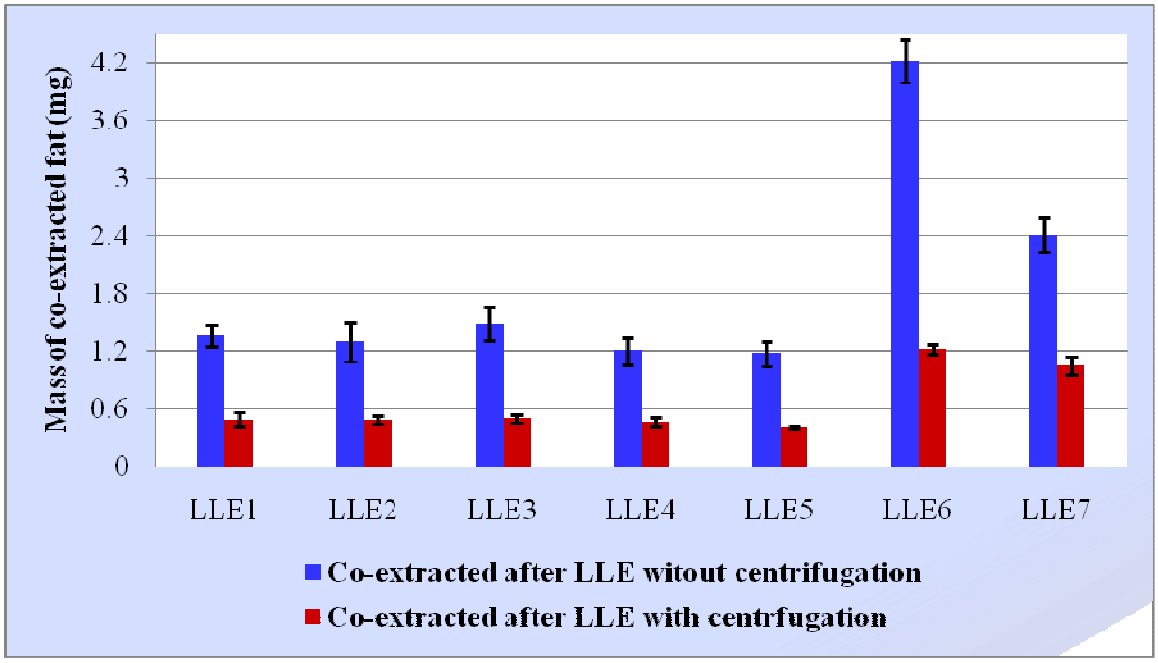


Figure 4.16: Effect of centrifugation on removal of co-extracted fat in LLE with different condition of solvent extraction.

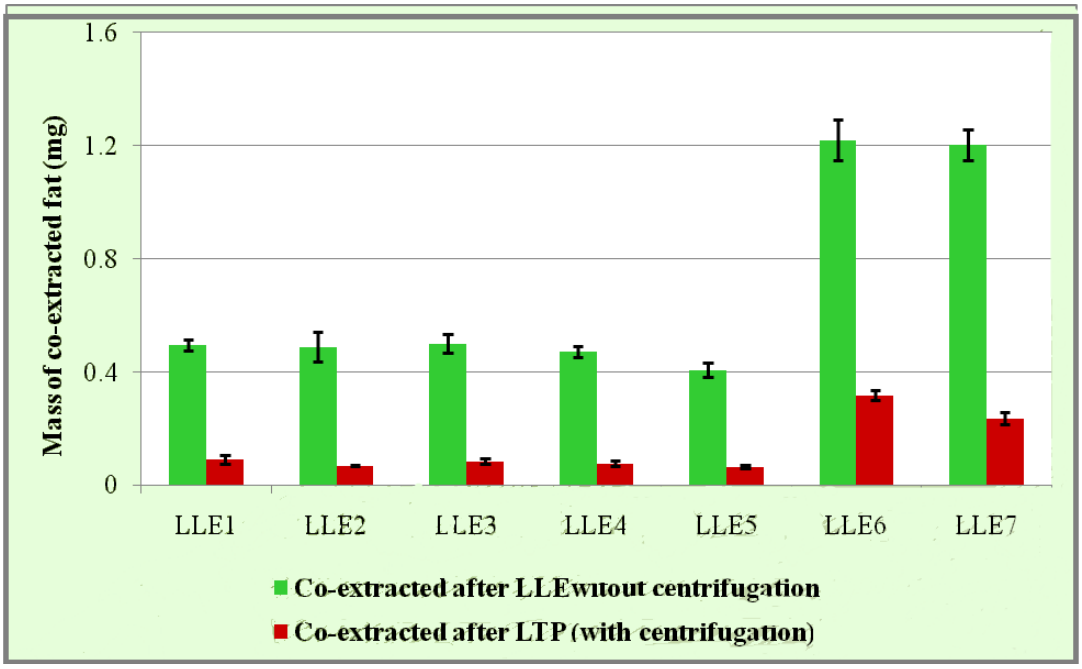


Figure 4.17: Effect of freezing out on removal of co-extracted fat in LLE with different condition of solvent extraction.

4.3.1.2 Optimization of conditions for UA-MSPD clean-up procedure

In addition to the extraction efficiency of residues from the matrix, the performance characteristic of a clean-up procedure is closely related to the generated data. The concentrated sample extracts prior to clean-up may contain a high content of co-extractants which can damage the LC column, as well as resulting in a matrix enhancement effect. Moreover, the co-extractants would accumulate in the injector and at the front end of the column which might increase the retention time of certain analytes. Therefore, the clean-up procedure is so important to remove co-extracted compounds that might interfere in the chromatographic determination or to be detrimental to the analytical instrumentation.

The operating conditions for the matrix solid-phase dispersion procedure were evaluated in order to achieve the highest recoveries of selected pesticides from oil

sample. Types and quantity of sorbents, and nature and volume of the eluting solvent are known to be key factors in MSPD, since they determine both the efficiency of the extraction and the purity of the final extracts.

4.3.1.2.1 Selection of MSPD sorbents

The selection of an appropriate MSPD sorbents is essential for the establishment of the MSPD method and it is dependent on the chemical nature of the target analytes. In this study, first the effects of the sorbent on the pesticide recoveries were evaluated, because it not only acted as adsorption separation material but also as a blending of solid support to disrupt and disperse of oil extract. In this case, two different dispersant sorbents, C_{18} and PSA were used while three clean up adsorbents namely alumina, florisil and graphitized carbon black (GCB) were tested. MeCN was used as the eluting solvent in order to find the most suitable sorbent material with higher recoveries and lower fat levels transferred in the final extracts. The preliminary assays were performed without sonication-assisted extraction. In this case, the palm oil samples were spiked at 50 ng g^{-1} and used for all experimental work involving optimization purposes. The extracts were analysed triplicate and injected three times (n = 9) during the analysis.

In evaluating the type of dispersing sorbent, the extraction column was prepared using PSA which was a weak anion exchanger sorbent with polar capability and 5 g of palm oil blend which was packed with florisil as a clean up sorbent, producing the colorless extract with minimal interferences. The obtained results were satisfactory for all pesticides studied as shown in Figure 4.18. The use of C_{18} as a solid support in MSPD extraction produced extracts with maximal interferences for most of the

pesticides studied. As can be seen from the obtained results, simultaneous extraction of all the studied compounds was unsuccessful when alumina was packed in the base of reversed-phase materials (C_{18}): palm oil blend. In these assays, the occurrence of a high recovery of pesticides could be attributed to the matrix effects and the presence of interfering endogenous compounds that enhance the chromatographic response to pesticides, indicating that perhaps this clean-up sorbent receives and retains more interferences which are subsequently desorbed from the column during the elution. The extract obtained from the MSPD column including a mixture of PSA / palm oil blend and alumina as the clean-up sorbent resulted in a chromatogram with higher background and interfering peaks from the palm oil. In this case, the obtained chromatogram had a large number of other peaks because of co-extracted interfering substance that resulted in high recovery of some pesticides. The extraction of pesticides was ineffective with average recovery of 44.2% when florisil was packed in the base of reversed-phase materials (C_{18}): palm oil blend. The extraction column prepared with PSA/ palm oil blend and GCB as a clean up sorbent, resulted the lower recovery for diuron and carbaryl. GCB has a strong affinity for planar molecules, and thus effectively removes pigments such as chlorophyll and carotenoids, as well as sterols present in foods (Anastassiades *et al*., 2003). The extraction column prepared with PSA/ palm oil blend and Florisil as clean up sorbent, produced clean extract and better chromatogram consequently obtained recoveries above 92.3% for all studied compounds except dimethoate that showed lower recovery (81.2%). Florisil is magnesium silicate containing 15% MgO and 85% SiO_2 that has often been used for the clean-up of apolar to semi-polar pesticides in fatty matrices due to its potential to retain polar matrix components such as lipids

(Kristenson *et al*., 2006). For this reason, elution was well performed and the polar fatty matrix components would not be co-extracted. Therefore, PSA and florisil were used as the dispersant and clean-up sorbent respectively.

