Solid Phase Extraction Techniques for the Analysis of Pesticides and Drugs in Biological Specimens

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by

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Dedication

In the name of Allah
my creator

I dedicate my work

to my beloved parents
Hj. Asri Abdul Ghani (deceased)
Hjh. Sapiah Johar
who raised me
with love and affection,
and guided me towards
the right direction

to my dearest wife
Norhalina,
you cherished up my life
with love and
understanding

and my kiddies
Muhammad Hafizuddin
Ahmad Arif
Siti Alyani
Siti Nazeerah,
You are the best there is.

Acknowledgments

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Abbreviations

mg/L	milligrams per litre
mg/mL	milligrams per millilitre
μg/mL	micrograms per millilitre
ng/mL	nanograms per millilitre
ng/μL	nanograms per microlitre
°C/min	degrees Centrigrade per minute
eV	electron volts
r^2	correlation factor
ELISA	enzyme linked immuno-sorbent assay
NPD	nitrogen-phosphorus detector
MS	mass spectroscopy
SPE	solid phase extraction
SPME	solid phase microextraction
HS	headspace
WHO	world health organisation
rpm	revolutions per minute
CE	capillary electrophoresis

SUMMARY

The aims of the project were to investigate the potential of solid phase extraction techniques for the detection and quantification of organophosphate pesticides in human biological specimens. Achievement criteria which were applied were that analytical methods should be sufficiently sensitive, specific, robust and cost effective for use in routine forensic toxicology, and, if possible, were inexpensive and within the economic and technical means of laboratories in developing countries. The procedures developed were based on established chromatographic and spectrometric techniques, drawing on analytical methods for environmental samples such as foodstuffs (which resemble target matrices such as autopsy blood and tissue) but also incorporated the more recently available technique of solid phase micro extraction (SPME). The project evaluated the application of these methods to real specimens from forensic cases, which occurred in Malaysia and elsewhere during the course of the study, as the incidence of pesticide poisoning cases in the United Kingdom is very low. The SPME technique was also evaluated for analysis of benzodiazepines in urine.

An overview is given of pesticides and organophosphates in particular, with respect to their chemistry, usage, metabolism and biological effects. Problems encountered in sample pretreatment and preparation for analysis are reviewed, with emphasis on techniques, which aim to reduce or avoid the use of environmentally damaging solvents.

This review also focuses on the history and theory of SPME. The SPME process has two steps, equilibrium partitioning of analytes between the SPME fibre coating and sample matrix and then transfer of absorbed analytes to the analytical instrument. In step 1, the extraction is considered to be complete after equilibrium is reached, i.e.the stage at which absorption is constant within the limits of experimental error and independent of further increase in extraction time. However, SPME is an equilibrium, but not an exhaustive, extraction. Parameters that contribute to extraction efficiency include sampling time, temperature, pH and ionic strength. Application of SPME under non-equilibrium conditions requires adequate control of these parameters.

In the initial method development study, SPME in combination with capillary column gas chromatography and nitrogen-phosphorus detection were evaluated as a method sequence for the analysis of organophosphate pesticides in whole blood and urine. Eleven pesticides were used initially in the study as model compounds representing the range of commonly-used substances and also representing different functional groups found in these phosphates. Two types of fiber were evaluated, with coatings of polydimethylsiloxane (PDMS) or polyacrylate (PA). Parameters affecting the adsorption of analytes on the SPME fibre were examined, including fiber pre-treatment, sampling time, temperature, the use of sample agitation, and modification of the ionic strength and pH of the samples. The best conditions for extraction involved a PDMS fiber along with a sample temperature of 90°C, with a conditioning time of 10 minutes, exposure time of 30 minutes and desorption time of 5 minutes. The addition of sodium chloride and hydrochloric acid increased the recovery of the organophosphates. All samples were agitated during the extraction process. The technique was later employed in urine as well as whole blood specimens. The primary detection and quantification technique was GC-NPD. The Limit of Detection and Precision was measured for validation purposes and were found to be in the range 1-50 ng/0.5 ml and 3.5-14 % RSD respectively. The developed method was subsequently applied to real cases involving fenthion and chlorpyrifos.

The method developed was tested in the Department of Chemistry in Malaysia to assess its viability and ruggedness. The proposed extraction procedure was maintained but modifications to the instrumentation involved dual column gas chromatography with twin nitrogen phosphorus detectors. Twenty-five specimens of post-mortem blood were examined using HS-SPME as a routine screening procedure for pesticides. Of these samples, 3 positive cases of malathion poisoning were identified and quantified.

A comparison was made of SPME and Solid Phase Extraction (SPE), another alternative technique for sample preparation which has become popular, for the extraction of organophosphates from blood and urine. Two type of SPE cartridge were evaluated: C_{18}-substituted silica and mixed-mode Bond-Elut® Certify. In the development of an SPE method, parameters affecting the extraction capabilities such as solubility, pH, salt effect were examined. An overall method of extraction was devised and validated including a limit of detection and precision study. The method

was applied to biological specimens from forensic autopsy cases. It was concluded that the two techniques are complementary and that SPME is particularly useful for dirty specimens containing many interfering substances.

An investigation was carried out to elucidate which factors contributing toward biodegradation of pesticides in aqueous matrices including water and blood in order to address the problem of specimen preservation between the medico-legal autopsy and the toxicological analysis. The HS-SPME technique was used in this study, which was carried out by exposing samples containing malathion under different conditions of pH and temperature and in the presence or absence of preservatives. The conclusion was that the rate of degradation of malathion can be reduced by lowering the storage temperature and adjusting the sample to a slightly acidic pH in the range 5-7. Fluoride was found to increase the rate of degradation.

Finally, the potential in forensic toxicology of analytical techniques based on direct immersion SPME was investigated using, as a representative system, the determination of benzodiazepines, especially diazepam, desmethyldiazepam, temazepam and oxazepam in urine. The technique was shown to be capable of producing reproducible and robust analytical methods and was applied successfully to urine specimens from different types of forensic case.

1. HISTORY AND BACKGROUND OF PESTICIDES

1.1. Introduction

In an effort to produce a more pleasant environment and abundant crops, man has developed and produced a variety of agricultural chemicals. One group of compounds are the pesticides. Their contribution to health and the economy are closely interrelated. They contribute directly to our health through control of certain vector-borne diseases; they contribute directly to the economy through increased production of food and fibre and through the protection of many materials during storage. Improved health has sometimes permitted a more prosperous and stable economy. In some countries, greater and more dependable production of food has eliminated famine and thus contributed as much to health as to the economy. Since their introduction in the 1930's, synthetic organic pesticides with insecticidal properties (some alkyl thiocyanates) and fungicidal properties (for example, dithiocarbamate) [1], the real beginning of the pesticide industry occurred with the introduction of DDT, leading to the subsequent synthesis of thousands of chemicals.

The introduction of organochlorine insecticides - chlorinated hydrocarbons such as BHC, dieldrin, aldrin, chlordane and others – led to a tremendous growth in the use of pesticides either in agriculture or elsewhere. Later, in the 1950's, the fungicide captan and the organophosphorus insecticide malathion were introduced. In the 1960's, with the discovery of the potential hazard of DDT and other chlorinated compounds, farmers stopped using organochlorine pesticides except for certain purposes, and switched in favour of using organophosphates and carbamates, which although more acutely toxic, do not persist in the environment. In 1972, the Environmental Protection Agency (EPA) revoked the use of DDT on all food sources in the United States of America. The World Health Organisation, however, still reserves the right to use DDT on particularly virulent outbreaks of malaria.

Herbicidal sulfonylureas, the synthetic fungicides metaxyl and triadimefron, and light-stable pyrethoid pesticides were introduced in the 80's. In the 90's due to the renewed interest in integrated pest management, intensified research took place on biological pest control methods and other alternatives to pesticide. The emergence of pest

management programs, a result of improved knowledge in host-pest interactions, has helped decrease insecticide use in major crop commodities such as corn, soybeans, cotton and wheat since the 1960s. Today, there is great interest in genetically engineered microbial agents, including the development of pest-resistant transgenic crops and other biological pest control methods [2].

1.2. Definition of a Pesticide

Most pesticides are chemicals used in agricultural for the control of pests, weeds, or plant diseases.

FAO (1986) defined a pesticide as "any substance or mixture of substances intended for preventing, destroying, or controlling any pests, including vectors of human or animal diseases, unwanted species of plants or animals causing harm during, or otherwise interfering with, the production, processing, storage, transport, or marketing of food, agricultural conditions, wood and wood products, or animal feedstuffs, or which may be administered to the animals for the control of insects, arachnids, or other pest in their bodies" [3].

The United States Environmental Protection Agency defines a pesticide as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest. Pests can be insects, mice and other animals, unwanted plants (weeds), fungi, or microorganisms like bacteria and viruses. Though often misunderstood to refer only to insecticides, the term pesticide also applies to herbicides, fungicides, and various other substances used to control pests. Under United States law, a pesticide is also any substance or mixture of substances intended for use as a plant regulator, defoliant or desiccant [4].

In Malaysia the term pesticide is defined in Section 2 of the Pesticide Act 1974 as (a) any of the substances listed in the First Schedule; or (b) any preparation containing any one or more of those substances; and includes (c) any preparation used, or capable, or purporting to be capable, of being used, or intended to be used, for preventing the attack or spread or for destroying -(i) fungi or other parasitic plants or

bacteria that affect or attack plants, fruits, animals or property; (ii) insects or other pests that affect or attack plants, fruits, animals or property; (iii) noxious animals or noxious birds; or (iv) weeds or other noxious plants; and (d) any substance purporting to be a pesticide but does not include contaminated food or any article listed in the Second Schedule [5].

1.3. Major uses of Pesticides

The agricultural and non-agricultural uses of pesticides represent a large number of different active ingredients. More than 725 "main entries", corresponding to chemicals and biological agents used as active ingredients which are in use or being developed, and 559 "superseded entries" which correspond to materials believed to be no longer manufactured or marked for crop protection use [1].

- Agriculture

This sector is considered to be the largest user of pesticides. In many parts of the world excessive loss of food crops to insects and other destructive pests contribute to obvious problems - famine and starvation.

- Non Agricultural/Public Health

The largest public health uses of pesticides are in malaria and rodent control programs. Nevertheless, weed control on highways is also a significant use. There is also a large usage by individual homeowners and gardeners. Pesticides have been incorporated into various types of paints as anti-fungal agents and also used in dry-cleaning processes for rugs and other fabrics.

It is clear that the opportunities for exposure to pesticides are numerous.

1.4. Classification of Pesticides

Pesticide is generic term and covers a wide range of compounds used in pest control. Based on current agriculture practices, they can generally be defined by their intended target organisms.

Table 1.1: Classification of Pesticides and Current Agriculture Usage

Category	Target Organism(s)	Examples
Pesticides	Any type of pest	
Insecticides	insects and related species	organophosphates, carbamates
Rodenticides	rats, mice, moles, and other rodents	warfarin, zinc phosphide
Herbicides	Weeds	paraquat, 2,4-D, glyphosate
Fungicides	Fungi and moulds	dithocarbamates, captan
Fumigants	Bacteria and viruses - gases that are used to sterilise products	methyl bromide ,hydrogen cyanide

1.5. Toxicity

Pesticides include such diversity of chemical types that it is not surprising that their toxicity covers a wide range. According to the World Health Organisation, pesticides can be classified into five categories according to their acute toxicity. These categories are: extremely hazardous (IA), highly hazardous (IB), moderately hazardous (II), slightly hazardous (III) and unlikely to present hazard in normal use (IV).

As examples, as little as 100 mg of mevinphos may cause death, whereas 1.5 kg of the herbicide aminotriazole is unlikely to endanger human life. Even with a particular class of pesticide the lethal dose may vary considerably. Thus, one system categorises these compounds by evaluating their LD50 in rats (i.e. dose required to kill 50% of

laboratory rats). The LD50 values are intended to be a guide to their relative toxicity's and should only be used in this manner.

1.6. The Pesticide Problem

By their very nature, most pesticides create some risk of harm to humans, animals, or the environment because they are designed to kill or otherwise adversely affect living organisms. At the same time, pesticides are useful to society because of their ability to kill potential disease-causing organisms and control insects, weeds, and other pests. Crop protection is a major industry. It is important for farmers, for manufacturers and for the research-based groups involved in agrochemical discovery and development. However, general perceptions often lean towards the contrary argument, that is, does using pesticides really help mankind or has it created more destruction and problems in this world? By looking at the effects of DDT, considering the damage caused by Agent Orange in Vietnam and the Bhopal tragedy in India, a negative perception of pesticides is obtained. Anything damaging and destructive has to be all bad.

The examination of recent literature reveals that the largest proportion of human toxicity data related to pesticide intoxication comes from developing nations. The World Health Organisation [6] estimated an annual world-wide total of some 3 million cases of acute, severe poisoning (including suicides) matched possibly by a greater number of unreported, mild-to-moderate intoxication cases with some 220,000 deaths. In Sri Lanka pesticide poisoning is the major public health problem [7]. Jeyaratnam [8], in a national survey of hospital cases in Sri Lanka, reported an incidence of 100,000 persons admitted to hospital annually for acute intoxication with almost 1000 deaths, out of a population of 12 million. In Thailand in 1983, an estimated 8268 pesticide-related intoxications occurred within an agricultural community of 100,000 workers [9]. A multi-hospital study by Yoram et al. found 859 cases treated for pesticide intoxication that resulted in seven deaths in Israel [10]. There were cases of moderate to severe pesticide ingestion in infants and children in Colorado, USA [11]. Malaysia is one of the countries that make use of pesticides for crop protection and enhanced crop productivity. But, by doing this, not only has the environment been polluted but also substantial numbers of pesticide-related poisoning

cases have occurred [12,13]. More than 1500 cases of severe accidental and self harm resulting from misapplication of these potent compounds have been reported since 1990-2000 [14]. Whether one examines reports from Costa Rica [15], Nicaragua [16] or Taiwan [17], the majority of the intoxications, accidental or intentional, can be attributed to organophosphorus and carbamate ester pesticides, with the exception of the herbicide paraquat, apparently an agent of choice of suicides in many countries. Whether or not a pesticide or its residues are hazardous depends on the type of pesticide and the inherent toxicity of the breakdown products. Well-publicised epidemics of poisoning have been reported in Iraq (organic mercury in feed grain), in Morocco (tri-o-cresyl phosphate in cooking oil), in Hong Kong (methamidophos in vegetables) and in the United States (tri-o-cresyl phosphate in alcoholic beverages) which have resulted from ingestion of contaminated foods and beverages [18,19, 20].

1.7. Rational and Theoretical framework

Insecticides represent one group of pesticides that are used in large quantities and have a history of causing toxic effects in humans, but amongst the other types of pesticides one can find several potent, injurious agents.

In Malaysia, as in many other countries in the Asian continent, pesticides are one of the most significant causes of fatal and non-fatal poisoning [21-23], exceeding those due to other substances such as prescribed or illicit drugs, which are more prominent in Europe and the United State [24,25]. These poisoning cases form an important part of the work of Forensic Toxicology Division in the Department of Chemistry, Malaysia and it is essential that an appropriate and adequate range of analytical procedures should be available for the detection and measurement of these substances in biological specimens. Such measurements form the basis of correct interpretation of the role played in causing injury or death [26]. In both clinical and forensic fields, the identification of parent substances can be difficult due to rapid metabolism in the body.

In the clinical field, there is a related interest in the rapid identification of substances involved in suspected poisoning cases, which is an important pre-requisite to remedial treatment: in this area, biochemical measurements such as cholinesterase inhibition are frequently used [27,28]. These techniques do not identy specifically what type of organophosphate pesticide was consumed. They have many limitations and are, at times, inadequate, non-specific and unpredictable [29].

At present, in the Forensic Field, there are a few studies, which have been published concerning the analysis of pesticides in autopsy specimens [see for example 30-32]. This is due to the relative rarity of such cases in countries with well-developed forensic toxicology services, whose main interest remains the analysis of drugs of different types. In contrast, an extensive literature has been published in the environmental field, concerning the detection and measurement of trace levels of pesticides in foodstuffs, water, soil and other specimens [33-38]. What is lacking is a systematic approach to conversion of these procedures from trace pesticide detection in environmental samples to methods which can cope with the peculiar problems encountered in the analysis of these pesticides in human biological specimens, especially autopsy specimens. There is a need to update methods to take into account new technology [39-41] although if possible this should be inexpensive and within the economic and technical means of laboratories in developing countries [26].