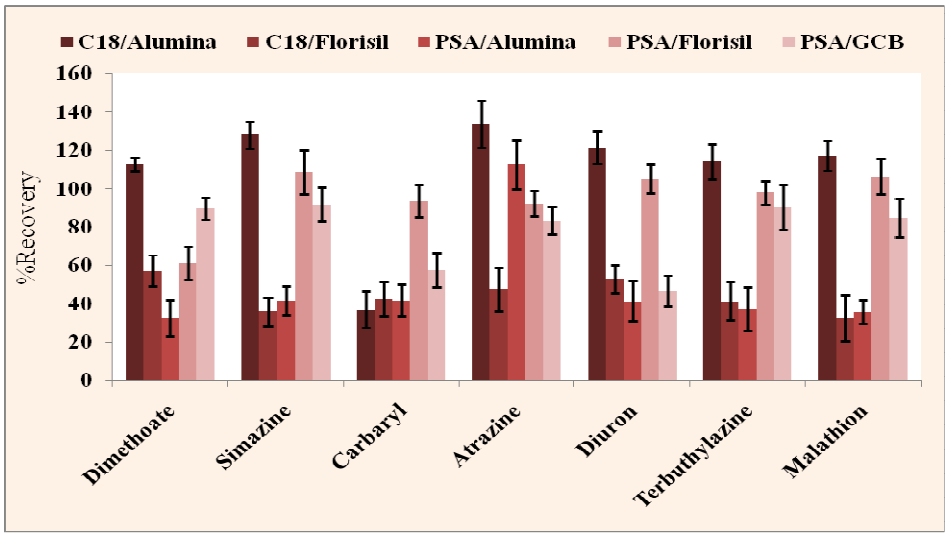


Figure 4.18: Mean percent recovery (50 ng g^{-1}) ± RSD (%) (n = 9) of selected pesticides in palm oil samples with different dispersing/clean-up sorbents.

4.3.1.2.2 Effect of MSPD sorbents mass

For further optimization, the PSA dispersant mass and florisil clean-up sorbent mass were varied to assess optimal conditions. In this study, four different masses of PSA (250, 500, and 750 mg) along with two different masses of florisil (100 and 250 mg) were evaluated to select the most appropriate mass for the method. The amount of sorbent used was found to have considerable influence; thus, the peak areas obtained and consequently the mean recovery obtained with 500 mg of PSA and 250 mg of florisil were higher than those provided by 250 mg of PSA and 100 mg of florisil. However, 750 mg of PSA and 250 mg florisil resulted in

chromatograms with fewer interferences and higher recovery. This study also revealed that the best results were obtained by using PSA/florisil at ratio 3:1 w/w and any further increased of the mass of florisil did not significantly improve the recovery of the pesticides studied as shown in Table 4.4.

Typical chromatogram obtained of spiked palm oil after UA-MSPD using different amounts of PSA / florisil are shown in Figure 4.19.

Overall results indicated that the best results were obtained using 5 g of palm oil, 750 mg of PSA as the dispersion phase, 250 mg of florisil as the clean-up sorbent and acetonitrile as the eluting solvent.

The extraction conditions in terms of with and without sonication were also evaluated. As has been shown in Table 4.4, a series of experiments were designed at three volumes of acetonitrile (5, 15, and 25 mL). As a result, 15 mL of acetonitrile and 15 min sonication assisted extraction in small column containing PSA and Florisil at ratio of 3:1, providing the cleanest extracts for all analytes of interest extracted from the palm oil matrix and they were recovered quantitatively with good reproducibility. According to these results, the elution of pesticides using 5-15 mL of acetonitrile showed a response enhancement ranging from 51.4% to 94.7%. No significant increase in the recoveries was observed when the volume of elution solvent increased.

Table 4.4: Influence of sonication assisted (UA) coupled with MSPD procedure on the pesticides recovery using different volume of MeCN as eluting solvent.

Compound	Recovery (%) (mean ± RSD)			
	PSA/Florisil (3:1 w/w)			
	Without sonication	With sonication		
		5	15	25 (mL)
Dimethoate	58.4 ± 9.6	41.3 ± 7.4	84.4 ± 6.4	85.1 ± 10.3
Simazine	87.3 ± 10.1	57.8 ± 9.4	94.7 ± 4.8	92.6 ± 8.4
Carbaryl	75.2 ± 6.2	51.4 ± 8.3	85.1 ± 10.2	87.5 ± 11.2
Atrazine	90.3 ± 12.1	54.1 ± 11.8	93.1 ± 7.5	92.4 ± 7.3
Terbuthylazine	89.6 ± 7.6	65.2 ± 6.7	87.6 ± 11.5	86.1 ± 9.5
Diuron	90.1 ± 6.9	61.8 ± 6.4	96.2 ± 9.3	91.5 ± 6.2
Malathion	84.7 ± 7.1	65.7 ± 12.5	94.3 ± 8.1	85.8 ± 7.6

MSPD column: 5.0 g palm oil + 0.75 g dispersant + 0.25 g clean up sorbent
Elution sovent: MeCN