The aim of the present project was to develop a set of analytical procedures for the detection and quantification of organophosphate pesticide in human biological specimens, which are sufficiently sensitive, specific, robust, and cost effective for the use in a routine forensic toxicology laboratory. These procedures were to be based on established chromatographic and spectrometric techniques, drawing on analytical methods for environmental specimens such as foodstuffs (which resemble target matrices such as autopsy blood and tissue). There was a need for the development of sample-handling strategies which are faster and more reliable (which means that the number of intermediate steps, such as transfers, evaporation and derivatization is diminished), easily capable of automation and as solvent-free as possible. They were to be developed to take into account recent advances in sample preparation and detection methods, incorporating newly available techniques such as solid-phase microextraction [41,42] and established solid phase extraction [43]. The project also

aimed to evaluate the application of these methods to real specimens from forensic cases, which occurred in Malaysia or elsewhere during the course of the study.

1.8. Aims

The main aim was to investigate the potential of solid phase micro extraction and conventional solid phase extraction in the analytical forensic toxicology of organophosphate pesticides and drugs. This was carried out by developing and evaluating a set of analytical procedures (sample preparation, extraction, sample cleanup) for the detection, identification and quantification of organophosphate pesticides in biological specimens (blood, urine) and other, alternative specimens such as vitreous humour/cerebrospinal fluid if possible. These techniques were required to be sufficiently sensitive, specific, robust and cost effective for the use in routine forensic toxicology laboratory in Malaysia.

1.9. Research Scope

The project was designed to encompass the following research strand:

a) Investigation of strategies for the rapid and specific extraction procedures for organophosphates and their acidic metabolites from biological specimens such as autopsy blood, urine, tissue, vitreous humour, cerebrospinal fluid, using solid phase microextraction/solid-phase extraction techniques;

b) Investigation of strategies for the use of solid-phase headspace sampling for volatile/semi-volatile pesticides using commercially available adsorbent probes in combination with gas chromatography with selective detectors such as the nitrogen-phosphorus detector (GC-NPD) and mass spectrometer (GC-MS), also evaluation of this approach in vitro as method of screening for pesticides;

c) Evaluation of screening and confirmatory quantitative analytical procedures for pesticides based on the instrumentation available such as gas and liquid chromatography, and gas chromatography-mass spectrometry;

d) Validation of the techniques developed in routine casework, using specimens obtained from the Malaysian laboratory and elsewhere.

2. ORGANOPHOSPHORUS COMPOUNDS

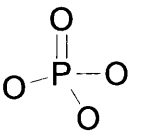
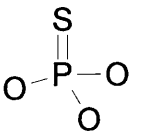
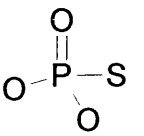
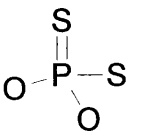
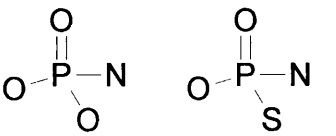
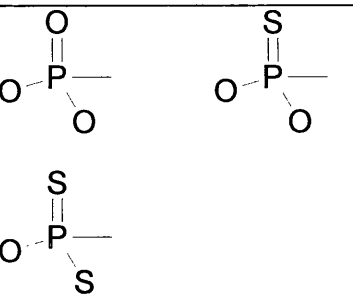
Organophosphorus pesticides (OP's) are widely used throughout the world as agricultural chemicals for the control of insects and other pests. Organophosphorus pesticides, which have been favoured over more persistent organochlorine pesticides due to their faster degradation rates, have played an important role in global agricultural chemistry for many years and are still widely, used today [44].

2.3. What are Organophosphates?

OP's were first recognised in 1854, but their general toxicity was not established until the 1930's. Tetraethyl pyrophosphate (TEPP) was the first OP insecticide, which was developed in Germany during World War Two as a by-product of nerve gas development [150]. OP's are all derived from phosphoric acid. They are generally among the most acutely toxic of all pesticides to vertebrate animals. They are also unstable and therefore break down relatively quickly in the environment. Altogether, over 100,000 OP compounds have been screened for their insecticidal properties, of which over 100 have been developed for commercial use. The Pesticides Trust holds details of 111 OP's on its active ingredient database. OP's are nerve poisons, which kill the target pest (usually insects). Most OP pesticides are insecticides, although there are also a number of related herbicide and fungicide compounds.

Most organophosphorus pesticide compounds are phosphates, phosphorodithioates (phosphorothionothiol) and phosphorothioates (phosphorothionates). Some others may fall in the following structural categories: phosphorothiolate, phosphorodithiolate, phosphoramide (phosphoramidate), phosphonate, phosphorodiamidate and phosphinate. OP's can be categorised into six classes based on the basic structure, as in Table 2.1.

Table 2.1 The Substructure Categories, the Structures and Some Examples of OP Pesticide Compounds

Category	Structure	Examples
Phosphate		monocrotophos
Phosphorothioate		Diazinon, parathion
Phosphorothiolate		profenofos
Phosphorodithioate		Malathion, ethion
Phosphoramide		Acephate, Methamidophos, Fenamiphos
Phosphonate		Trichlorfon, Leptophos, Fonofos

General Formula:

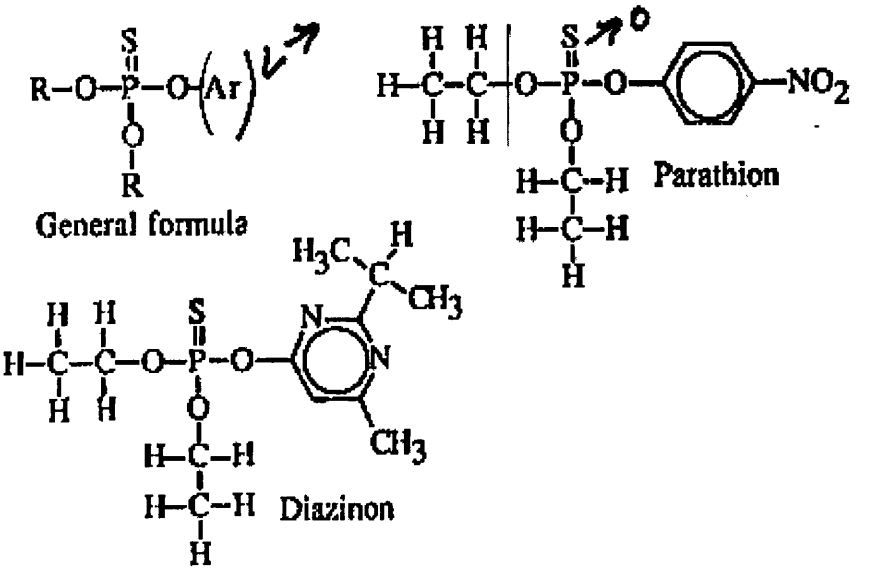


Figure 2.1 General formula for organophosphates

2.4. Uses and production

OP's are marketed by many of the world's major agrochemical companies. Some of the main agricultural products are Hostathion (triazophos), Metasystox-R (oxydemeton-methyl), Dursban and Lorsban (chlorpyrifos), Sumithion (fenitrothion) and Actellic (pirimiphos-methyl) [1]. OP's have a wide range of pest control applications as contact, systemic and fumigant insecticides. Whilst widely used in agriculture, they are also used against household and catering establishment pests. They are used against head lice in humans and a number of ectoparasites in domestic animals. The aerial application of OP's (such as dimethoate) is permitted in the UK to control cereal and vegetable pests. Recently OP's have been in the news because of health concerns following their use in sheep dips, and as insecticides in military premises, on equipment and even on personnel during the Gulf War. The latest issue of Current Research Monitor provides a full list of OP's on the market.

2.5. Toxicity

Organophosphates poison insects and mammals primarily by phosphorylation of the acetylcholinesterase enzyme (AChE) at nerve endings. The enzyme is critical to normal control of nerve impulse transmission from nerve fibres to muscle and gland cells, and also to other nerve cells in autonomic ganglia and in the brain. Some critical proportion of the tissue enzyme mass must be inactivated by phosphorylation before symptoms and signs of poisoning become manifest. At sufficient dosage, loss of enzyme function allows accumulation of acetylcholine (ACh, the neurotransmitter substance) at cholinergic neuroeffector junctions (muscarinic effects), at skeletal nerve-muscle junctions and autonomic ganglia (nicotinic effects), and in the brain. At cholinergic nerve junctions with smooth muscle and gland cells, high ACh concentrations cause muscle contraction and secretion, respectively. At skeletal-muscle junctions, excess ACh may be excitatory (cause muscle twitching), but may also weaken or paralyse the cell by depolarising the end plate. In the brain, high ACh concentrations cause sensory and behavioural disturbances, loss of co-ordination and depressed motor function. Depression of respiration and pulmonary oedema are the usual causes of death from organophosphate poisoning. Recovery depends ultimately on the generation of new enzyme in all critical issues.

Organophosphates are efficiently absorbed by:

- ingestion
- inhalation
- dermal/skin penetration

To a degree, the occurrence of poisoning depends on the rate at which the pesticide is absorbed. Breakdown occurs chiefly by hydrolysis in the liver and rates of hydrolysis vary widely from one compound to another. In the case of certain organophosphates whose breakdown is relatively slow, significant temporary storage in body fat may occur. Many organophosphates readily undergo conversion from thions (P=S) to oxons (P=O). Conversion occurs in the environment under the influence of oxygen and light, and in the body, chiefly by the action of liver microsomes.

Oxons are more toxic than thions, but oxons breakdown more readily than thions. Ultimately both are hydrolysed at the ester linkages, yielding alkyl phosphates and leaving groups as the principle metabolites [29,45,46].

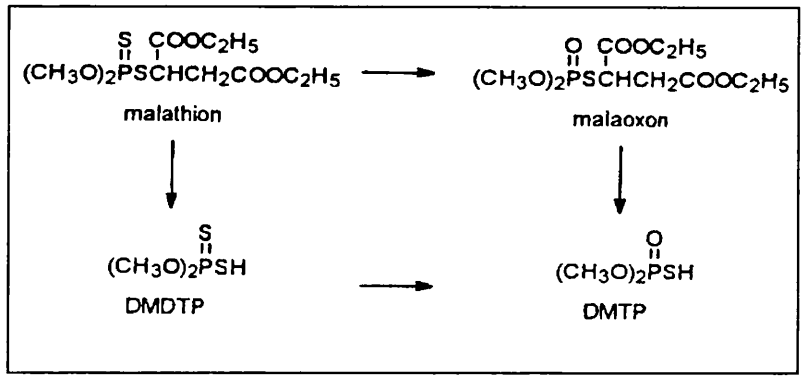


Figure 2.2 Metabolism of malathion in humans

Within one to two days of initial organophosphate binding to acetylcholinesterase, some phosphorylated acetylcholinesterase enzyme can be dephosphorylated (reactivated) by the oxime antidote pralidoxime [47]. As time progresses, the enzyme-phosphoryl bond is strengthened by loss of one alkyl group from the phosphoryl adduct. Pralidoxime reactivation is thereafter no longer possible ("ageing", Figure 2.3).

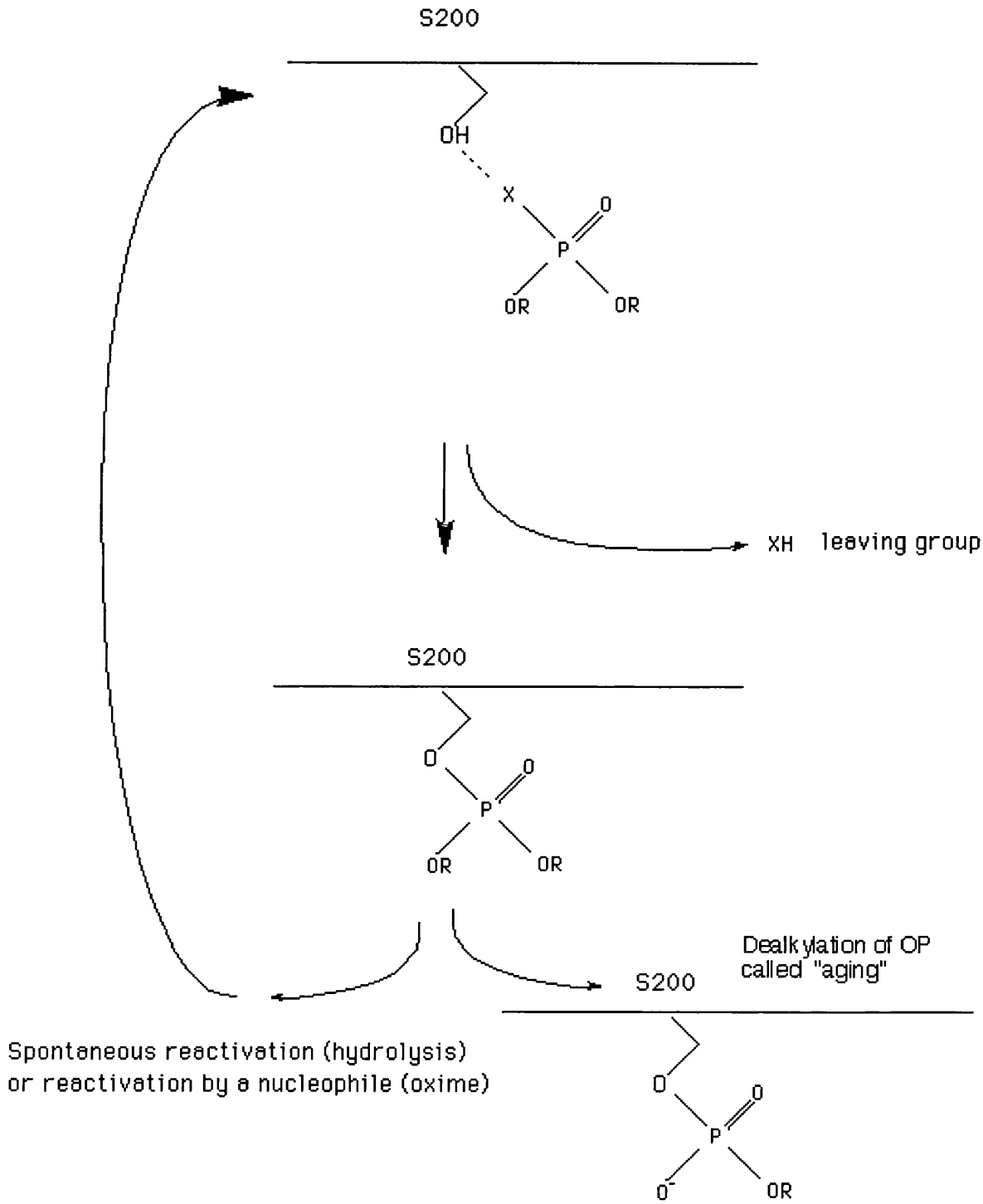


Figure 2.3 The "ESTHER" Chemical Mechanism of Acetylcholinesterase Inhibition. The classical description of inhibition of acetylcholinesterase by organophosphate.

Rarely, certain organophosphates have caused a different kind of neurotoxicity consisting of damage to the axons of peripheral and central nerves and associated with inhibition of "neurotoxic esterase"(NTE). Manifestations have been chiefly weakness

or paralysis and paresthesia of the extremities, predominantly the legs, persistent for weeks to years. Most of these rare occurrences have occurred 8-21 days after an acute poisoning episode of the anticholinesterase type [48], but some have not been preceded by acute poisoning. Only a few of the many organophosphates used as pesticides have been implicated as causes of delayed neuropathy in humans. Thus, Environmental Protection Agency guidelines require that organophosphate and carbamate compounds, which are candidate pesticides, be tested in susceptible animal species for this neurotoxic property.