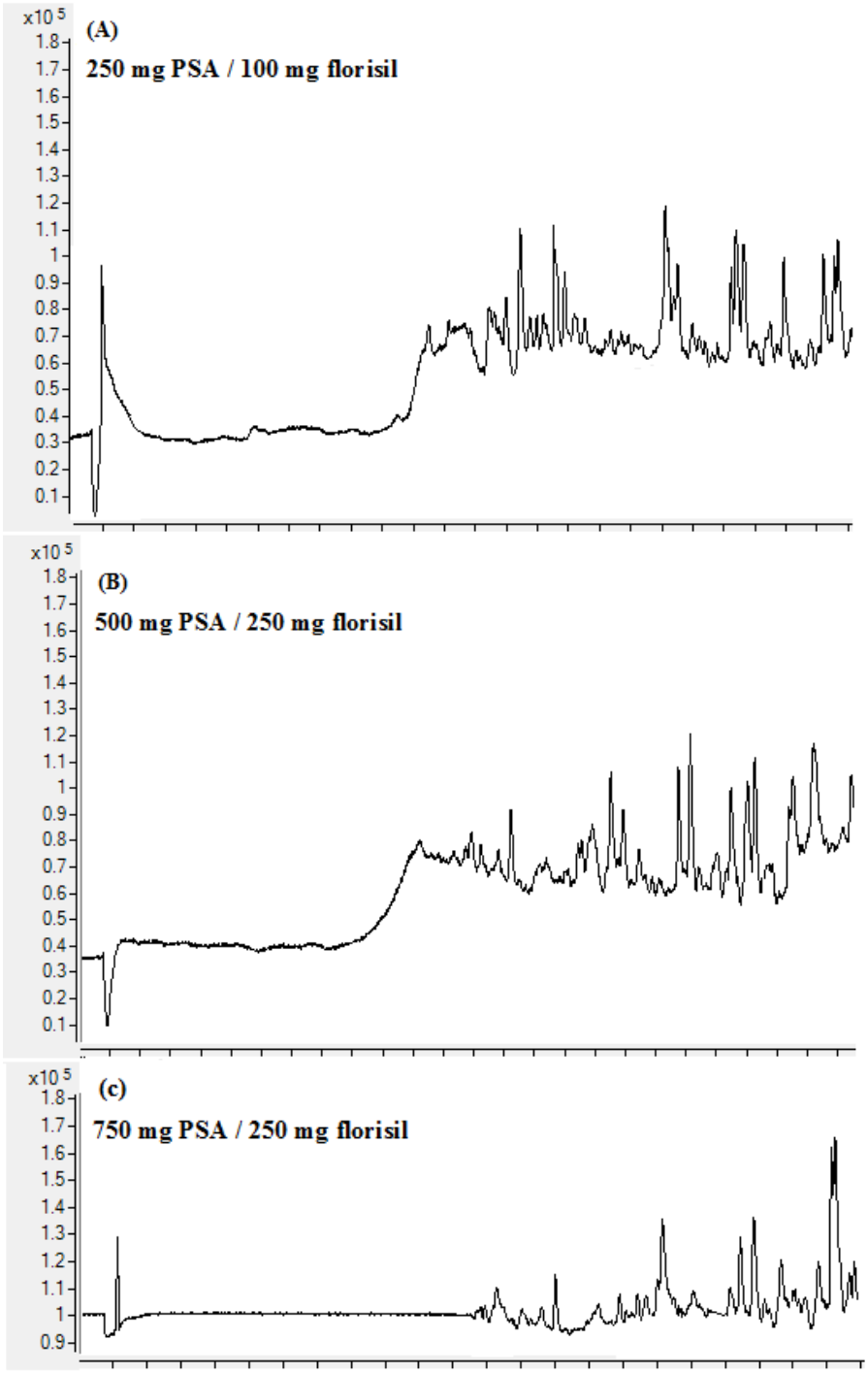


Figure 4.19: Chromatogram obtained of spiked palm oil after UA-MSPD using different amounts of PSA / florisil for pesticides-free oil samples (A) 250 mg PSA + 100 mg florisil, (B) 500 mg PSA + 250 mg florisil, (C) 750 mg PSA + 250 mg florisil

4.3.1.3 Analytical performance

4.3.1.3.1 Recovery studies

To evaluate the effectiveness of the extraction method, different recovery studies were carried out. In this case, untreated oil samples were spiked with appropriate volumes of composite working standard solution at three different concentration levels: 25, 50, and 100 ng g^{-1}. Six replicates were carried out at each spiking level to determine the mean recovery (%) and relative standard deviation (RSD %). Most values of the relative standard deviations of the analysed samples (n = 6) were in general less than 10% that could be attributed to the experimental error. The obtained results for mean recoveries (%), RSD (%) and relative error (%) of all investigated compounds at three concentration levels are shown in Table 4.5. For all compounds in all samples, the mean recoveries lie within an acceptable range from 68.5 to 109.4% with relative standard deviation values from 5.4% to 14.2% for palm oil and from 71.8 to 112.4% with relative standard deviation values between 6.2% and 13.1% for olive oil, respectively.

Table 4.5: Mean percent recovery ± RSD (%) obtained by UA-MSPD procedure of the spiked palm oil[a] and olive oil[b] sample for the pesticides studied.

Pesticide	Mean recovery ± RSD (%)[a] Concentration level (ng g^{-1})			Mean recovery ± RSD (%)[b] Concentration level (ng g^{-1})		
	25	50	100	25	50	100
Dimethoate	68.5 ± 8.9	82.2 ± 10.3	80.7 ± 7.1	71.8 ± 6.5	84.4 ± 9.7	87.8 ± 11.2
Simazine	83.4 ± 9.3	104.6 ± 7.7	91.1 ± 5.8	89.6 ± 7.1	107.6 ± 9.8	110.5 ± 8.9
Carbaryl	77.2 ± 13.3	89.1 ± 12.0	93.4 ± 8.7	75.7 ± 6.2	87.3 ± 12.5	92.9 ± 9.6
Atrazine	73.3 ± 5.4	95.8 ± 8.8	87.2 ± 10.4	82.9 ± 8.5	105.9 ± 13.1	112.4 ±10.2
Diuron	81.3 ± 8.1	94.7 ± 9.3	86.4 ± 8.4	79.5 ± 6.2	91.2 ± 8.8	90.4 ± 11.3
Terbuthylazine	71.8 ± 6.2	91.1 ± 7.9	85.3 ± 8.2	80.6 ± 7.3	96.6 ± 11.4	91.7 ± 12.5
Malathion	79.2 ± 5.8	109.4 ± 14.2	106.7 ±10.2	74.4 ± 7.2	92.8 ± 13.3	93.5 ± 9.4

[a]; palm oil
[b]; olive oil

4.3.1.3.2 Precision and accuracy

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the standard deviation (s) or the percent relative standard deviation (RSD) for a statistically significant number of samples. The precision requires several components including repeatability and reproducibility to describe its behaviour. Repeatability (intra-day) is the ability of an analytical method when repeated several times in a single day by a single analyst to give the same result. In contrast, reproducibility (inter-day) is defined as the ability of an analytical method to provide the same answer on different days and if possible by different analyst and possibly even in different laboratories.

Repeatability of the developed analytical method to obtain precision was calculated by analyzing six extractions of olive and palm oil samples spiked at the three concentration levels (50, 500, and 2500 ng g^{-1}) measurement in six replicates in single day and in six different days, as intra-day and inter-day precision study. Tables 4.6 and 4.7 show the RSDs (%) obtained from these assays.

Accuracy was calculated by comparing the obtained concentrations from spiked samples and expected concentrations after analyzing the samples. Linear regression analysis was conducted on linearity curves of the calculated concentrations versus the expected concentrations. A slope of 1.0 indicated 100% accuracy.

4.3.1.3.3 Linearity

Linearity was determined by matrix-matched standard solutions of palm oil (in triplicate) using sample treatment methodology at seven concentration levels between 5 and 1000 ng g^{-1}. The slope and intercept values, together with relative standard deviations were estimated using regression analyses. The responses of all compounds extracted with UA-MSPD method were linear in the range under study in with the regression coefficients higher than 0.9983. The results obtained for each pesticide in both samples (olive oil and palm oil) are also included in Tables 4.6 and 4.7, respectively.

4.3.1.3.4 Detection and quantification limits

The instrumental limit of detection and limit of quantitation were determined from the injection of matrix-matched standard solutions with low concentration levels giving a signal-to-noise ratio of 3 and 10, respectively. The

obtained results are summarized in Tables 4.6 and 4.7 for both samples (olive oil and palm oil) respectively**.** These LOD and LOQ levels are considerably low since they are far below the maximum residue level regulations established for selected pesticides in this study. These results demonstrate the high sensitivity of the proposed method based on UA-MSPD and LC-TOF-MS for the detection and quantification of the selected pesticides in olive and palm oil.

4.3.1.3.5 Matrix effects (ME)

Matrix components can provide variation in the detector response to pesticides. Matrix effect can reduce or enhance the response of the detector and it can be evaluated by comparing the detector response for pesticide standards prepared in solvent with that for standards prepared in sample extract. In this study, these possible effects were evaluated by comparing the slopes obtained in the calibration with matrix-matched standards and those obtained with solvent-based standards, to calculate matrix slope / solvent slope ratio for each pesticide (see Tables 4.6 and 4.7). A value <1 indicates signal suppression due to the matrix, while values >1 involve enhancing effect of the matrix on analyte signal. Regarding the obtained result, quantitation of pesticides was performed with matrix-matched calibration, using the same matrix as the sample analyzed.

The percentages of signal suppression or enhancement (calculated by formula: matrix slope / solvent slope ratio × 100 – 100) are also shown in Tables 4.6 and 4.7. Depending on the decrease/increase in the percentage of the slope, different ME could be observed. Negative values indicate signal suppression of the matrix, while positive results show enhancement due to the matrix. The obtained positive values in

more cases showed an enhancement signal for both olive and palm oil extracts except carbaryl and malathion in the case of palm oil and dimethoat, simazine, terbuthylazine in the case of olive oil indicated signal suppression respectively. As a result, no matrix effects were observed more than ±20% signal enhancement and suppression in all cases except diuron (+22.3%) in the case of palm oil. Therefore, low temperature precipitation followed by UA-MSPD clean up procedure can be effective to remove interfering species from the olive oil and palm oil samples.

Table 4.6: Method precision expressed as the RSD%, calibration data, matrix effects expressed as the average standard deviation (RSD %) and the ratio between the calibration curve slopes of matrix-matched standards and solvent-based standards, LOD and LOQ of the pesticides analysed in palm oil samples by LC-QTOF-MS

Pesticide	Solvent		Matrix		ME (Δ%)	RSD (%) Spiking level (ng g^{-1})						LOD (ng g^{-1})	LOQ (ng g^{-1})
	Slope	R^2	Slope	R^2		50		500		2500			
Dimethoate	13352	0.9989	14994	0.9992	1.12 (+ 12.3)	5.3[a]	6.5[b]	3.4[a]	5.4[b]	3.1[a]	4.3[b]	2.0	6.1
Simazine	10474	0.9994	10876	0.9990	1.04 (+ 3.8)	5.0[a]	7.2[b]	4.3[a]	5.5[b]	3.8[a]	6.8[b]	2.7	8.2
Carbaryl	69046	0.9996	63523	0.9989	0.92 (- 8.7)	4.7[a]	5.9[b]	2.7[a]	7.3[b]	2.9[a]	3.1[b]	2.0	6.1
Atrazine	12050	0.9984	12411	0.9988	0.82 (- 17.5)	9.5[a]	13.2[b]	5.0[a]	12.8[b]	2.0[a]	4.3[b]	1.5	4.6
Diuron	22563	0.9993	27595	0.9987	1.22 (+ 22.3)	4.8[a]	6.7[b]	4.1[a]	6.6[b]	3.1[a]	4.6[b]	0.8	2.1
Terbuthylazine	41022	0.9995	46485	0.9992	1.13 (+ 13.3)	8.3[a]	10.6[b]	8.7[a]	9.0[b]	6.5[a]	11.7[b]	1.0	3.0
Malathion	22762	0.9993	21574	0.9990	0.94 (- 5.2)	6.1[a]	8.2[b]	5.1[a]	10.4[b]	4.0[a]	10.3[b]	1.1	3.1

[a:] Repeatability (intra-day) n = 6.
[b:] Reprodusibility (inter-day) n = 6.

Table 4.7: Method precision expressed as the RSD%, calibration data of matrix matched standard, matrix effects expressed as the average standard deviation (RSD %) and the ratio between the calibration curve slopes of matrix-matched standards and solvent-based standards, LOD and LOQ of the pesticides analysed in olive oil samples by LC-QTOF-MS

Pesticide	Solvent		Matrix		ME (Δ%)	RSD (%) Spiking level (ng g^{-1})						LOD (ng g^{-1})	LOQ (ng g^{-1})
						50		500		2500			
	Slope	R^2	Slope	R^2									
Dimethoate	17396	0.9992	17943	0.9994	1.03 (+ 3.14)	4.7[a]	5.7[b]	3.4[a]	7.4[b]	3.1[a]	4.3[b]	2.1	5.7
Simazine	41239	0.9994	48956	0.9991	1.18 (+ 18.7)	6.4[a]	8.1[b]	4.3[a]	5.5[b]	6.8[a]	8.8[b]	0.6	2.1
Carbaryl	57442	0.9991	53889	0.9996	0.94 (- 6.2)	3.7[a]	4.5[b]	2.7[a]	7.3[b]	2.9[a]	3.1[b]	2.0	5.3
Atrazine	10501	0.9992	8553	0.9988	0.81 (- 18.5)	8.3[a]	10.1[b]	5.0[a]	12.8[b]	2.0[a]	4.3[b]	5.4	14.2
Diuron	13878	0.9983	15973	0.9990	1.15 (+ 15.1)	10.3[a]	14.1[b]	9.8[a]	11.2[b]	7.5[a]	8.6[b]	1.2	3.0
Terbuthylazine	16997	0.9989	19013	0.9992	1.12 (+ 11.8)	9.7[a]	11.6[b]	8.7[a]	10.4[b]	5.8[a]	7.7[b]	1.2	3.3
Malathion	1023	0.9997	929.7	0.9999	0.90 (- 9.3)	7.2[a]	8.5[b]	6.1[a]	9.8[b]	5.0[a]	7.1[b]	1.0	2.8

[a:] Repeatability (single-day) n = 6.
[b:] Reprodusibility (inter-day) n = 6.

4.3.1.4 Application of the method to real sample

The proposed method based on low temperature precipitation (LTP) followed by matrix solid-phase dispersion-sonication was applied for the determination of seven multiclass pesticides in olive and palm oil. Two different brands of extra virgin olive oil and palm oil samples were purchased from local markets in Kuala Lumpur city of Malaysia.

Simazine, diuron and atrazine residues with a concentration of 6.5 ng g^{-1}, 3.5 and 6.5 ng g^{-1} were detected in olive oil sample respectively. A concentration of 8.5 ng g^{-1} of dimethoate and 5 ng g^{-1} of malathion were present in the palm oil samples. Although the EU Regulation (EC) 396/2005, establishes (Annexes II and III) maximum residue levels (MRLs) for some of the studied pesticides in olives for oil production (Codex Alimentarius Commission, 1996). There are no harmonized MRLs established for pesticide residues in olive oil yet (Gilbert-Lópeza *et al*., 2010). On the other hand, the National Committee on Agricultural Commodity and Food Standards issued a Notification entitled the Thai Agricultural Standards on Pesticide Residues: Maximum Residue Limits (TAS 9002-2006) for palm oil on 31 July 2006 which was published in the Royal Gazette (Thai Agricultural Standard (TAS 9002-2008) (see Table 4.8). The results showed that, no pesticide residues were found at concentrations above the permitted MRL published for pesticide residues for food samples.

Table 4.8: Pesticide residues detected in real samples of both olive oil and palm oil.

Pesticides	[*]MRL ($ng\ g^{-1}$)	[a]Residues found ($ng\ g^{-1}$)	[b]Residues found ($ng\ g^{-1}$)
Dimethoate	[a]50	n.d	8.5
Simazine	[b]100	6.5	n.d
Carbaryl	[a]25000	n.d	n.d
Atrazine	[b]50	6.5	n.d
Diuron	[a,b]200	3.5	n.d
Terbuthylazine	[b]50	n.d	n.d
Malathion	[a,b]500	n.d	5

[*]: MRL for pesticides in olive & palm oil
[a]: Olive oil
[b]: Palm oil
n.d: not detected

4.3.2. Optimization approach of d-SPE procedure

Since fats are not very soluble in MeCN, a certain quantity of them will be co-extracted and these remaining matrix constituents would possibly interfere with the determination and deteriorate the LC-QTOF-MS system performance. Therefore, to solve this problem and to remove the remaining fat an additional dispersive solid-phase extraction (d-SPE) clean up is necessary. In clean-up procedure, the use of magnesium sulphate yields the largest recoveries of pesticides (especially very polar pesticides) because it facilitates the partitioning of polar analytes into the organic phase by effectively reducing the volume of the aqueous phase.

In this method after LLE and freezing out step and separation of the solvent and oil as described in Section 3.7.3, 3 mL aliquot of the obtained acetonitrile extract from

the freezing-out step was separated from the precipitates by decantation and filtration into a PTFE centrifuge tube containing 100 mg of anhydrous magnesium sulphate (to remove the residual water), 150 mg of PSA sorbent (to remove various polar organic acids, polar pigments, some sugars and fatty acids), 50 mg of GCB sorbent (to remove sterols, and pigments such as chlorophyll and beta-carotene) then analyzed by LC-QTOF-MS. Different dispersing sorbents such as PSA, C_{18}, GCB and mixture of PSA and GCB were used on d-SPE technique in order to find out materials available for the performance of the pesticides determination with higher recoveries and lower fat levels transferred in the final extracts. The palm oil samples spiked at 50 ng g^{-1} were applied for all optimization purposes. The extracts were analysed in triplicates measurements and injected three times (n = 9).

The respective mean recoveries of studied pesticides are shown in Figure 4.20. As we can see, the respective mean of recoveries of the pesticides determined by LC-QTOF-MS ranged from 53.5 to 73.6% for clean-up on C_{18}, from 70.4 to 86.3% for clean-up on GCB except diuron (48.9%), from 79.5 to 91.6% for clean-up on PSA and from 91.8 to 104.7% for clean-up on bulk of PSA and GCB except in the case of diuron (70.1%).