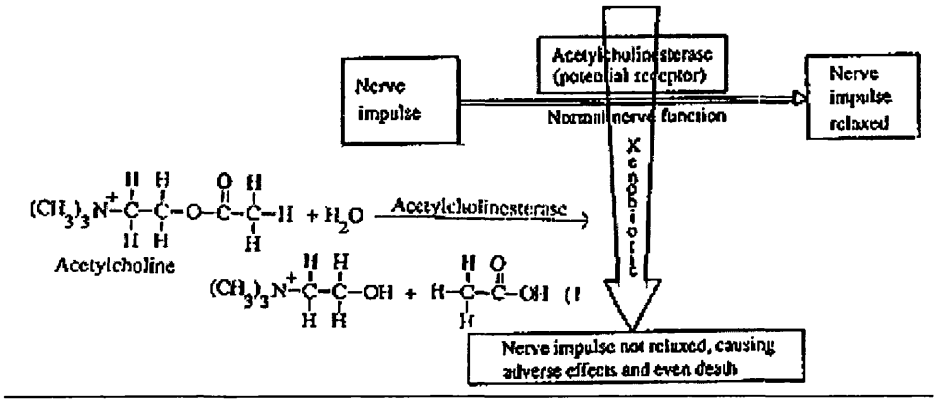


Figure 2.4 Mechanism of inhibition of acetylcholinesterase

Other specific properties of individual organophosphates may render them more hazardous than is suggested by basic toxicity data. By-products can develop in malathion stored long-term which strongly inhibit the hepatic enzymes operative in malathion degradation, thus enhancing its toxicity. Certain organophosphates are exceptionally prone to storage in fat tissue, prolonging the need for antidote as stored pesticide is released back into the circulation. Animal studies have demonstrated potentiation of toxic effects when two or more organophosphates are absorbed simultaneously: Enzymes critical to the degradation of one are inhibited by the other [49]. Whether this interaction is a significant factor in human poisoning is not known.

3. ANALYTICAL TOXICOLOGY

3.1. Introduction

Pesticides are important and diverse environmental and agricultural species. They constitute a large number of widely differing organic compounds many of which are positional, geometrical and optical isomers (chiral and achiral). Also pesticides differ in their degree of ionisation, polarity and solubility in water. Once applied in the environment (including humans), pesticides usually undergo degradation, metabolism and dilution (in humans, metabolism). Thus the determination of various pesticides and their metabolites at trace levels in environmental and human biological matrices has always required separation methods of high resolving power, high separation efficiency, high sensitivity and unique selectivity [50-66].

In the clinical and forensic science fields, the identification (qualitative) and quantification of one or more chemicals are essential in the case of victims whose cause of illness or death is considered to be related to intoxication with drugs or poisons. The main interest is being focused on methodologies capable of rapidly, accurately and sensitively detecting the chemicals and is highly dependent on the development of new analytical instruments, if possible, or techniques. Also taken into consideration is the cost effectiveness of the method, especially in developing countries.

3.2. Chromatographic and Related Techniques for the analysis and Detection of Pesticides

Chromatographic techniques are the common methods of choice for the determination of pesticide residues and their metabolites in biological specimens [43, 45, 46]. Pesticides of wide range polarities often need to be measured at low concentrations in complex biological specimens (autolysed and putrefied samples). Generally the determination of pesticides in biological specimens involves the sampling, sample pre-treatment, and proper analysis involving identification, confirmation and quantification.

Separation of pesticides by chromatographic methods is now routine prior to determining individual components [32,52, 53].

Gas chromatography (GC) and liquid chromatography (LC) are the methods of choice to separate complex mixtures prior to detection [32,43]. GC for a long time has been the preferred method for the analysis of pesticides. Their strong hydrophobic character, their sufficient volatility, and their high thermal stability make them easily amenable to GC analysis using highly sensitive detectors i.e. flame ionisation detector (FID), alkali flame ionisation detector/nitrogen-phosphorus detector (AFID/NPD), electron capture detector (ECD), flame photometric detector (FPD) and the mass spectrometer (MS). Recently capillary electrophoresis [50], LC-MS [54] and enzyme-linked immuno-sorbent analysis (ELISA) [55] have also become new techniques for the analysis of pesticides.

Table 3.1 Examples of methods that have been used for the determination of organophosphorus pesticides in biological specimen.

Pesticide group	Matrix	Extraction method	Detector	Ref.
Organophosphate	Blood CSF, Vitreous Humor, Urine	Liquid-liquid	GC-FTD GC-MS	56
Organophosphate	Blood Liver	SPE	HPLC-DAD GC-MS	57
Carbamate	Blood Gastric Contents	Liquid-liquid	TLC GC-MS	58
Organophosphate, Synthetic Pyrethroids	Plasma Urine	SPE	GC-FID	59
Organophosphate	Blood	Liquid-liquid	GC-MS	60
Organophosphate	Blood, Urine Stomach Contents	Liquid-liquid	GC-FPD HPLC-UV GC-MS	61

Organophosphate Organochlorine Pyrethroids, Carbamate	Blood	SPE	GC-MS	62
Organophosphate	Blood Liver	Liquid-liquid	GC-MS	63
Organophosphate	Serum Urine	Liquid-liquid	GC-NPD GC-MS	64
Organophosphate Organochlorine Carbamate	Blood Tissue Stomach Contents	Liq-liq	GC-FTD GC-FID GC-MS	65
Polychlorinated biphenyl	Serum	SPME	GC-μECD	66

3.3. Sample Handling Techniques (Extraction and Clean-up of Samples)

Many biological specimens cannot be analysed without preliminary sample preparation because either they are low in concentration or the matrix is too complex. Preconcentration is not applicable as most samples received are limited in amount. Even if there are sufficiently large amounts of sample (e.g. kidney and liver) to overcome the limitation of the detection systems, the extract is often too complex for efficient separation by the chromatographic column at low detection levels, so that additional treatment is required before separation. The objective of sample pre-treatment is to provide a sample fraction enriched in all pesticides of interest, and as free as possible from other matrix components. This pre-treatment, which can be achieved in one to three different steps, consists of ;

- extracting traces of analytes of interest from the biological media,
- concentrating these traces,
- removing the other matrix components which have been co-extracted and co-concentrated and which may interfere in the chromatographic analysis (i.e. clean-up).

It is always important to consider the strong interdependence of the various steps of the whole analytical procedure, i.e. sample handling, separation and detection. There is no unique strategy for the pre-treatment of biological specimens for pesticide analysis. It mainly depends on the nature of the solutes to be determined (e.g. volatility or polarity), on the nature of the matrix and on the concentration range required or expected.

Interference removal is a critical step, which is strongly related to the concentration of the analytes of interest and on the nature of the matrix (biological specimen) encountered. Despite the advances in separation and quantification techniques, sample pre-treatment is still the weakest link and is the time-determining step in the whole analytical procedure, accounting for about the two thirds of the total analysis time. It is also the primary cause of errors and discrepancy. Many sample preparation practices are based on usage of common organic solvents, both for liquids (liquid-liquid extraction) and for solids (Soxhlet extraction, ultrasonic extraction0 [31, 42, 56]. The traditional sample preparation methods are typically timed consuming, have multi-step procedures that are prone to losing analytes, and use toxic organic solvents. Also, a new awareness of their hazards has resulted in international initiatives to stop the production of many organic solvents.

Thus, there is a need for the development of sample-handling strategies which are faster, more reliable (which means that the number of intermediate steps, such as transfers, evaporation and derivitisation is diminished) and capable of automation and are as solvent-free as possible.

3.4. Solvent-free sample preparation techniques

The operating principle of any sample preparation method is partitioning of the analytes between the sample matrix and extracting phase. Sample preparation techniques that use little or no organic solvent have been available for some time. They can be classified as in Figure 3.1 [67]. They are:

- Gas phase sample preparation methods, including:

  Headspace (static and dynamic)

  Supercritical fluid extraction (SFE)

- Membrane extraction, including:

  Low-Pressure Gas Stripping

  High-Pressure Gas Stripping

- Sorbent Extraction

  Solid-Phase Extraction (tubes, disks)

  Solid-Phase Microextraction (fibre form)

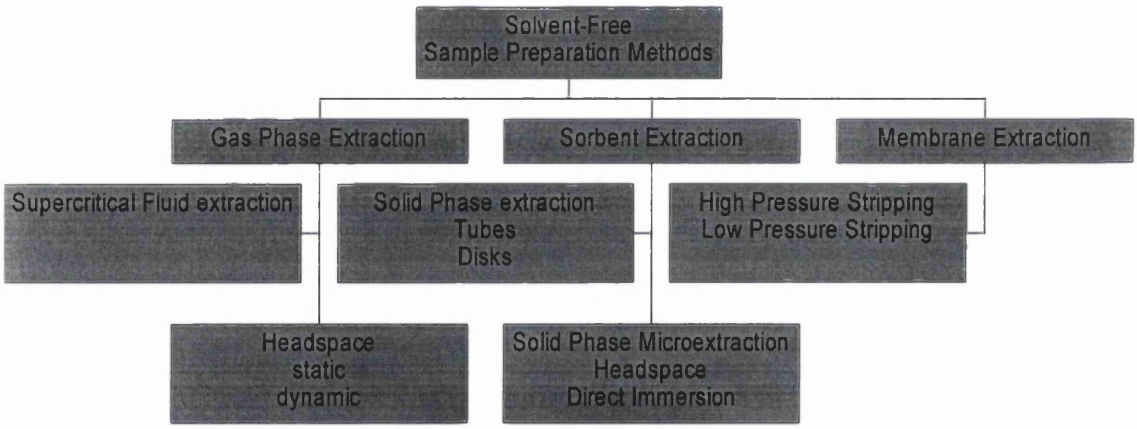


Figure 3.1 Classification of the solvent-free sample preparation

4. SOLID PHASE MICROEXTRACTION (SPME)

4.1. Overview

In 1990, SPME was introduced by Arthur and Pawliszyn [39]. This method is a new, fast, and simple analytical technique which employs a stationary phase, typically of polydimethylsiloxane, coated on a fused-silica fiber to extract analytes from aqueous or gaseous (headspace) samples in sealed vials. The subsequent analyses are currently performed by GC and HPLC and the analytes are desorbed in the injector of the gas or liquid chromatograph. This method represents a further advance as a solvent-free alternative to the extraction of organic compounds from water samples [68-70]. The technique was first applied to the trace determination of volatile organic compounds such as substituted benzenes, toluene and xylenes (BTEX) and chlorinated hydrocarbons [71-73]. Then, with the availability of new fibres, various groups of semi-volatile compounds such as phenols, polycylic aromatic hydrocarbons and polychlorinated biphenyls were successfully extracted from aqueous samples [74-77]. Later, the method was applied to other environmental pollutants such as organophosphorus pesticides, nitrogen-containing pesticides, triazine and 2,6-dinitroaniline pesticides and metolachlor [78-84].

SPME was applied to analysis of halogenated volatiles in food [86], and then drugs in human biological fluids, such nicotine and cotinine in human urine [87], tricyclic antidepressants in urine [88], local anaesthetics in blood [89] and methadone in urine [90].

Headspace SPME had been considered suitable only for extraction of volatiles but the results on tricyclic antidepressant [88] and amphetamines [91] seem to open the applicability of headspace SPME to a number of other solid drugs and poisons of medium-sized molecular weights such as phencyclidine [9 2], organophosphates and carbamate in blood and urine [93-94] and organochlorine compounds in urine [95].

4.2. Description and set-up

An outstanding feature of the SPME sample-preparation method is its simplicity. Organic pollutants are adsorbed from aqueous or gaseous (headspace) samples by the solid phase coating on the silica fibre support. The analytes are then directly transferred to the injector of the gas chromatograph using a modified syringe assembly where they are thermally desorbed and analysed. In contrast to conventional extraction methods, the total amount of extracted sample is used for the determination by GC or HPLC. The SPME device used a conventional syringe (from Hamilton or other supplier), that makes the system portable, and is shown in Figure 4.1.

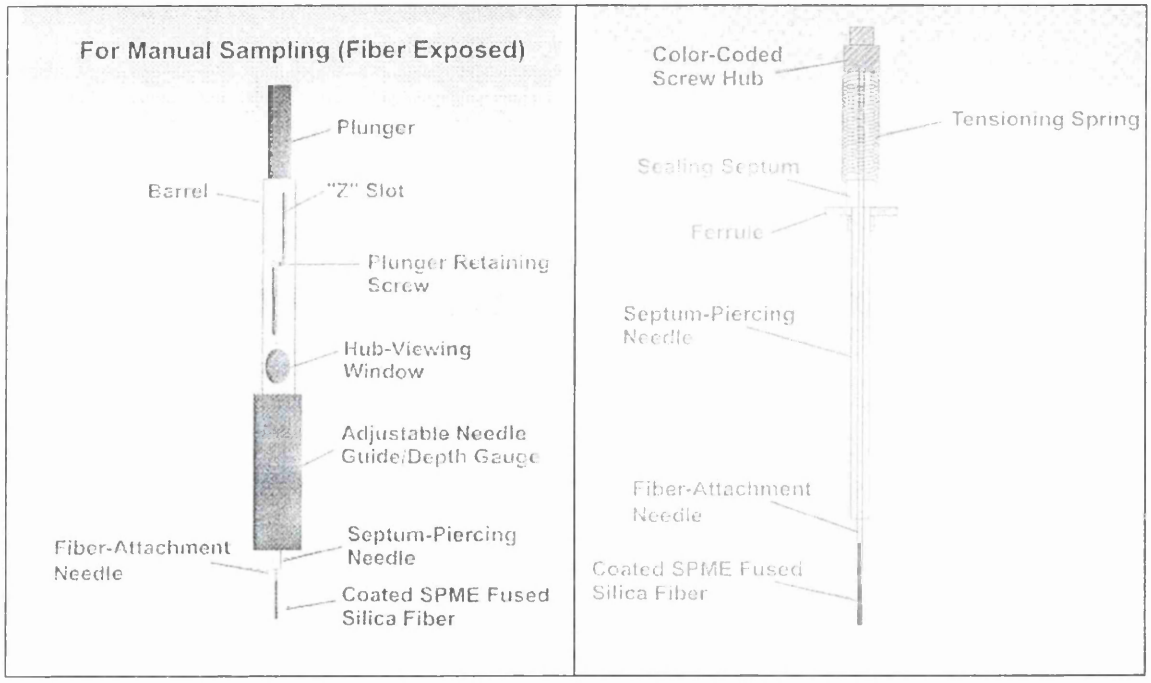


Figure 4.1 Schematic of the SPME device (holder and fibre assembly)

The bottom centimetre of the fibre is coated with stationary phase. The fibre is glued with high temperature epoxy resin into a length of stainless steel rod, which runs up through the syringe needle. When the plunger retracted, the fibre is drawn into the needle and this protects the fibre when the needle is used to pierce the septum of a vial or the GC injector port. The plunger can then be pushed down to expose the fibre to the sample or to the GC carrier gas during the thermal desorption step [67].

The miniature cylindrical geometry of this apparatus permits rapid mass transfer during extraction and then desorption of the concentrated extract into the GC. In the first step, the coated fibre is exposed to the sample or its headspace and the target analytes partition from the matrices into the coating. The fibre with the concentrated analytes is then transferred to the GC where the compounds are thermally desorbed separated and quantified. No special thermals desorption module and no modification of the GC is needed. Temperature programmable, split-splitless and on-column injectors can and have been used for SPME desorption. Organic solvents are completely eliminated in this procedure. The volume of biological sample used is typically in the range 0.5-1.0 ml.

Table 4.1 SPME Fibres (From Supelco Sigma-Aldrich Co.1997)

Phase Types
Non-Polar Fibres Polydimethylsiloxane (PDMS) : 100μm, 30μm, 7μm
Polar Fibres Polaracrylate : 85μm Carbowax®-divinylbenzene (CW-DVB) : 65μm Carbowax-Templated resin (CW-TPR) HPLC Only : 50μm
Bi-Polar Fibres PDMS-DVB : 65μm Carboxen™-PDMS (CAR/PDMS) : 75μm PDMS-DVB HPLC only (Special More Durable) : 60μm

4.3. Approaches to extraction (Extraction Modes)

4.3.1. Direct Immersion

In this mode, the coated fibre is inserted directly into the sample and analytes are transported directly from the sample matrix to the extracting phase.