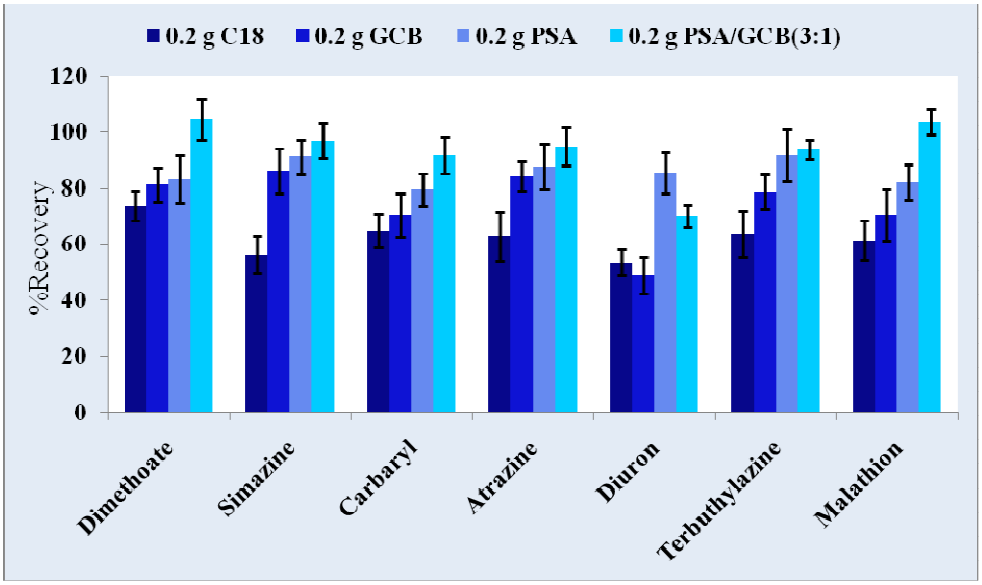


Figure 4.20: Mean percent recovery and RSD (%) of the studied pesticides in palm oil sample using LTP and d-SPE procedure with different clean-up sorbent.

GCB has a strong affinity for planar molecules, and thus effectively removes pigments such as chlorophyll and carotenoids, as well as sterols present in foods. The obtained low recovery for diuron (with average recovery 48.9%) when GCB was used as a dispersant indicated that, this compound was not completely retained in GCB phase during the clean up procedure, can be explained due to its planar structure. C_{18} as clean-up sorbent resulted the chromatogram with higher background and interfering peaks from the palm oil. PSA is known to exhibit a strong retaining activity for sugars, fatty acids and other organic acids. All pesticides assayed fell within the acceptable recoveries with RSD values below 8.5% when a bulk of PSA and GCB were used as the clean-up sorbent. Therefore, as shown in Figure 4.20, although d-SPE clean-up on PSA gave clean chromatogram from the extract however, a bulk of PSA and GCB (3:1 w/w) showed the cleanest chromatogram from the extract with lowest interfering and gave the highest mean recoveries from

91.8 to 104.7%. This mixed sorbents also presented better recoveries and lower RSDs% which are between 3.8 and 6.5% in relation to the other solid supports.

To assay the effect of GCB content in the clean-up sorbent on d-SPE efficiency, a mixture of PSA/GCB at ratio 25, 50, and 100 of GCB were investigated. The results obtained are shown in Figure 4.21. The recoveries of pesticides studied increased with an increase in GCB up to 50%. No significant changes observed with an increase in the content of GCB in most cases. Therefore, the extracts obtained using PSA/GCB at ratio 3:1 w/w furnished a transparent and colorless solution with minimal interferences for pesticides studied. In all subsequent experiments, 150 mg of PSA and 50 mg of GCB (PSA: GCB 3:1 w/w) were used as sufficient dispersion adsorbent and clean-up adsorbent, respectively.

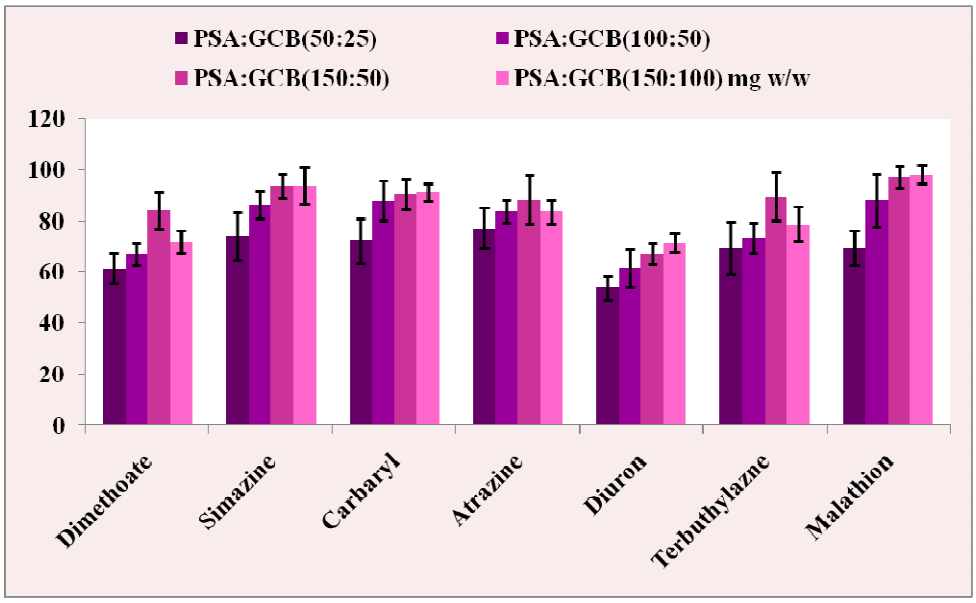


Figure 4.21: Effect of GCB content in the clean-up sorbents (PSA/GCB w/w) on the extraction efficiency of pesticides studied in palm oil using d-SPE procedure (MeCN as extracting solvent).

The typical chromatogram obtained by LC-QTOF-MS of the spiked palm oil; blank palm oil, and olive oil; blank olive oil, extracted using LTP followed by d-SPE procedure have been shown in Figures 4.22 and 4.24, respectively.

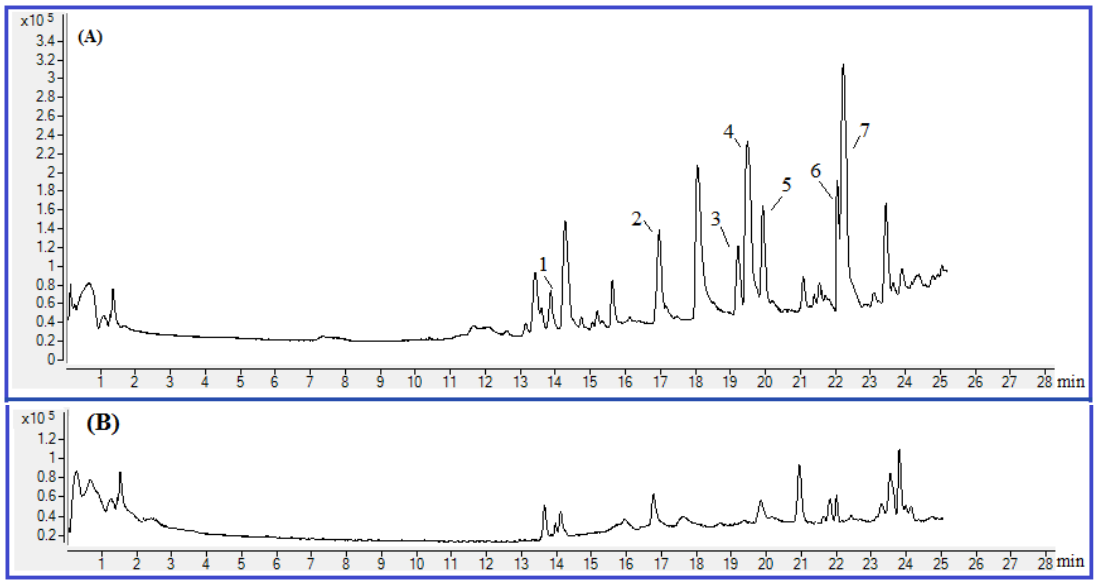


Figure 4.22: Typical Chromatograms obtained by LC-QTOF-MS of: (A) spiked palm oil sample with pesticides at 25 ng g^{-1}, (B) blank palm oil, Peak identification: (1) dimethoate; (2) simazine; (3) carbaryl; (4) atrazine; (5) diurone; (6) terbuthylazine; (7) malathion.

Comparative study between two chromatograms obtained from the extracts after only freezing-out clean-up and additional d-SPE showed some chromatographic problems such as peak suppression of dimethoate and retention time shifts of dimethoate, simazine and malathion (see Figure 4.23 (A), (B), (c)).