During the dynamic adsorption immersion process of SPME (two phase system i.e. the sample matrix and the SPME polymer), mass diffusion from the matrix to the SPME polymer film is considered to be the rate determining step in reaching an adsorption equilibrium, and a steady-state diffusion is assumed for SPME in an effectively agitated sampling medium. Mathematical treatment of the adsorption process generates an expression that can describe experimental adsorption /time profiles of the SPME process.

4.3.2. Headspace Configuration

In the headspace mode, the analyte needs to be transported through the barrier of air before it can reach the coating. This modification serves primarily to protect the fibre coating from damage by high molecular weight and other non-volatile interferences present in the sample matrix, such as humic materials or protein.

In Headspace SPME, three phases are involved: the condensed phase, the headspace gas phase and the SPME polymer film. There will be two interfaces: the condensed/gas phase and the gas/polymer phase. The rate-determining step can be either the analyte evaporation from the condensed phase to the headspace or the analyte diffusion from the SPME polymer film surface into its inner layer. Mass transfer in the gas phase is considered to be a fast process.

4.3.3. Membrane Protection Approach

The principle of indirect SPME involves extraction through a membrane which acts as a barrier to protect the fibre against damage, similar to that afforded by headspace when dirty samples are analysed.

4.4. Theoretical Treatment

The principle behind SPME is the equilibrium partition process of the analyte between the fibre coating and the aqueous solution and headspace above it; therefore, analytes are not completely extracted from the matrix. The ideal way to carry out a quantitative analysis when applying SPME is to transfer an analyte completely from a sample matrix to the adsorbing polymer film, but this will be a time consuming process. In practice, once equilibrium is reached, the extracted amount is constant within the limits of experimental error and independent of further increase in extraction time. Thus, if the analytes of interest have small coating/aqueous and coating/gaseous headspace partition coefficients, sampling times could be very fast. In the situation where the compounds have large coating/aqueous and coating/headspace partition coefficients, it is the case of exhaustive extraction and equilibrium is highly unlikely to be reached. Thus in general, a linearly proportional relationship between the amount of the adsorbed analyte and its initial concentration in the sample matrix before partition equilibrium was proposed and proven [96-98].

4.4.1. Basic Principles of Solid Phase Microextraction

SPME methods have 2 steps: partitioning of analytes between the coating and the sample matrix and then desorption of the concentrated extracts into an analytical instrument [67]. Figure 4.2 and Figure 4.3 show schematic extraction and desorption procedure for SPME.

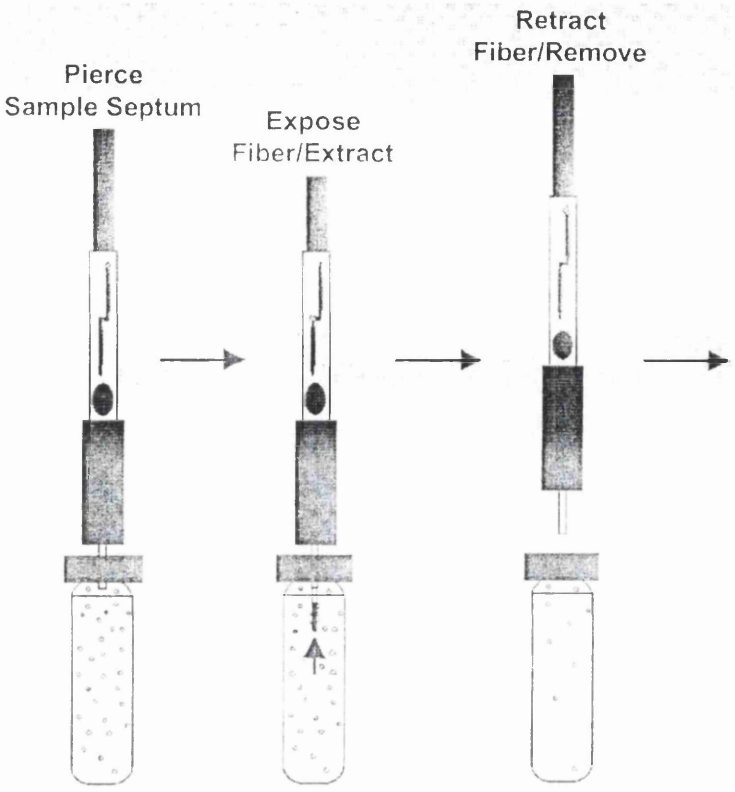


Figure 4.2 Extraction Procedure for SPME

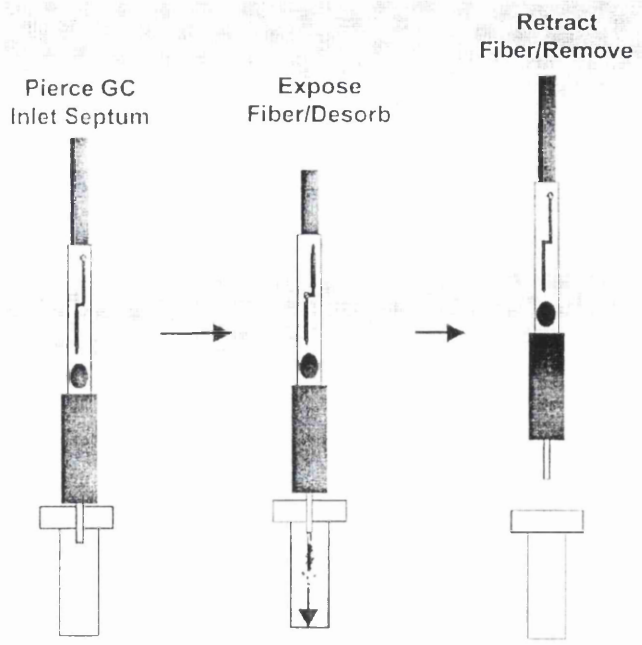


Figure 4.3 Desorption Procedure for SPME

The extraction principles can be explained/described using:-

- Thermodynamic process
- Kinetics process

4.4.1.1.Thermodynamics

SPME is a multiphase equilibration process. Frequently the extraction system is complex, as in a sample consisting of an aqueous phase with suspended solid material/particles having various adsorption interactions with analytes plus a gaseous headspace. Some analysis problems have specific factors to consider, such as biodegradation processes or walls of the sampling vessel adsorbing analytes significantly. For simplification an assumption must be made that is only two or three phases will be considered, namely the fibre coating, the gas phase or headspace, and a homogenous matrix such as pure water, and limited to partitioning equilibrium involving liquid polymeric phases such as PDMS. During extraction, analytes migrate among the two/three phases (depending on whether direct immersion or headspace extraction) until equilibrium is reached.

Because of the physicochemical properties of, for example polydimethylsiloxane (PDMS, melting point:-50°C, glass transition temperature:-126°C), which is most often applied in SPME, the extraction obeys the rules of liquid-liquid equilibrium:

$$\text{Analyte (aqueous)} \Leftrightarrow \text{Analyte (fibre)}$$

$$K^{fw} = C_f/C_W \qquad (1)$$

Where K^{fw} is the equilibrium constant of liquid-liquid equilibrium, C_f is the equilibrium concentration of the analyte in the coating and C_W is the equilibrium concentration of the analyte in the aqueous matrix. The equation (1) can be written as:

$$K^{fw} = n_f V_W/n_W V_f. \qquad (2)$$

And because $n_O = n_f + n_W$ a rearrangement is possible to

$$n_f = (K^{fw} n_O V_f)/(K^{fw} V_f + V_W) \qquad (3)$$

where n_f is the number of molecules in the fibre in equilibrium, n_W is the number of molecules in the aqueous phase in equilibrium, n_O is the number of molecules in the aqueous phase prior to SPME, V_f is volume of coating and V_W is volume of aqueous phase. It is evident from equation (3) that the basis for the quantitative method is given because of the linear relationship between n_O and n_f. However, SPME is an equilibrium extraction but not an exhaustive extraction. A simple rearrangement of equation (3) gives an expression for the recovery of SPME in equilibrium, which is also the maximum recovery {equation (4)}.

$$\text{Maximum recovery} = n_f / n_O = (K^{fw} V_f)/(K^{fw} V_f + V_W) \qquad (4)$$

This equation can be applied for samples with no headspace.

In headspace SPME, equation (3) is extended to equation (8) after rearranging equation(5), equation(6) and equation(7)

$$K^{hW} = C_h/C_W \qquad (5)$$

$$K^{fh} = C_f/C_h \qquad (6)$$

The mass of an analyte extracted by the polymeric coating is related to the overall equilibrium of the analyte in the three-phase system. Since the total mass of an analyte should remain the same during the extraction as the initial amount, we have:

$$C_0 V_W = C_f^{\infty} V_f + C_h^{\infty} V_h + C_W^{\infty} V_W \qquad (7)$$

$$n_f = (K^{fh} K^{hW} V_f n_O)/(K^{fh} K^{hW} V_f + K^{hW} V_f + V_W) \qquad (8)$$

K^{fh} and K^{hW} can be calculated with Henry's Law constants in coating (H_f) and in the water (H_W), respectively [Refer to equation (9) and (10)].

$$K^{hW} = H_W/RT \quad (9)$$

$$K^{fh} = RT/H_f \quad (10)$$

The vapour pressures in aqueous samples (P_W) and coating (P_f) are given by equations (11) and (12).

$$P_W = H_W/C_W \quad (11)$$

$$P_f = H_f/C_f \quad (12)$$

Also

$$K^{fW} = H_W/H_f = K^{hW}.K^{fh} \quad (13)$$

From equation (1) and equations (9)-(12) and since in equilibrium $P_W = P_f$, an alternative equation for the amount extracted can be derived.

$$n_f = (K^{fW} V_f n_O)/(K^{fW} V_f + K^{hW} V_f + V_W) \quad (14)$$

$$\text{Maximum recovery (HS-SPME)} = n_f/n_O = (K^{fW} V_f)/(K^{fW} V_f + K^{hW} V_f + V_W) \quad (15)$$

Accordingly, the recovery of HS-SPME should be much lower than that of direct SPME (Refer to equation 16).

$$\text{Maximum recovery (HS-SPME)/ Maximum recovery (direct SPME)} = 1/1+\{(K^{fW} V_f)/(K^{fW} V_f + V_W)\} \quad (16)$$

The values of K^{fW} are influenced by temperature, salt, pH and organic solvents. The dependence of K^{fW} on temperature is expressed by equation (17) where $K^{f}_{O}{}^{W}$ is the equilibrium constant at T_O and ΔG^{fW} is the free enthalpy of the transfer of analyte between the two phases:

$$K^{fW} = K^{f}_{O}{}^{W} \exp -(\Delta G^{fW}/R).(1/T-1/T_O) \quad (17)$$

$$\Delta G^{fW} = G^{f} - G^{W} \quad (18)$$

$$\ln (K^{fW}/ K^{f}_{O}{}^{W}) = (-\Delta G^{fW}/R).(1/T-1/T_O) \quad (19)$$

Because of the interference of organic molecules with the intermolecular interactions of water the free enthalpy in water (G^W) is always higher than in PDMS (G^f). Thus, according to equation (18) ΔG^{fW} should be negative except for, perhaps, rare cases with high entropy value. It can be concluded that K^{fW} decrease with increased temperature and, therefore, the amount of analyte extracted and the recovery of SPME also decreases.

The relationship between K^{fW} and salt concentration (c_S) can be expressed with equation (20) where K_O^{fW} is K^{fW} at $c_S=0$ and k_S is a specific constant [99]. The higher the concentration of salt the higher is K^{fW} and the amount of analyte extracted [100]

$$\mathrm{Ln}(K^{fW}/K_O^{fW}) = k_S c_S \qquad (20)$$

The relationship between K^{fW} and pH can be described with equation (21) when acids are extracted, where K_O^{fW} is K^{fW} of the undissociated form. The analyte is better extracted at low pH.

$$\mathrm{Ln}\{(K_O^{fW}/(K^{fW})-1\} = \mathrm{pH}-\mathrm{p}K_a \qquad (21)$$

Equation (22) can be used when bases are extracted. The analyte is better extracted at higher pH.

$$\mathrm{Ln}\{(K_O^{fW}/(K^{fW})-1\} = \mathrm{pH} + \mathrm{p}K_a - 14 \qquad (22)$$

Finally the presence of an organic solvent in the aqueous sample usually decreases K^{fW} [101].

4.4.1.2. Kinetics

The relationship of SPME with time as shown in Figure 4.4, for example, was mathematically described in a model, which used several prerequisites with regard to geometry, size of sample and access of analyte molecules to the fibre [102]. If all

analyte molecules have access to the coating, i.e. perfectly agitated model, the time to equilibrium (t_e) is given by equation (23) with r_O being the outer radius of the coating, r_I the inner radius of the coating and D_f the diffusion coefficient of the analyte in the coating. Taking into account the experimental errors, it can be assumed that t_e is reached within 95% ($t_{95\%}$) of the maximal amount extracted. Otherwise, the theoretical t_e is infinitely long according to the model used:

$$t_e=t_{95\%}=(r_O-r_I)^2/2D_f \qquad (23)$$

Not all analyte molecules have simultaneous access to the coating in a more realistic approach. This is described in a model using a hypothetical boundary layer of radius δ with no agitation. Perfect agitation occurs only in the sample outside the boundary layer. The radius δ of this static layer depends on the rate of agitation. The higher the rate the lower is δ and vice versa. The time to maximal extraction can be calculated using equation (24) where D_W is the diffusion coefficient of the analyte in water:

$$t_e=t_{95\%}=3.\delta K^{fW}(r_O-r_I)/D_W \qquad (24)$$

It is concluded that the time of extraction is increased with an increase in K^{fW}, an increase in fibre thickness (r_O-r_I) or with lower diffusion coefficients of the analyte molecule in the sample (D_W). The time of extraction can be decreased with an improved agitation method, thus by decreasing δ. In the case of perfect agitation the minimal time of extraction is reached and t_e only depends on the geometry of the fibre and the diffusion coefficient of the analyte in the fibre [equation (23)]. However it is emphasised that equilibrium is not prerequisite for a quantitative method. The time of extraction t_e is independent of the concentration of the sample. The relative number of molecules extracted at a distinct time (n^t/n_f) is also independent of the concentration of analyte. Finally the absolute number of molecules extracted at a distinct time (n^t_f) is linearly proportional to the concentration of analyte [102]

In HS-SPME, equation (23) is also valid for the estimation of t_e if the aqueous phase and the HS are perfectly agitated. Several variables have to be taken into account for the estimation of t_e in the case of practical agitation [equation (25)]: thickness of

coating, HS and aqueous phase (L_f, L_h and L_W, respectively), revolution rate of the stir bar (N), D_W and diffusion coefficient of the analyte in HS (D_h) as well as K^{hW} and K^{fW}. A simple model was applied with the assumption of only one-dimensional diffusion and R only slightly smaller than the radius of the vial [103].

$$t_e = t_{95\%}$$
$$= 1.8.\{L_h/K^{hW}.(D_h + 2.10^{-5}NR^2) + L_W/1.6(D_W + 0.03NR^2)\}.K^{fW}L_f \quad (25)$$

The dynamics of such models have been mathematically modelled for both stirred and unstirred solutions, by equations for the diffusive and connective transport of analyte in the sample matrix and the extractive phase. The model predicts that rapid extractions are possible if the solution is completely mixed as represented in Figure 4.4 [103].