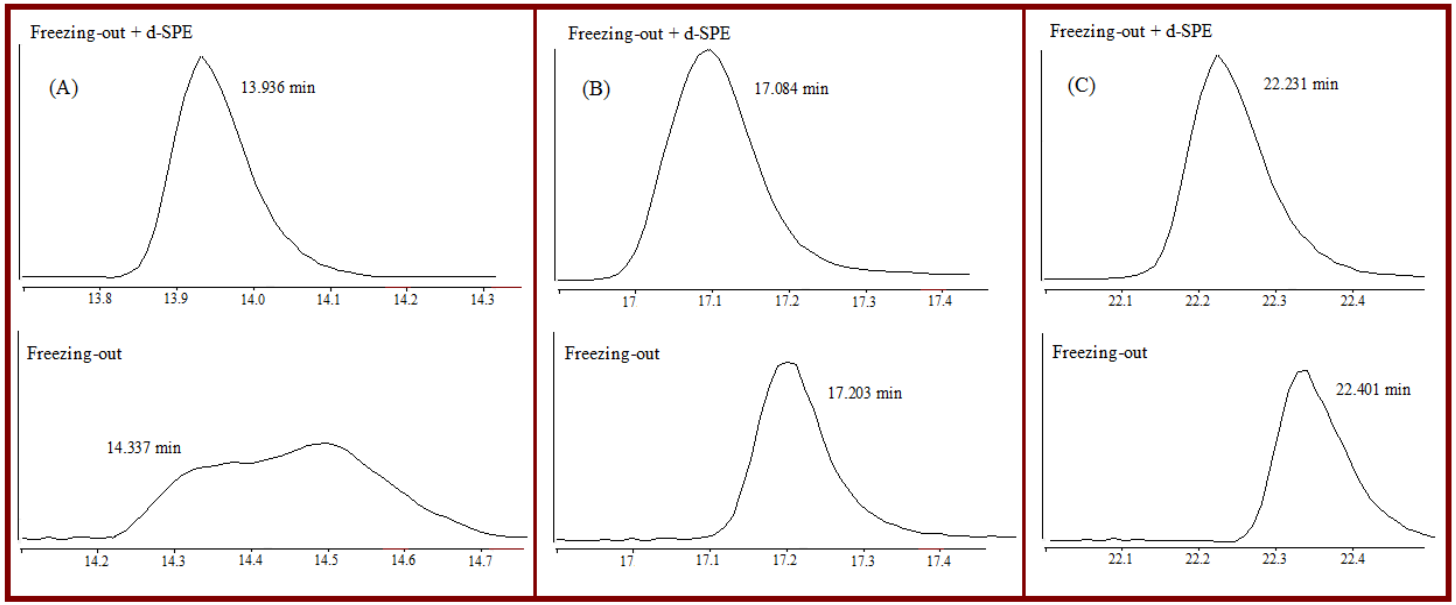


Figure 4.23: Effect of clean-up on peak shape and retention time of (A) dimethoate, (B) simazine, and (C) malathion.

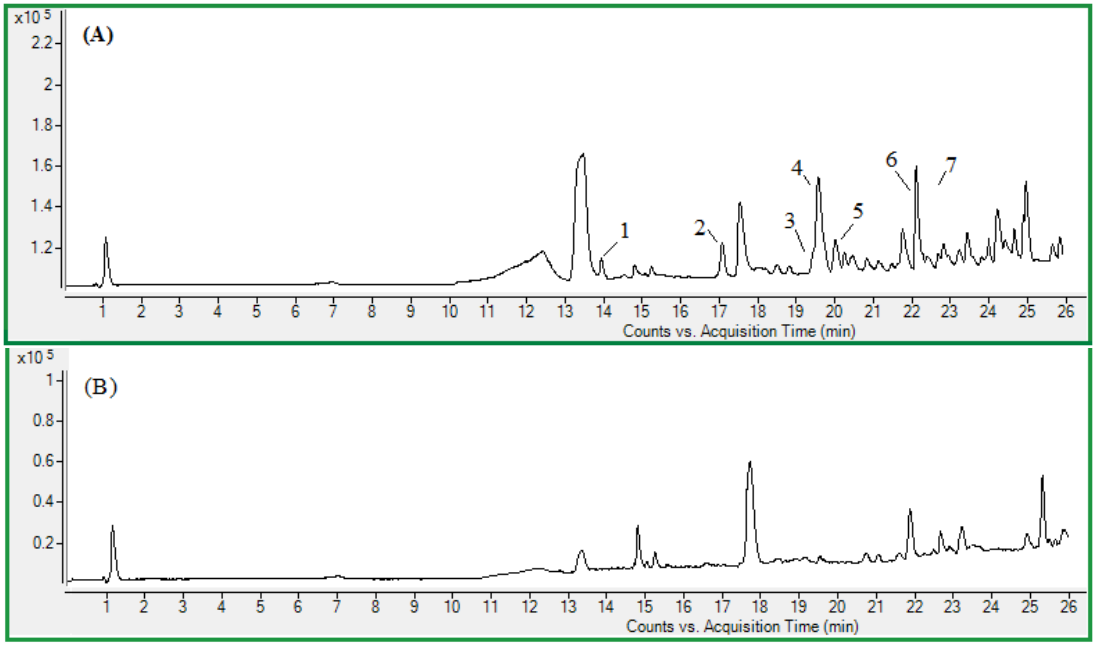


Figure 4.24: Typical Chromatograms obtained by LC-QTOF-MS of: (A) spiked virgin olive oil with pesticides at 25 ng g^{-1}, (B) blank virgin olive oil, Peak identification: (1) dimethoate; (2) simazine; (3) carbaryl; (4) atrazine; (5) diurone; (6) terbuthylazine; (7) malathion.

4.3.3 Analytical performance

4.3.3.1 Recovery studies

Recovery studies were performed by spiking untreated palm oil samples with the appropriate volumes of composite working standard solution at three different concentration levels: 25, 50, and 100 ng g^{-1}. Six replicates were carried out at each spiking level to determine the mean recovery (%) and relative standard deviation (RSD %). Most values of the relative standard deviations of the analysed samples were in general less than 10% that could be attributed to the experimental error. The obtained results for the mean recoveries and RSD (%) of all pesticides at three concentration levels are shown in Table 4.11. the lower fortification level (25 ng g^{-1})

gave lower recovery and higher RSDs than the higher level (100 ng g^{-1}). The mean recoveries lie within an acceptable range from 75.6% to 107.4% with relative standard deviations values from 5.2% to 14.1% for olive oil and mean recoveries ranged from 76.4% to 107.2% with relative standard deviations of 6.1-15.2% for palm oil sample except diuron. The overall mean recovery and RSD show the adequacy of the whole method.

4.3.3.2 Accuracy

Once the parameters that affect the LLE and d-SPE clean-up procedure were optimized, a method validation process was performed by establishing the basic analytical requirements of the performance to be appropriate for quantitative determination of selected pesticides in oil samples. Validation refers to the measures taken in order to test and describe whether a method in respect to its accuracy, use, implementation and sources of errors operates at all time in accordance with expectations and laid down requirements.

Data that are obtained as a result of analyzing a chemical sample should be assessed for accuracy and precision. In this aspect, accuracy refers to the ability of an analytical technique or analyst to portray the real quantity of an object. Another term which is used to illustrate the accuracy is relative error. Mathematically, the accuracy is the average relative deviation of the analysis of a set data from the mean of the population. The % relative error is:

$$\% \text{ Relative Error} = \frac{\frac{\sum \overline{X} - \text{True}}{\text{True}}}{N} \times 100$$

As we can see in Tables 4.9 and 4.10, the relative errors were ranged between -2.21 and 4.72% for olive oil and between -3.28 and 5.31% for palm oil when n = 6.

Table 4.9: Mean percent Recovery ± RSD (%) and accuracy (relative error %) (n = 6) obtained by LTP followed by d-SPE procedure of the spiked olive oil sample for the pesticides studied.

	Mean recovery ± RSD (%)			**Relative error (%)**
Pesticides	**Concentration level (ng g^{-1})**			
	25	**50**	**100**	
Dimethoate	75.6 ± 7.9	104.7 ± 12.3	106.4 ± 10.1	2.76
Simazine	85.8 ± 11.2	91.4 ± 8.1	76.3 ± 6.5	3.38
Carbaryl	75.7 ± 6.8	89.4 ± 7.3	72.6 ± 7.7	4.72
Atrazine	88.3 ± 8.2	91.3 ± 9.5	107.4 ± 9.6	-2.21
Diuron	52.3 ± 6.4	61.9 ± 5.2	64.4 ± 6.1	-1.85
Terbuthylazine	76.1 ± 9.2	92.8 ± 8.4	87.2 ± 8.1	3.42
Malathion	75.9 ± 8.5	93.4 ± 7.6	97.5 ± 11.3	1.63

Table 4.10: Mean percent Recovery ± RSD (%) and accuracy (relative error %) (n = 6) obtained by LTP followed by d-SPE procedure of the spiked palm oil sample for the pesticides studied.