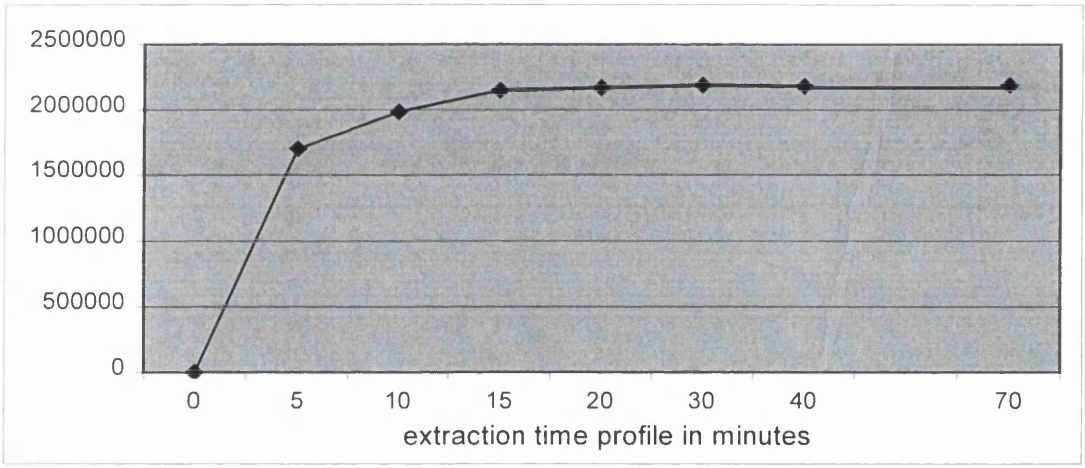


Figure 4.4 Theoretical extraction-time profile when diffusion in the coating determines the extraction rate.

Without mixing, the time required to reach equilibrium is limited by diffusion in the aqueous/water phase. With inefficient mixing, an unstirred layer of water remains next to the fibre, which limits the rate of sorption because the analytes must first diffuse across the static layer.

But in practice, stirring with a magnetic bar fails to provide perfect mixing, so the static water layer increases equilibrium times to a few minutes [89]. Therefore, extractions are usually performed under stirred conditions.

It can also be seen from the above equation that the mass adsorbed and the linear range depend on the partition coefficient and volume of stationary phase. Therefore, the choice of stationary phase is important. The equilibrium time depends on the partition coefficient. The higher the K^{fW} value, the larger is the amount extracted at equilibrium. The disadvantages are that adsorption to the stationary phase is quite different in different compounds and because of this sometimes-low recoveries are obtained [90].

For semi- and rather non-volatile compounds, as are most of the analysed GC-amenable pesticides, the fibre is generally immersed in the aqueous samples.

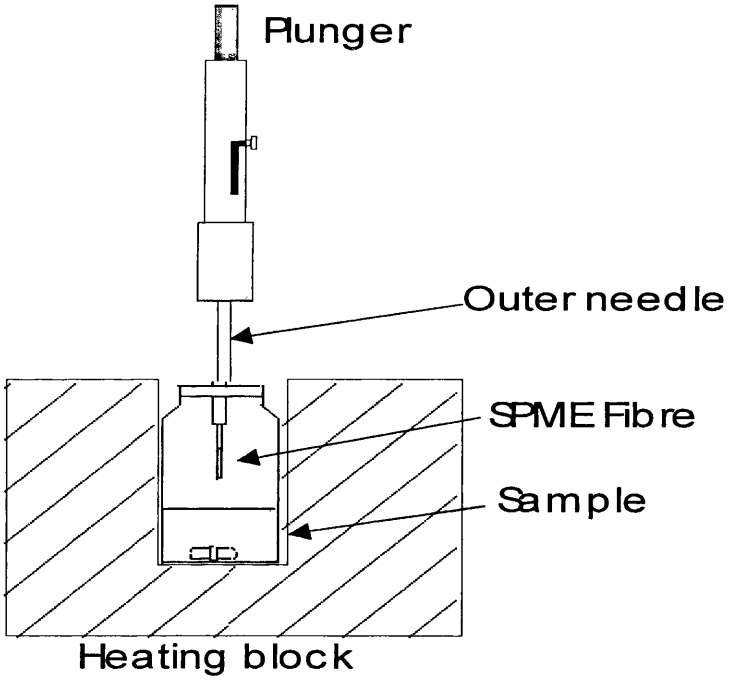


Figure 4.5 Schematic illustration of headspace SPME method

The headspace can be used to sample target organic compounds in very complex matrices such as oily or greasy water and human blood. In those cases, direct SPME sampling will lead to the fibre coating being enclosed by the grease or impaired by large protein molecules. The headspace technique is preferred for volatile analytes, especially in dirty matrices (Figure 4.5).

4.5. Stages in SPME Method Development

4.5.1. Extraction strategy :

- Coating selection
- derivatisation reagent selection
- Extraction mode selection
- Agitation mode selection
- Selection of a manual vs. automated system

4.5.2. Hardware :

- Selection of separation and/or detection technique
- Optimisation of desorption conditions

4.5.3. Initial optimisation :

- Optimisation of sample volume
- Determination of extraction time profile (pure matrix)*
- Determination of extraction time
- Calculation of the distribution constant
- Optimisation of extraction conditions (pH, salt, temperature)

4.5.4. Calibration and validation :

- Determination of linear dynamic range of the method for a pure matrix under optimum extraction conditions
- Selection of the calibration method
- Optimisation of extraction conditions for heterogeneous samples (hair, nail)
- Verification of equilibrium time, sensitivity, and linear dynamic range for complex sample matrices
- Method precision
- Method detection limits
- Validation

- Automation

The selection of the fibre is guided by the nature of the analytes; the non-polar polydimethylsiloxane phase is preferred for the extraction of non-polar pesticides with a very low solubility in water such as organochlorine and some non-polar organophosphorus compounds whereas the more polar polyacrylate fibre is more appropriate for the more polar nitrogen-containing pesticides.

Practically, for compounds of very low volatility, sampling the liquid rather than the headspace is often preferable but if the matrix contains salts and protein or high concentrations of humic acid, headspace is desirable.

Mixing the sample is effective in increasing the response for compounds of low volatility, but it is not necessary for volatile (84)

4.5.5. Effect of the various parameters;

- Desorption conditions.

Optimal desorption conditions are determined by trying various temperatures for different lengths of time. The time and temperature required to desorb the pesticides successfully from the fibre into the column head.

- Time profiles, equilibrium and extraction times.

Equilibrium times are usually determined from the profiles, which consists in reporting on the graph the chromatographic peak areas obtained, or amount extracted, versus the extraction time, from a few minutes to several hours. The equilibrium is reached when a further increase in extraction time does not result in a significant increase in the detector response.

- Partition coefficients (K)

The K value indicates that there is a correlation with solubility in water: the smaller the K values; the more water-soluble is the analyte. Therefore, by reducing the solubility of a given analyte in blood or urine (mostly water), the amount extracted

might be increased. The effect might be observed by varying the ionic strength and/or pH.

- Solvent effect (e.g. methanol)

It is conceivable that high content of organics (e.g solvent) precludes an efficient extraction. Thus, an increased the methanol contents could have an effect on the peak area of the analyte of interest [79,104].

- Sample Volume

Sample volume is an important parameter affecting quantitative results, and contrary to a common belief, it is negligible only in a few cases [72].

The question arises whether a shorter time than the equilibrium time can be selected. However, this might affect the sensitivity and precision of the method. It is therefore important that the extraction time be monitored carefully, because when equilibrium is not established, slight deviations in the extraction times may result in deviation in the amounts extracted.

Also, the modification of the ionic strength and the pH might have an impact on the sorption (affinity) of the analyte for the fibre coating. The more soluble the analyte is in water, the lower will be the affinity of the analyte to the fibre coating. An assessment needs to be made of whether the amount of the analyte extracted by the fibre can be increased by reducing the solubility of the analyte, which can be achieved by the addition of salt or by pH adjustment.

5. METHOD DEVELOPMENT FOR HEADSPACE SOLID PHASE MICROEXTRACTION (HS-SPME) IN ORGANOPHOSPHORUS PESTICIDE ANALYSIS.

5.1. Introduction

In recent years, much attention in analytical chemistry has been paid to sample preparation techniques, especially those, which minimise the consumption of organic solvents. One of the most promising of these is solid phase microextraction (SPME). Since Pawliszyn's introduction of this technique some ten years ago, it has gained momentum not only in the area of trace determination of volatile organic compounds in water but also for drug/pesticide analysis in biological specimens such as blood and urine. The outstanding feature of the SPME sample preparation method is its simplicity. It is a solvent-free technique, if used for headspace analysis, which is preferred in practice, especially for dirty matrices.

Organic compounds are absorbed from headspace samples by the solid/liquid coating on a silica fibre. The analytes are then directly transferred to the injector of the gas chromatograph using modified syringe assembly where they are thermally desorbed and analysed. The amount of analytes absorbed by the polymeric coating is related to the overall equilibrium of analytes in the three-phase system (liquid polymeric coating, headspace, and aqueous solution). Also the kinetics of the mass transport, in which analytes move from the aqueous phase to the headspace and finally to the coating, must also be addressed because it is this process that determines the sampling time of the headspace SPME technique.

Application of SPME to the determination of pesticides in biological samples (blood and urine) has not been fully explored, although a few publications have appeared describing its use to measure pesticides in blood and urine [93, 105, 106, 107]. To ensure reproducibility the matrix needs to be carefully controlled. Conditions, which affect its performance, are assessed, as are the problems, which may arise from its use. Finally, applications to real case studies are made, highlighting the method's sensitivity and precision, and the range of samples where SPME can be used

successfully. In this section will be discussed aspects of the technique that could be applied provided all the necessary steps are taken into consideration.

During Method Development many protocols and steps have to be taken into consideration. This is to achieve the maximum capabilities of the technique introduced. It includes extraction strategy, hardware used, optimisation and finally calibration and validation.

Optimisation for the extraction and equilibrium can be varied and enhanced in a number of ways. Various steps in optimisation and the effects of some parameters must be considered.

Optimisation of the procedure consists of: -

- Selecting the phase type and the thickness of the phase;
- Optimising desorption times and temperatures;
- Optimisation of the sorption process by generating time profiles for each analyte in order to select the exposure time of the fibre under static condition/stirring condition;
- Determination of extraction time;
- Optimising the extraction conditions (temperature, pH, salt);
- Validation;
- Determining the Limit of Detection and Precision;
- Testing the linearity of the method.

5.2. Instrumentation and Apparatus

Hewlett-Packard Gas Chromatograph Model 5890 Series II fitted with a nitrogen phosphorus detector, 0.75 mm splitless insert and HP1capillary column (30 m x 0.53 mm and 0.88 μm phase thickness). The column oven was programmed with oven temperature starting at 100°C (held 2 minutes) ramped at the rate of 10°C per minute to 300°C (held 3 minutes). The injector temperature was 250°C and detector temperature was 280°C.

An assembly for SPME, with replaceable extraction fibre coated with 100 μm polydimethylsiloxane (PDMS) or 85 μm polyacrylate (PA) was obtained from Supelco UK.

Amber headspace vials (4.0 ml volume) were used with screw septum caps fitted with PTFE/Silicone Septa. A Corning hot plate (setting from 25-550 °C)/stirrer (60-1100 rpm) was used with SPME sampling stand (holding 8 vials) and PTFE covered magnetic stirrers (10 x 3 mm). All these items were purchased from Supelco Sigma-Aldrich Com. Ltd.

5.3. Reagents and Standards

Sodium chloride and hydrochloric acid were AR grade. Standards of Malathion, Parathion, Diazinon, Fenthion, Ethion, Quinalphos, Fenitrothion, Prothiofos, Profenofos, Methyl-Parathion, Chlorpyrifos, Coumaphos were supplied by Promochem Limited (UK). Stock solutions (1 mg/ml) were prepared individually by dissolving appropriate amounts in methanol. Blank blood for preparation of blood standards used time-expired red blood cells from the blood transfusion service.

5.4. Methods

5.4.1. Headspace SPME Set-up

The polydimethylsiloxane (PDMS)-coated fibres for SPME were pre-treated overnight in the GC injection port at 250 ^{0}C (polyacrylate fibres at 300 °C) prior to usage, to remove contaminants from the fibres. The fibres were subsequently tested for carry-over by injecting the fibre after every run.

5.4.2. Working Standards Solution

Eleven organophosphate working standards were prepared in methanol at a concentration of 10 μg/ml by diluting an appropriate amount from the stock solution

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5.4.3. Fibre comparison

A comparison was made between polydimethylsiloxane and polyacrylate fibres with respect to their affinity for organophosphates; the fibres were exposed to blood containing organophosphate pesticides at a concentration of 1000 ng/ml (each) at a sample temperature of 90 °C.

5.4.4. Optimisation of Desorption Conditions (carry-over).

The SPME fibre was exposed to 1.0-ml blood samples containing organophosphate pesticides at a concentration of 1000 ng/ml for 20 minutes and was then injected into the gas chromatograph and left for various time intervals (1 minute, 2 minutes, 3 minutes, 4 minutes and 5 minutes). The peak height was recorded and chromatograms were checked for carry-over and for the presence of interfering peaks.

5.4.5. Extraction time profile

Individually the eleven standards were spiked into blood at a concentration of 1000 ng/ml. 1ml blood was placed in a 4.0 ml amber vial. The vial was then placed in a vial receptacle and placed over a heated hot plate/stirrer at 80°C. One series of vials were stirred and a second series were not. After heating for 10 minutes, the septum-piercing needle of the SPME was passed through the septum (Figure 4.5). The pre-treated fibre was pushed out from the needle and exposed in the headspace for periods ranging from 5 minutes to 70 minutes to allow the adsorption of the compounds. After exposure, the fibre was withdrawn into the needle and pulled out from the vial. It was then injected into the injection port of the gas chromatograph. The fibre was exposed for 5 minutes to ensure complete desorption of the compounds. The same procedure was carried with vials heated at 90°C on the stirrer hotplate.

5.4.6. Additives and pH Adjustment

Four sets of 5 vials were set up, each vial containing 0.5-ml blood spiked with 1000 ng of the analytes.The first set was diluted with 0.5-ml distilled water. The second set

was diluted with 0.5-ml 0.1M HCl. The third set was diluted with 0.5-ml of saturated saline, and the forth set was diluted with 0.5-ml water, 100 μL 0.1M HCl and 100 μL saturated sodium chloride.

5.5. Results and Discussion

5.5.1. Coating Selection

Two commercially available fibres, PDMS and PA, were compared. The first stationary phase has liquid properties while the second one is solid. These differences basically affect the absorption equilibrium times for the analytes. Faster diffusion and consequently lower equilibrium times must be expected when using the liquid PDMS than when using the solid PA. On the other hand, PDMS is non-polar whereas PA is more polar. Because of this, the affinity for the selected pesticides, which have intermediate polarities, should be stronger using the PA fibre.

Bearing in mind these aspects, comparison of the two fibres was made by extracting samples of 500ng of each pesticide/ml blood during 30 minutes. This time was chosen because it allows the extraction of a new sample during chromatographic analysis of the precedent sample.

As shown in the bar graphs in Figure 5.1, the extracted amount was greater with PA for most target pesticides compared to PDMS. However the PDMS was chosen to perform the rest of the experiments because the equilibrium condition was attained in a shorter time i.e. 30 minutes for malathion and diazinon (see Figure 5.3 and Figure 5.4.) compared to using PA [81].which needed more then 100 minutes to attain equilibrium. Also, most published scientific papers acknowledge the use of PDMS fibres [93, 105, 107].

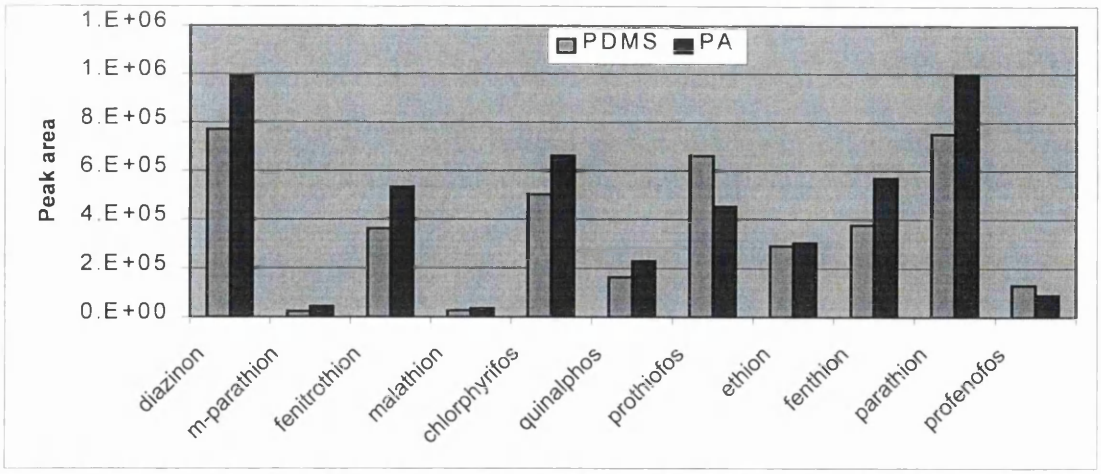


Figure 5.1 Extraction performance between PA and PDMS fibre coating from blood spike with 1000 ng/ml organophosphorus pesticides each.

5.4.1. Agitation versus non-agitation

The dynamics of the extraction process have been studied in depth by Louch et al. [69], obtaining mathematical models of the process. One of the conclusions obtained is that in a real situation the extraction is controlled by the efficiency of the mixing technique. Stirring was optimised by using the speed settings on the stirrer/hotplate (settings 1-10, which correspond to approximately 60-1100 rpm). It was set at no.10 for standardisation of subsequent tests. By analysing 1000ng of each pesticide per ml blood stirred and unstirred, it was found that the unstirred samples gave a higher GC peak for most pesticides (Figure 5.2). However, stirred samples were expected to reach equilibrium much faster (Figure 5.3 and Figure 5.4.). Thus, all subsequent analyses were stirred using magnetic stirring bars.

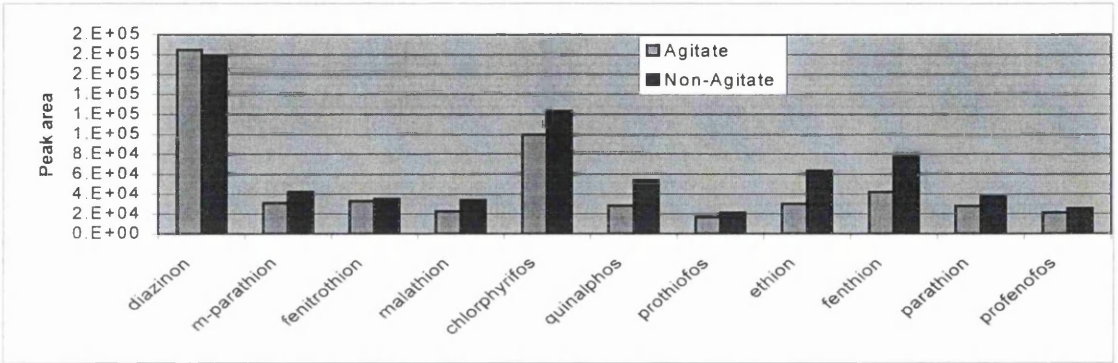


Figure 5.2 Comparison extraction efficiency between agitation and non-agitation

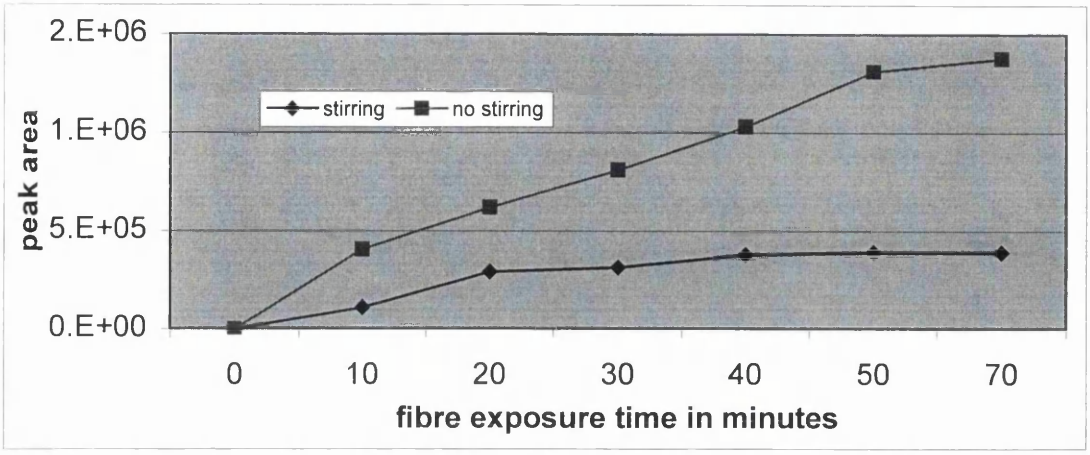


Figure 5.3 Extraction time profile for malathion with respect to agitation and non-agitation at 80°C

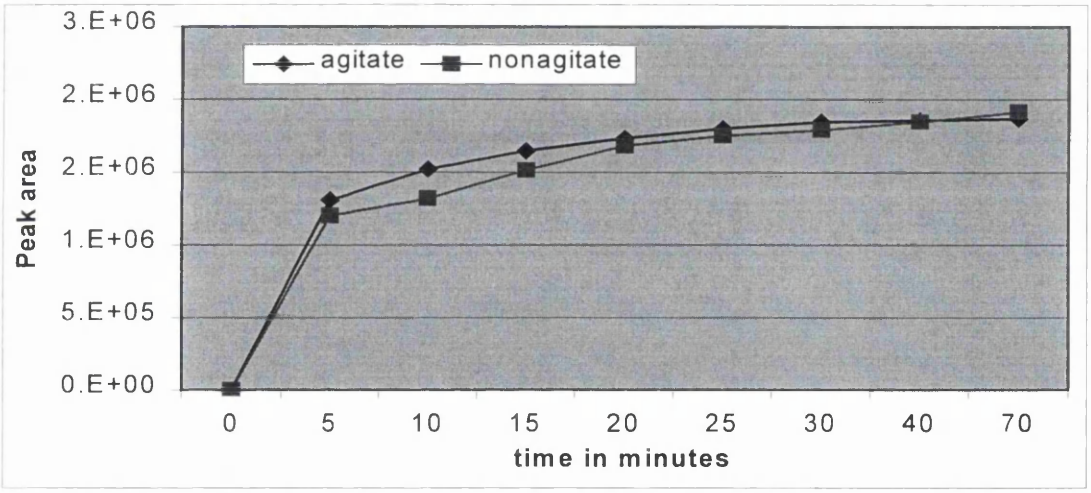


Figure 5.4 Extraction time profile for diazinon with respect to agitation and non-agitation at 80°C

5.4.2. Temperature

The effect of temperature was studied by sampling 500 ng/ml of the studied pesticides under different conditions (80°C and 90°C). Figure 5.5 of bar graph showed that response was better at higher temperature for most pesticide except fenitrothion. Therefore 90°C was used as sample extraction temperature in subsequent work.In practice, there are two parameters directly affected by temperature. The diffusion of the analytes in the blood (aqueous phase) increases as temperature rises. Thus, the

extraction, limited basically by mass transfer, is more efficient at higher temperatures. However the absorption is an exothermic process and increasing temperature has a negative effect on malathion but not on diazinon (Figure 5.6 and Figure 5.7).

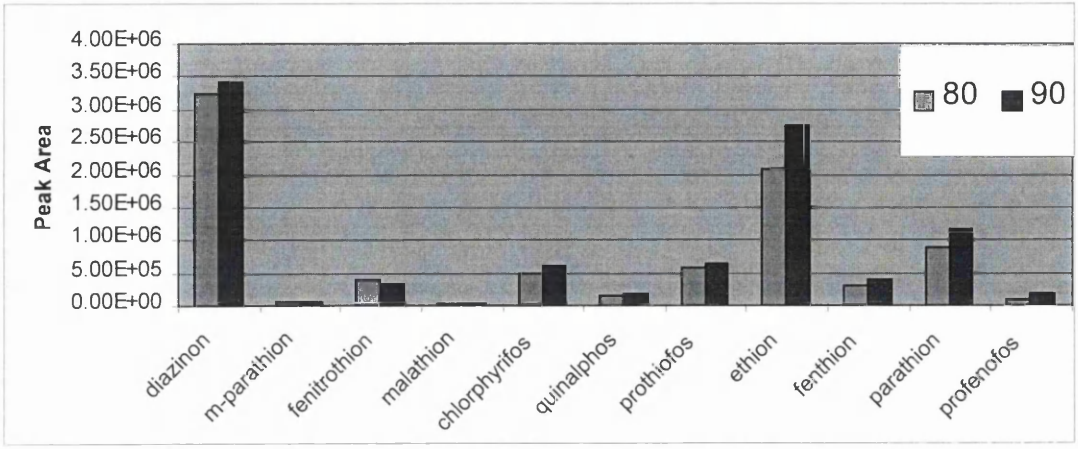


Figure 5.5 Effect of temperature on absorption.

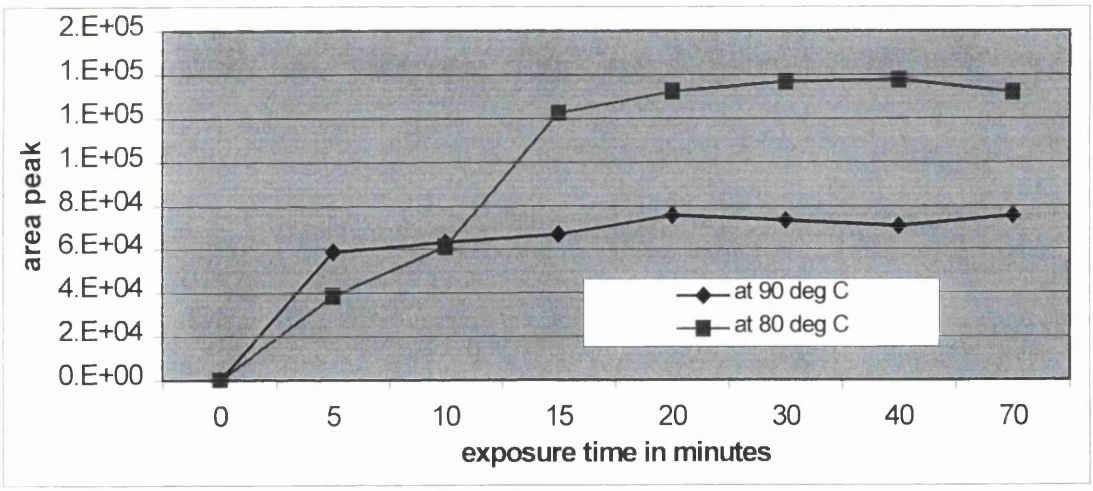


Figure 5.6 Extraction time profile with respect to temperature for malathion

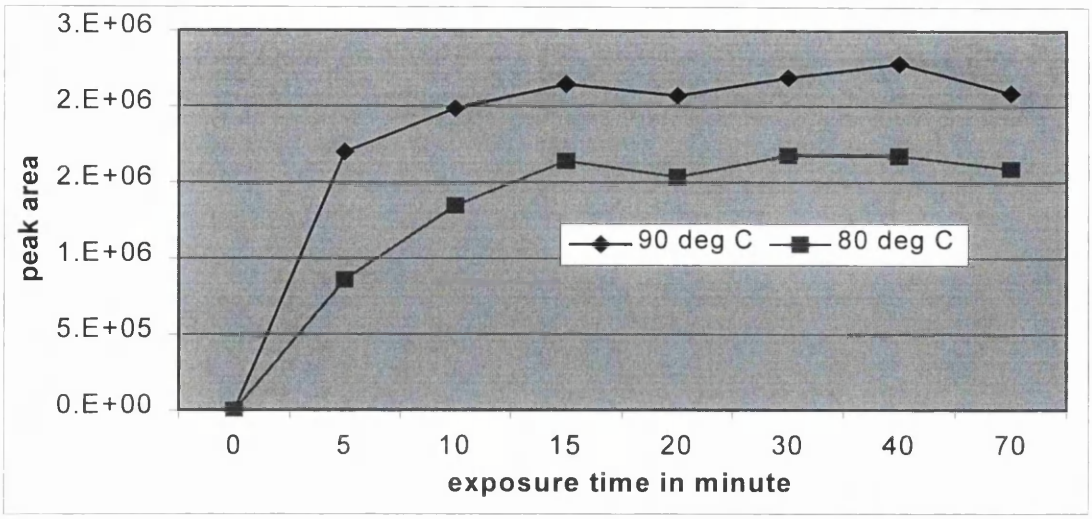


Figure 5.7 Extraction time profile with respect to temperature for diazinon

5.4.3. Absorption-time profiles

The absorption-time profile for each organophosphate pesticide was studied by monitoring the GC peak area counts as a function of exposure time. Figure 5.8 represents the performance of the PDMS fibre for all analytes of interest, showing that some analytes reached the equilibrium after 30 minutes (see also Figure 5.6 and Figure 5.7 for malathion and diazinon respectively). Diffusion of analytes is the limiting step in the absorption process, so high molecular-mass compounds are expected to have longer equilibrium times than low molecular mass analytes because of their smaller diffusion coefficients e.g. ethion (Figure 5.9)

Although the advantages of using the equilibrium time as the absorption period are interesting (due to precision), the need for a practical time for the analysis must also be taken into consideration. Thus a time of 30 minutes was taken as the preferred exposure/extraction period.

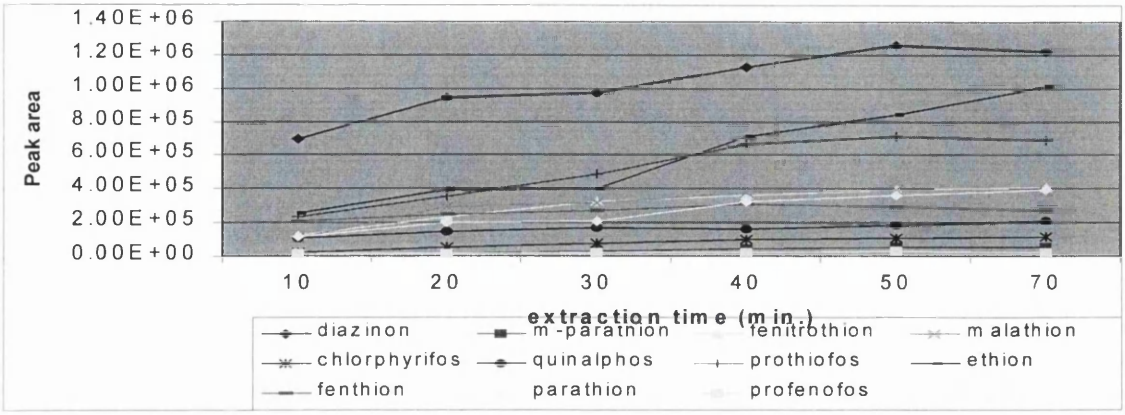


Figure 5.8 Extraction time profile

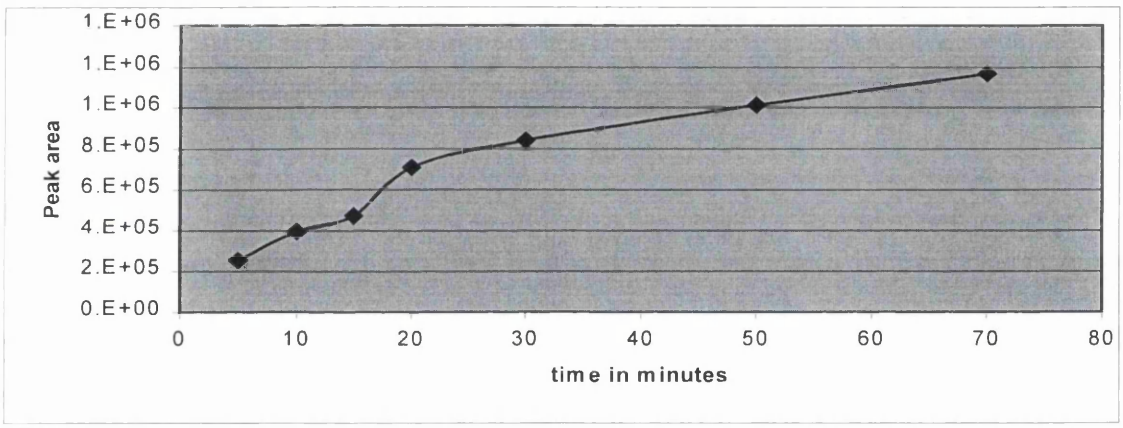


Figure 5.9 Extraction time profile for ethion

5.4.4. Effect of pH adjustment and salt addition

The effects of ion strength and pH on the extraction of analytes were investigated simply by adding saturated NaCl and using 0.1M hydrochloric acid (HCl). Figure 5.10 represents the effect of these parameters on the relative extraction efficiency for selected pesticides. Lowering the pH increases the extraction for most of the pesticides except diazinon and methylparathion. The efficiency of extraction is enhanced because the compounds are kept undissociated.