	Mean recovery ± RSD (%)			**Relative error (%)**
Pesticides	**Concentration level (ng g^{-1})**			
	25	**50**	**100**	
Dimethoate	77.3 ± 9.4	92.9 ± 11.6	90.5 ± 10.1	2.23
Simazine	80.6 ± 8.9	87.4 ± 6.4	75.2 ± 6.3	1.83
Carbaryl	78.5 ± 7.1	104.3 ± 12.7	107.2 ± 14.2	5.31
Atrazine	81.8 ± 7.2	93.9 ± 10.4	83.6 ± 7.4	1.91
Diuron	61.5 ± 5.4	63.4 ± 6.7	62.1 ± 7.2	-3.28
Terbuthylazine	76.4 ± 8.2	87.5 ± 9.4	91.3 ± 10.7	4.52
Malathion	81.6 ± 7.8	106.4 ± 14.0	96.8 ± 11.7	3.67

4.3.3.3 Precision, linearity and lower limit values

The developed d-SPE method was evaluated in terms of intermediate precision (between-day RSD). In this case, repeatability of the developed analytical method to obtain precision were calculated by running six extractions of palm oil samples spiked at the three concentration levels (50, 500, and 2500 ng g^{-1}) for six-replicate measurement within a single day and within six different days, as intra-day and inter-day precision study. The repeatability of the developed method, expressed as relative standard deviation (RSD) was in most cases 5-15 %. Tables 4.11 and 4.12

show the RSDs (%) obtained from these assays. These results were similar to those obtained in repeatability of the UA-MSPD method.

The linearity of the method using matrix matched standards using d-SPE sample treatment methodology was evaluated at seven concentration levels ranging from 5 to1000 ng g^{-1}. The calibration curves showed correlation coefficients higher than 0.992. Analytical method detection limit was calculated to noise ratio of 3 (S/N = 3) for all pesticides and method quantification limits, corresponding to the concentrations giving the value of ratio S/N = 10, were also calculated (see Tables 4.11 and 4.12)

These LOD and LOQ levels are considerably low since they are far below the maximum residue level regulations established for selected pesticides in this study. These results demonstrate the high sensibility of the proposed method based on d-SPE and LC-QTOF-MS for the detection and quantification of the selected pesticides in both palm oil and olive oil.

4.3.3.4 Matrix effects

The study of the ratio of the slopes, in solvent and in matrix, provided information about the matrix effects. Depending on the decrease/increase in the percentage of the slope, different matrix could be observed. Matrix effect was calculated as described in 4.2.1.3.6. As can be seen in Tables 4.11 and 4.12, most of compounds showed signal enhancement for both samples except carbaryl, atrazine and malathion that indicated signal suppression. Carbary and malathion showed higher matrix effects when d-SPE method was used as the clean-up procedure (slope ratios values of 0.71, 0.77 in palm oil and 0.69, 0.76 in olive oil were obtained for

carbaryl and malathion, respectively) in comparison with the obtained results when UA-MSPD procedure was used for both olive oil and palm oil. It is might be due to the capability of UA-MSPD method to extract non-polar interfering species that compete with analytes for ionization in the ESI source, originating ion suppression in such way that analyte response diminished in matrix-matched standards, compared to analyte responce in solvent based standard. The overall results showed the ME less than ±20% signal enhancement and suppression in most cases except diuron (+24.3%) in the case of olive oil and carbaryl (-28.5%, - 31.2%) and malathion (-22.6%, - 24.3%) in both palm oil and olive oil, respectively.

Table 4.11: Method precision expressed as the RSD%, calibration data of matrix matched standard, matrix effects expressed as the average standard deviation (RSD %) and the ratio between the calibration curve slopes of matrix-matched standards and solvent-based standards, LOD and LOQ of the pesticides analysed in palm oil samples by LC-QTOF-MS

Pesticide	**Solvent**		**Matrix**		**ME (Δ%)**	**RSD (%)**						**LOD**	**LOQ**
						Spiking level (µg kg^{-1})						**(µgkg^{-1})**	**(µgkg^{-1})**
	Slope	**R^2**	**Slope**	**R^2**		**50**		**500**		**2500**			
Dimethoate	84414	0.9996	95487	0.9999	1.13 (+ 13.1)	7.1[a]	6.3[b]	4.4[a]	5.8[b]	3.1[a]	4.3[b]	3.5	5.2
Simazine	17884	0.9992	20927	0.9996	1.17 (+ 16.9)	6.1[a]	7.0[b]	5.3[a]	6.5[b]	3.8[a]	6.8[b]	2	7
Carbaryl	45732	0.9997	32681	0.9993	0.71 (- 28.5)	4.3[a]	6.9[b]	5.7[a]	8.0[b]	3.8[a]	4.1[b]	1.5	3
Atrazine	48290	0.9993	42359	0.9990	0.88 (- 12.3)	7.5[a]	10.4[b]	6.0[a]	9.8[b]	5.2[a]	2.6[b]	1.5	3.6
Diuron	16318	0.9985	19263	0.9991	1.18 (+ 18.04)	7.8[a]	10.3[b]	2.2[a]	4.0[b]	5.4[a]	6.6[b]	5	9
Terbuthylazine	31757	0.9987	37040	0.9990	1.16 (+16.6)	7.1[a]	12.5[b]	7.7[a]	10.3[b]	5.5[a]	13.2[b]	2	5
Malathion	49124	0.9997	38021	0.9998	0.77 (- 22.6)	4.8[a]	7.6[b]	8.1[a]	11.9[b]	6.8[a]	9.1[b]	1	2.5

[a:] Repeatability (single-day) n = 6.
[b:] Reprodusibility (inter-day) n = 6.

Table 4.12: Method precision expressed as the RSD%, calibration data, matrix effects expressed as the average standard deviation (RSD %) and the ratio between the calibration curve slopes of matrix-matched standards and solvent-based standards, LOD and LOQ of the pesticides analysed in olive oil samples by LC-QTOF-MS

Pesticide	Solvent		Matrix		ME (Δ%)	RSD (%) Spiking level (µg kg^{-1})						LOD (µg kg^{-1})	LOQ (µg kg^{-1})
						50		500		2500			
	Slope	R^2	Slope	R^2									
Dimethoate	26813	0.9996	29281	0.9990	1.09 (+ 9.2)	3.7[a]	5.3[b]	5.6[a]	9.0[b]	4.7[a]	5.1[b]	1.2	3.2
Simazine	39471	0.9992	42685	0.9987	1.08 (+ 8.2)	8.2[a]	10.3[b]	6.7[a]	7.5[b]	7.8[a]	9.5[b]	5.6	17.8
Carbaryl	42231	0.9993	29042	0.9990	0.69 (- 31.2)	2.8[a]	3.3[b]	5.1[a]	8.0[b]	7.0[a]	10.1[b]	1.2	3.2
Atrazine	14056	0.9982	13586	0.9991	0.97 (- 3.6)	2.4[a]	3.1[b]	4.2[a]	8.8[b]	5.4[a]	6.8[b]	0.6	1.9
Diuron	13610	0.9990	16916	0.9993	1.24 (+ 24.3)	8.0[a]	10.7[b]	6.8[a]	12.6[b]	5.5[a]	9.2[b]	1.7	4.5
Terbuthylazine	26624	0.9987	26718	0.9991	1.18 (+18.5)	11.2[a]	14.0[b]	7.7[a]	12.1[b]	6.8[a]	7.9[b]	0.5	2.2
Malathion	32793	0.9995	24816	0.9992	0.76 (- 24.3)	6.6[a]	9.5[b]	6.3[a]	8.7[b]	5.2[a]	6.3[b]	1.1	3

[a:] Repeatability (single-day) n = 6.
[b:] Reprodusibility (inter-day) n = 6.

4.3.3.5 Application of the method to real sample

The proposed method based on low temperature precipitation followed by dispersive solid-phase extraction was applied for the determination of seven multiclass pesticides in both olive and palm oil. Two different brands of virgin olive oil and two different brands of palm oil were purchased from local markets in Kuala Lumpur city, Malaysia.