Theoretically the addition of salt should enhance the extraction but from the results shown in Figure 5.10 salt addition lowers the response, except for diazinon and methylparathion. However, the combination of both salt and acid additives does give good precision on the overall method of extraction [93, 105].

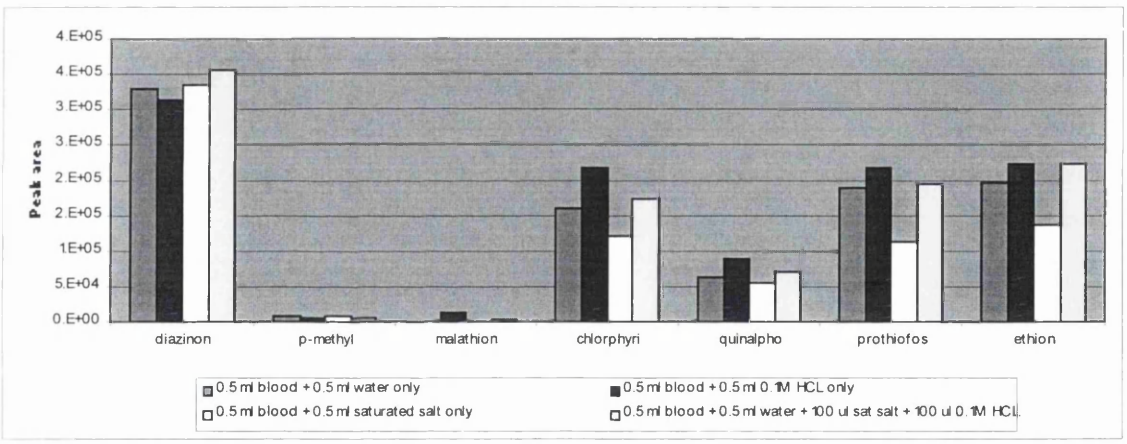


Figure 5.10 Effect of pH and salt adjustment

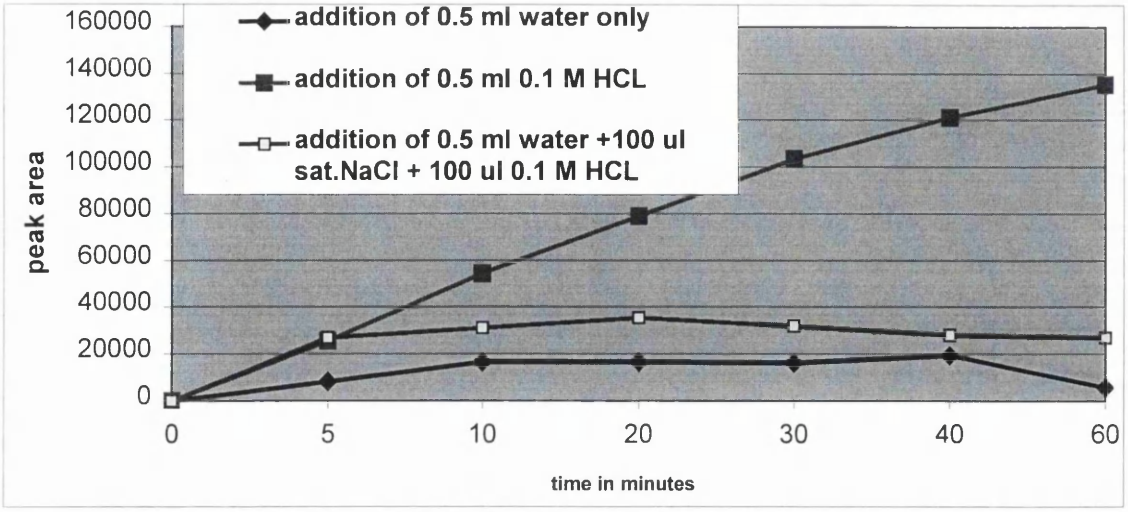


Figure 5.11 Effect of acid and salt addition on extraction time profile for malathion in blood at 90°C with stirring

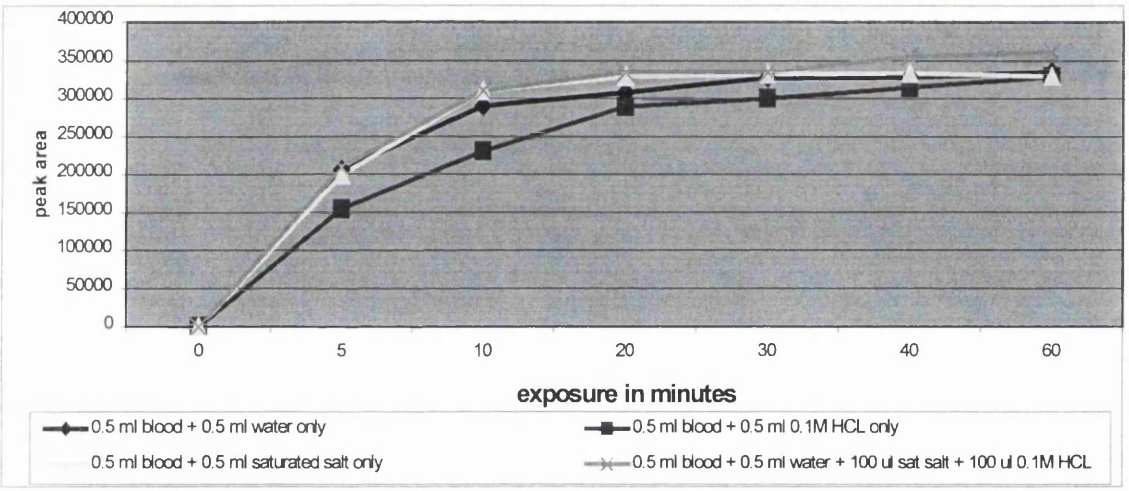


Figure 5.12. Effect of acid and salt addition on extraction time profile for diazinon in blood at 90°C with stirring.

Figure 5.11 shows the effect of only using acid addition for malathion analysis. Even though a higher response was observed, the equilibrium was not reached after 70 minutes compared to the situation in which addition of salt and pH adjustment were both included. Equilibrium was attained after 30 minutes. For diazinon there was not much difference with respect to equilibrium time, which was attained after 20 minutes in whatever conditions (Figure 5.12).

5.4.5. Final protocol for extraction of organophosphorus pesticides.

Spiked blood samples and real case blood samples, volume 0.5 ml, were diluted with 0.5 ml distilled water followed by addition of 100 μL of 0.1M HCL and 100 μL saturated sodium chloride solution. Extraction time consisted of 10 minutes sample conditioning and 30 minutes fibre exposure to the sample headspace. The sample temperature was set at 90°C. All samples were agitated at speed level 7 (on the hotplate stirrer) to obtain consistency. The same conditions were applied to urine.

5.4.6. Limit of Detection (LOD), Precision and Recoveries

For the LOD, dilutions of the stock standard solutions were used to prepare low concentrations of organophosphate pesticides in spiked blood samples, and these were extracted and absorbed using the protocol described above. In this case the LOD was considered to be the concentration that gave a signal to noise ratio of 3.

The precision of the method was determined by analysing five replicate samples consecutively. The same technique was applied to urine samples.

The recoveries were calculated by comparing the peak areas obtained from the extracts of the spiked blood with that obtained from non-extracted authentic pesticides dissolved in methanol and injected directly into the gas chromatograph (10 ng each standard).

These results are summarised in Tables 5.1 - 5.3.

Table 5.1 Limit of Detection and Precision Study (n=5) in 0.5 ml blood based on proposed procedure

Pesticide	Limit of Detection (ng/0.5 ml)	Precision Study (RSD%) @ 200 ng/0.5 ml Day 1	Precision Study (RSD%) @ 200 ng/0.5 ml Day 2
Chlorpyrifos	10.0 ng	5.2	6.5
Diazinon	1.0 ng	3.5	4.8
Ethion	10.0 ng	6.2	4.2
Fenithrothion	15.0 ng	10.5	14.0
Fenthion	5.0 ng	4.9	3.8
Malathion	50.0 ng	6.7	4.4
Methyl Parathion	40.0 ng	12.4	10.0
Parathion	30 ng	9.3	7.3
Profenofos	50 ng	5.9	8.1
Prothiofos	30 ng	5.3	3.5
Quinalphos	40 ng	7.1	6.1

Table 5.2 Limit of Detection and Precision Study (n=5) in 0.5 ml urine based on proposed procedure

Pesticide	Limit of Detection (ng/0.5 ml)	Precision Study (RSD %) @ 50 ng/0.5 ml Day 1	Precision Study (RSD%) @ 50 ng/0.5 ml Day 2
Chlorpyrifos	5.0	5.4	3.7
Diazinon	1.0	4.3	5.5
Ethion	5.0	6.3	3.9
Fenithrothion	5.0	7.4	6.4
Fenthion	5.0	5.6	7.7
Malathion	30.0	5.9	6.2
Methyl Parathion	20.0	7.9	8.1
Parathion	50.0	6.3	6.4
Profenofos	10.0	5.3	5.4

Prothiofos	20.0	4.9	4.9
Quinalphos	30.0	5.5	4.6

Table 5.3 Recoveries for various pesticides each at a concentration of 200 ng/0.5ml blood and 50 ng/0.5 ml urine

Pesticides	Recoveries (%) Blood	Recoveries (%) Urine
Chlorpyrifos	3.4	6.0
Diazinon	5.8	9.2
Ethion	4.1	27.3
Fenithrothion	0.9	10.1
Fenthion	2.7	21.4
Malathion	0.4	6.0
Methyl Parathion	0.5	3.5
Parathion	0.7	1.1
Profenofos	0.5	0.8
Prothiofos	0.3	0.7
Quinalphos	0.4	0.9

Figure 5.13 and Figure 5.14 show the chromatograms obtained with blank and spiked blood samples respectively. Background noise and interference from the matrix were minimal and did not interfere with the peak of interest. Figure 5.15 and Figure 5.16 illustrated the chromatogram of a blank and spiked urine sample respectively.

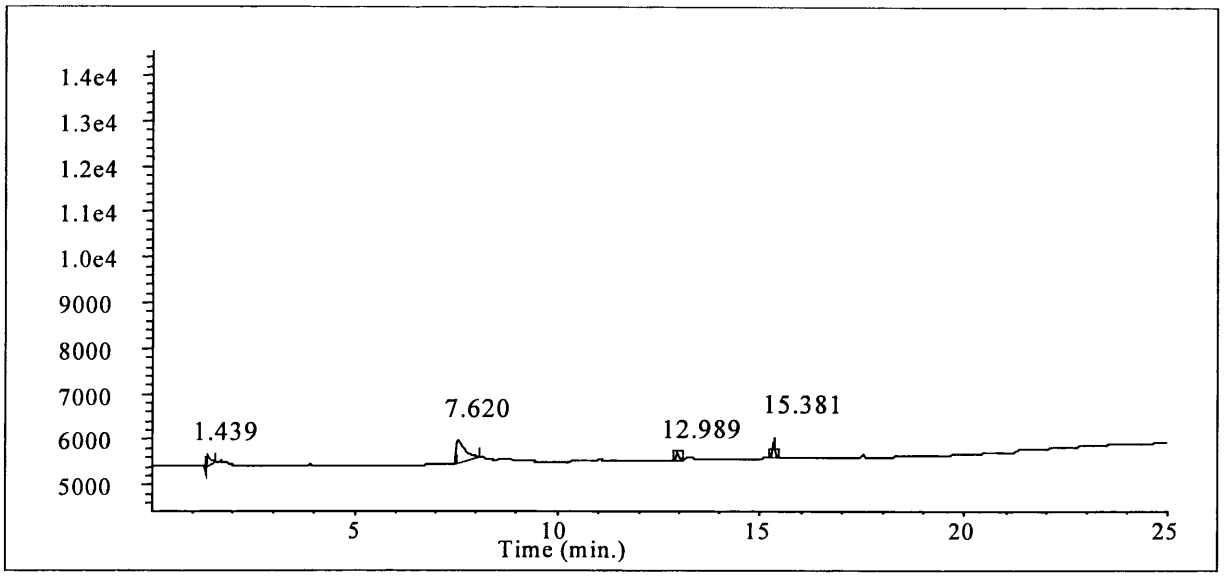


Figure 5.13 Typical Chromatogram of blank blood by HS-SPME

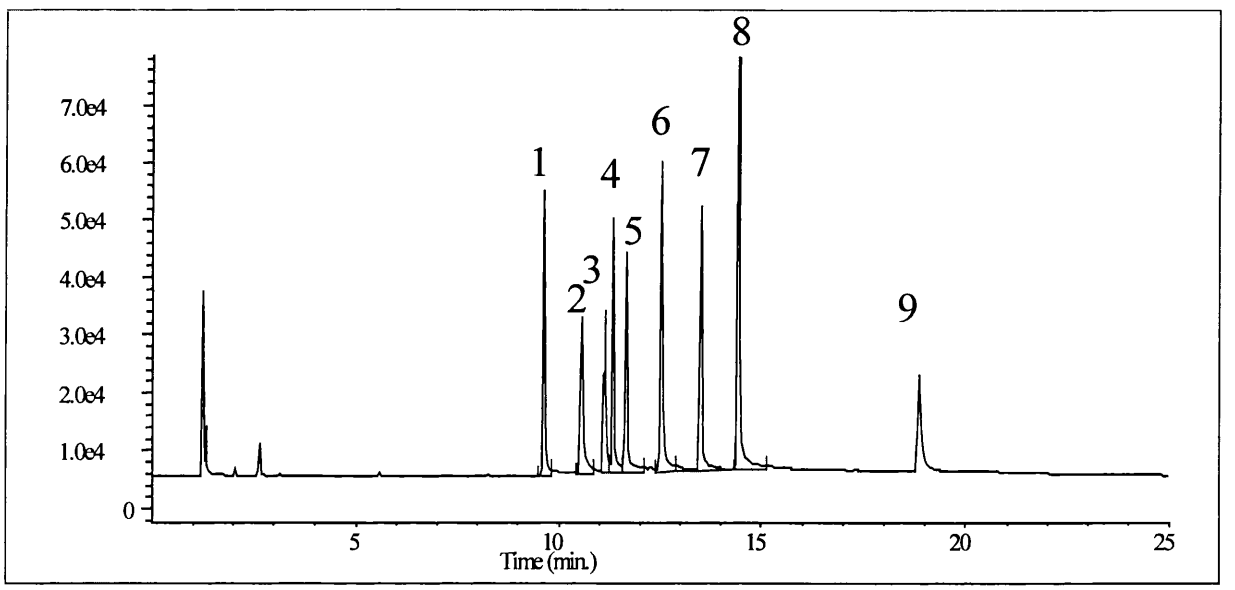


Figure 5.14 Chromatogram of standard mixture (200ng/0.5ml blood) by HS-SPME

Key: 1=diazinon, 2=methyl-parathion, 3=fenitrothion, parathion, 4=malathion, 5=fenthion, chlorpyrifos, 6=quinalphos, 7=profenofos, prothiofos, 8=ethion., 9= coumaphos