Simazine and atrazine residues with concentrations of 3.5 ng g^{-1} and 6.5 ng g^{-1} were detected in olive oil sample. A concentration of 9.5 ng g^{-1} of dimethoate and 5 ng g^{-1} of malathion were present in the palm oil samples. The results showed that, no pesticide residues were found at concentrations above the permitted MRL published for pesticide residues for food samples.

Table 4.13: Pesticide residues detected in real samples of both olive oil and palm oil.

Pesticides	**[*]MRL (ng g^{-1})**	**[a]Residues found (ng g^{-1})**	**[b]Residues found (ng g^{-1})**
Dimethoate	[a,b]50	n.d	9.5
Simazine	[a]100	3.5	n.d
Carbaryl	[b]20	n.d	n.d
Atrazine	[a]50	6.5	n.d
Diuron	[a,b]200	n.d	n.d
Terbuthylazine	[a]50	n.d	n.d
Malathion	[a,b]500	n.d	5

[*]: MRL for pesticides in olive & palm oil
[a]: Olive oil
[b]: palmoil
n.d: not detected

CHAPTER V

CHAPTER V: CONCLUSION

4.1 Conclusion

Pesticide analysis in foodstuffs is a challenging application involving the simultaneous trace analysis of a wide range of agrochemicals. Considering that the presence of trace amounts of both pesticide residues and their degradation products could be potential health hazards, they have to be controlled. Monitoring pesticide residues in food is therefore of great interest to ensure "food safety" in terms of pesticide residue levels. For this reason, numerous regulations such as the European Union directives have set maximum residue limits (MRLs) for pesticides in food.

In pesticide analysis, the present trend in the development of multi-residue methods is towards a methodology which allows proper control of a large number of pesticides in a unique analysis, as well as capability of providing an average of more than 80% with good reproducibility, is basically the main applied strategy.

Analysis of pesticide residues in fatty matrices is yet a challenging issue, because of the inherent complexity of the matrix. This fosters the development of strategies to isolate/extract the pesticide fraction from the whole fatty matrix. In fact, it is very difficult to avoid the co-extraction of fatty material, especially when some of the pesticides which are usually targeted are fat-soluble non-polar compounds (e.g. organochlorine), and tends to concentrate and remain in the fat. Therefore, to obtain high recoveries of most multi-class pesticides in ideally fat-free extract, an additional clean-up step is usually necessary prior to subsequent steps in the analytical process. The choice of sample treatment is related to the complexity of the matrix, the nature of analytes targeted in the method and the detection method.

The use of non-dedicated cleanup procedures can foster the occurrence of matrix-effects during the detection step in both GC and LC methods. The matrix-effects are generally due to the influence of co-eluting compounds on the actual ionization process, which is well remove before the analyte ions enter the mass analyzer. Therefore, matrix-effects must be solved prior to analyte ionization, for instance by eliminating the sample constituents responsible of the matrix-effects, which would involve improvements on sample treatment and/or the chromatographic separation. As an alternative, lowering the injected sample volume or performing dilutions of the sample extract might help to overcome this problem, although this decreases the overall method performance in terms of limits of detection (Gilbert-López *et al*., 2009).

The objective of this study was the development and validation of the two fast, reliable and easy to carry out multi-residue method such as LTP followed by d-SPE or UA-MSPD, for the determination of pesticide residues belong to different class of pesticides including organophosphates, carbmates, triazines and phenylureas and different chemical uses such as insecticides and herbicides.

In the first sample treatment methodology, LLE technique was selected as the more suitable method for the routine analysis of pesticide residues in oil sample. This procedure has some advantages such as low cost, nonspecific instrumentation demands and ease of carrying out. After LLE, at first, the co-extract fat in organic solvent was reduced by centrifugation based on the difference of the mass of oil and of the extraction solvents. Next, due to the significant difference of melting points between fat (below 40 °C) and studied pesticides (normally above 250 °C), the co-extracted fat can be separated from pesticides by freezing. The co-extracted fat in organic extract is precipitated as frozen at −20 °C in the freezer, while pesticides are still dissolved in cold

organic solvent. Thus, frozen co-extract fat can be easily discarded by centrifugation. After centrifugation and freezing the fat filtration, most of the remaining co-extract fat is removed by MSPD clean up. Among different dispersive and clean-up sorbents, PSA (750 mg) and florisil (250 mg) under 15 min ultrasonic bath at room temperature was used to maximize recovery of the pesticides contained in oil samples while eliminating most of the interfering matrix components.

In second sample treatment method, LLE coupled with centrifugation followed by d-SPE was successfully applied to determine seven selected pesticides in both olive and palm oil. The d-SPE procedure using 150 mg of PSA and 50 mg of GCB (3:1 w/w) clean-up sorbent was carefully optimized in order to obtain high recoveries and lower fat levels transferred in the final extracts.

The high sensitivity attained by rapid resolution LC-QTOF-MS. The hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer showed high sensitivity for confirmation of the pesiticide residues with good reproducibility and low values of LOQs. In this way, the identification by LC-QTOF-MS is talented with the retention time mached, accurate mass of the protonated molecules $[M + H]^+$, along with the accurate mass of the main fragment ion and the characteristic chlorine isotope cluster present in some of cases. For quantitation purposes, peak areas of the extracted ion chromatograms (XICs) of the protonated molecules ($[M+H]^+$) were used for most of the species except when the relative intensity of sodium adducts ($[M+Na]^+$) was higher than that of the protonated molecule in the selected conditions (fragmentor voltage 190 V; nebulizer pressure 40 psig; drying gas 9 L min^{-1}; gas temperature 300 °C; skimmer voltage 65 V) such as malathion or the relative intensity of characteristic fragment ion was higher such in the case of dimethoate and carbaryl.

The high intensity of protonated pseudomolecular ions made them possible to achieve high specificity and therefore high specific analysis can be performed by monitoring these ions for quantitative purposes. No significant differences were observed in the mass accuracy obtained in the matrix matched standards when compared with that obtained with standards in pure solvent. Therefore, we can deduce that the accurate mass measurements have capability for the unequivocal confirmation of these species in oil matrices at different concentration levels.

The two methods gave satisfactory analytical performance parameters for the most of the targeted pesticides and analysis of real samples proved its feasibility for the intended purpose. Considering the results obtained from recovery study showed that enhanced solvent extraction techniques such as Ultrasonic-Assisted Extraction (UAE), provided a more efficient contact between the sample and solvent due to an increase of both pressure (which favours penetration and transport) and temperature (improves solubility and diffusivity), so can easily result in higher recoveries. On the other hands, for pesticides determination, ultrasonic energy has been reported to speed, low solvent consumption and improved the extraction efficiency during solid–liquid extraction (SPE), also when the cartridge was placed in an ultrasonic bath. Ultrasonic-assisted matrix solid-phase dispersion (UA-MSPD) in comparison with classic MSPD, improves the general extraction efficiency, decreases the RSDs and allows complete sample treatment within a few minutes.

Satisfactory recoveries with good sensitivity were obtained for different classes of pesticides assayed, which presages the application of the two proposed methods for the multi-residues analysis of pesticides in the tested samples. The results obtained from linearity, precision, accuracy matrix effects and detection and quantification limits

illaustrate the potential of LC-QTOF-MS for the rapid screening of agrochemicals in fatty vegetable samples. The ruggedness and potential of the proposed methods were demonstrated by analyzing two brands market purchased samples with excellent selectivity and sensitivity. The LOQs achieved by the methods that are in good agreement with the limit values established by EU Regulation (EC) 396/2005 and Thai Agricultural Standards on Pesticide Residues (TAS 9002-2006) for olive and palm to make oil productions respectively. These LOQs, combined with the low LODs, proved that the proposed procedures are suitable for the accurate determination of the target analytes at levels set in current legislations.

In comparative study of application of two proposed method, LLE/LTP followed by d-SPE combined with electrospray TOF-MS showed slightly better recoveries with lower relative standard deviations for all pesticides except diuron due to its planar structure however, UA-MSPD illustrated minor matrix effects of the most investigated compounds in both olive and palm oil samples. In addition, d-SPE method requires a small sample size, and offers considerable savings in terms of solvent consumption, cost of materials and sample manipulation. This procedure was proven to be effective, economical and fast compared with the other procedures, so it has a slight advantage on overall performance over UA-MSPD procedure. Although every oil sample showed matrix effect that was influencing the analyte signal, it was successfully eliminated using matrix- matched standards.