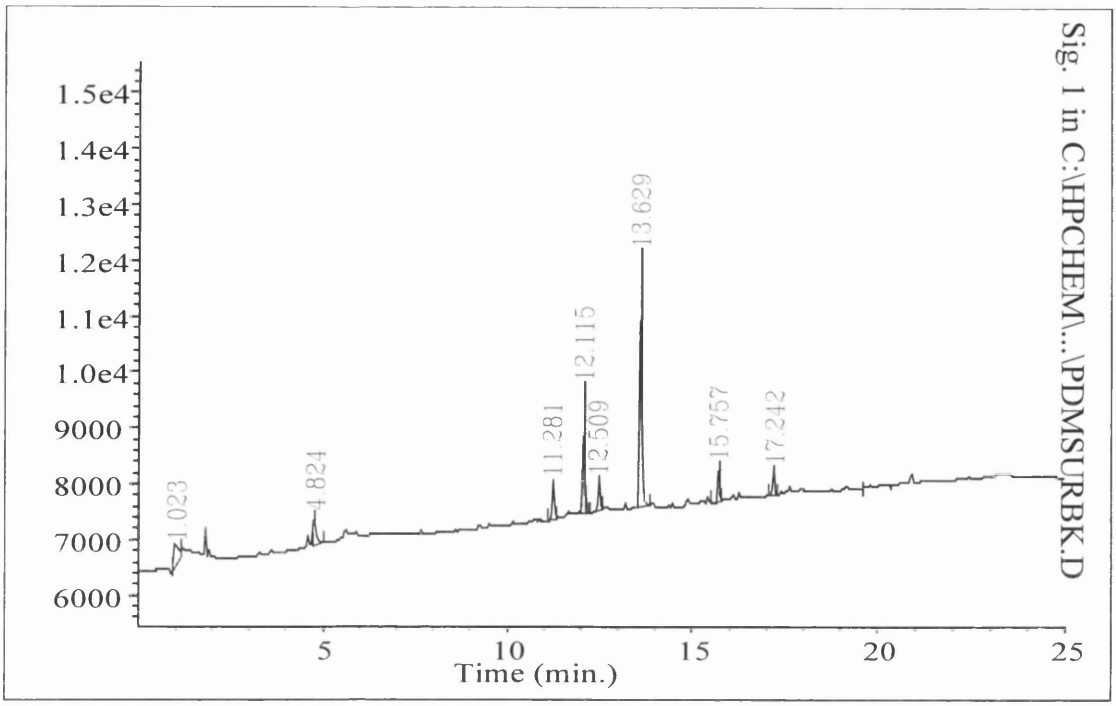


Figure 5.15 Chromatogram of blank urine by HS-SPME

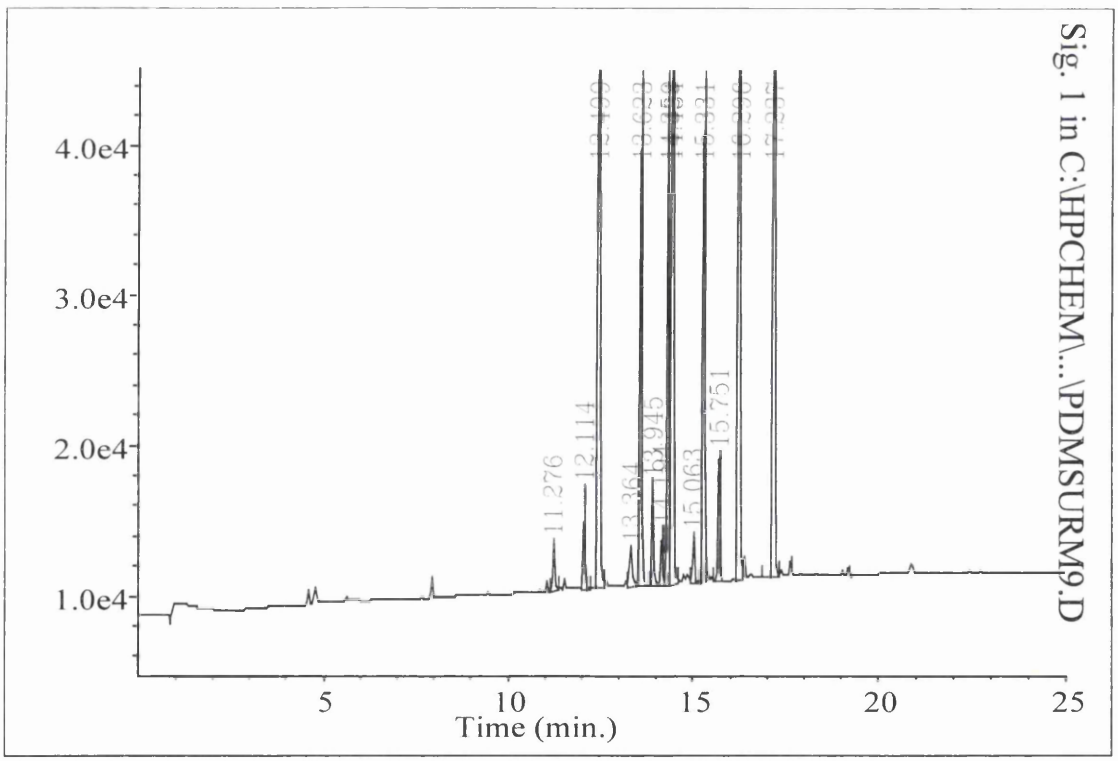


Figure 5.16 Chromatogram of spike mix standard (50ng/0.5ml urine) by HS-SPME

The sampling method (headspace or immersed), the pH, salt content, temperature, volume of the sample, sorption and desorption time frame affect results from SPME.

The selection of the fibre is guided by the nature of the analytes; generally the non-polar polydimethoxysilane is preferred for the extraction of non-polar pesticides with a very low solubility in water such as organochlorine pesticides and some non-polar organophosphorus compounds whereas the more polar polyacrylate fibre is considered to be more appropriate for the more polar nitrogen-containing pesticides.

In the present work, a PDMS 100μm fibre was selected because of its polarity, the thickness of the coating on the fibre, and the high sample capacity, which render it suitable for a wide variety of applications [65, 73, 84]. Also, equilibrium is achieved much faster compared to the more polar phases such as polyacrylate.

The extraction time depends on the partition coefficient of the analyte and on agitation of the sample, and is generally shorter in headspace [62]. Practically, for compounds of very low volatility, sampling the liquid rather than the headspace is often preferable but if the matrix contains salts and protein or high humic acid, headspace is desirable [62].

Mixing the sample is effective in increasing the response for compounds of low volatility, but it is not necessary for volatiles [84] which is also shown in Figure 5.4.Optimising a solvent-free method is quite complex because in sorbent extractions the extracting phase does not interact directly with the matrix and in headspace the interaction is very weak. Heating can release analytes from matrix, thus sample temperatures chosen were 80°C and 90°C. The analytes are expected to be quite stable at such temperatures. But the sample temperature has a double impact at higher temperatures: diffusion coefficients in blood (mostly water) are higher and extraction time is shorter, but partition coefficients are also lower (Figure 5.3 and Figure 5.4).

Heating alone is sometimes inadequate because analytes are bound too strongly or the matrix or analytes are unstable at higher temperature. Addition of a soluble salt into the sample increases the ionic strength of the solution. This makes organic compounds less soluble and the partition coefficients can be significantly higher, but this not the case with malathion as shown in Figure 5.10. Combination of pH adjustment (since extraction is more effective if the compounds are kept

undissociated) and addition of salt showed an increase in response and also the equilibrium was attained much faster Figure 5.11 and Figure 5.12.

It is important to remember that in SPME neither complete extraction of analytes nor full equilibrium are necessary but consistent sampling time, temperature, headspace volume are crucial for reproducibility. Precision can be a problem if careful timing is not observed (which can be overcome by using automated SPME) whereas carry-over was not a problem with using PDMS fibre. Precision (RSD), detection limit (LOD and recovery were tabulated in Table 5.1, Table 5.2 and Table 5.3.

5.6. Conclusion

Solid phase microextraction is an emerging sample preparation technique, which is very suitable for the analysis of not only volatile but also semi volatile substances such as pesticides in biological samples. It offers complete elimination of organic solvents from the extraction process and thus greatly simplifies it. It is also rather inexpensive and rapid. As extraction and concentration steps are combined, the whole of the extracted analyte is introduced into the analytical system, in this case gas chromatography, and the detection limit are quite reasonable for most of the pesticides analyse. The technique was later applied for qualitative and quantitative study on to real cases shown in Chapter 5, Chapter 6 and Chapter 11 and Chapter 12. The method was also used in Chapter 10.

6. HEADSPACE SOLID PHASE MICROEXTRACTION (HSSPME) IN CASES OF FENTHION POISONING.

6.1. Introduction :

Fenthion (O, O-dimethyl-O-[3-methyl-4-(methylthio)-phenyl]-thiophosphate) is an organophosphate insecticide. Fenthion as an insecticide (Trademark: Lebaycid®) was discovered in the late 50s and is one of the oldest organophosphates still on the market [108]. It offers a broad spectrum of activity as well as fast knockdown action coupled with long residuality. Due to these unique product-specific features fenthion is still an indispensable tool for farmers today [109]. However, use of fenthion is likely to pose unreasonable risks to human health and the environment and very high toxicity to birds, fish and aquatic invertebrates [110, 111]. Fenthion is used mainly in rice against hoppers, bugs and stemborers and in fruiting crops (e.g. olives, citrus, and stone fruit) against fruit flies. Fenthion is registered in more than 30 countries throughout the world.

6.2. Materials and Methods

6.2.1. Reagents and standards.

Fenthion and Diazinon were purchased from Promochem Limited, Hertz, England. Stock solutions (1 mg/ml) were prepared by dissolving an appropriate amount in methanol. Standard working solutions of 10 μg/ml of each were prepared by diluting the stock solution. Blank blood for preparation of blood standards used time-expired red blood cells from the blood transfusion service.

Authentic case samples of blood were obtained from the Chemistry Department, Kuala Lumpur and from the office of the Forensic Medical Examiner, Mauritius.

6.2.1. Apparatus

A manual assembly for SPME with replaceable extraction fibre, coated with 100 μm polydimethylsiloxane or polyacrylate 85 μm was from Supelco UK. Amber headspace vials (4.0 ml volume) were used with screw septum caps fitted with PTFE/Silicone Septa. A Corning hot plate (setting from 25-550 °C)/stirrer (60-1100 rpm) was used with SPME sampling stand (holding 8 vials) and PTFE covered magnetic stirrers (10 x 3 mm). All these items were purchased from Supelco Sigma-Aldrich Com. Ltd.

6.2.2. Gas Chromatography NPD and Mass Spectrometry

Gas Chromatography used a Hewlett-Packard Model 5890 Series II chromatograph equipped with a nitrogen-phosphorus detector, and an HP-1 capillary column (30 m x 0.25 mm i.d. x 0.25 μm phase thickness). Oven/Column conditions were: 100°C for 2 minutes, rising to 300°C at 10°C/minute and held for 10 minutes. The temperature of the injection port was set at 250°C and detector at 280°C. Splitless injection mode was used for the first 5.0 minutes.

GC-MS was carried out using a Thermo-Finnigan Trace GC linked to a Trace MS, with an HP-5 X-link column (5%Ph, 95% Me Silicone, 30 m x 0.32 mm x 0.25 μm film thickness). The column temperature was set at 100°C for 2 minutes and then programmed from 100°C to 300°C at 10°C/minute (held for 3 minutes). The temperature of the injection port was set at 250°C and interface at 250°C. The ionisation energy was 70eV in the EI+ mode.

6.2.3. Headspace SPME Set-up

The polydimethylsiloxane and polyacrylate-coated fibres for SPME were pre-treated overnight in the GC injection port at 250°C and 300°C respectively prior to use to remove contaminants from the fibres. They were subsequently tested for carry-over by injecting the fibre after every run.

6.2.4. Optimisation of Desorption Conditions

Fibres exposed to 1.0-ml aliquots of blood containing fenthion at a concentration of 200 ng/ml for 30 minutes were injected into the gas chromatograph and left for various time intervals (1 minute, 2 minutes, 3 minutes, 4 minutes, and 5 minutes). The chromatographic peak height was recorded. The chromatograms were also checked for carry-over and interfering peaks.

6.2.5. Extraction time profile

A comparison was made of extraction temperature (set at 80°C and 90°C) *versus* absorption time profile. The fibre was exposed to the sample headspace at various absorption times (ranging from 5-70 minutes) with and without stirring. Conditioning time to attain the desired temperature was also measured. One millilitre (1 ml) of blood containing 200 ng Fenthion was placed in a 4-ml vial. A PDMS fibre was exposed to the headspace for different times (Figure 4.5).

6.2.6. Additives and pH Adjustment

Four (4) sets of 5 vials were set up. Each vial contained 0.5-ml blood spiked with 200 ng of Fenthion. The first set was diluted with 0.5-ml distilled water. The second set was diluted with 0.5-ml 0.1M HCl. The third set was diluted with 0.5-ml of saturated saline, and the forth set was diluted with 0.5-ml water, 100 µL 0.1M HCl and 100 µL saturated sodium chloride.

6.2.7. Procedure Developed

6.2.7.1.Standard Calibration curve

0.5-ml of blood was spiked with fenthion at concentrations in the range from 50-400 ng/0.5ml and 60 ng (6µl of 10ng/µL standard solution) diazinon as internal standard and transferred to a 4.0-ml headspace vial with magnetic stirrer. Then 100 µl of 0.1M HCL and 100 µl of saturated NaCl solution were added and the vial was capped. The vials were placed in a vial receptacle and placed over a heated hot plate/stirrer at 90°C

set at maximum stirrer speed. After heating for 10 minutes, the septum-piercing needle of the SPME was passed through the septum (Figure4.5). The pre-treated fibre was pushed out from the needle and exposed in the headspace for 30 minutes to allow the adsorption of the analytes. The fibre was withdrawn into the needle and pulled out from the vial. It was then injected into the port of a capillary gas chromatograph where the fibre was exposed for 5 min. to ensure complete desorption of the analytes. This method was adopted after obtaining the results from the preliminary studies described above.

6.3. Results and Discussion

6.3.1. Evaluation of the extractions condition

Temperature plays an important factor in determining the rate of extraction. At higher temperatures the analyte was more rapidly partitioned from the matrix into the headspace and also the partition coefficient increased in favour of the headspace. The analyte concentration in the headspace therefore increased and thus the quantity of analytes absorbed on the fibre also increased. This could be observed at a temperature of 90°C. The time for fibre exposure (extraction) determines the equilibrium condition at which 95% of the analyte will be extracted. SPME is a dynamic equilibrium process and not an exhaustive technique of extraction. From the graph the equilibrium was attained after 30 minutes of fibre expose to the environment.

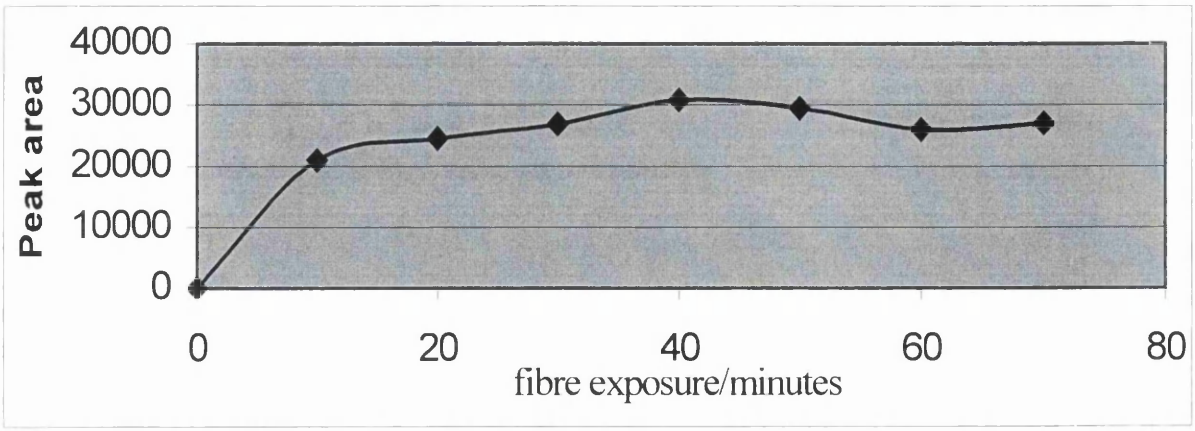


Figure 6.1 Extraction time profile.

6.3.2. Optimising the extraction by varying the pH and salt concentration.

Addition of salt, diluting the specimen with distilled water and adding of acid individually did not make any difference to the extraction efficiency. However, the combination of all three produced significant enhancements in term of sensitivity i.e. extracted amount.

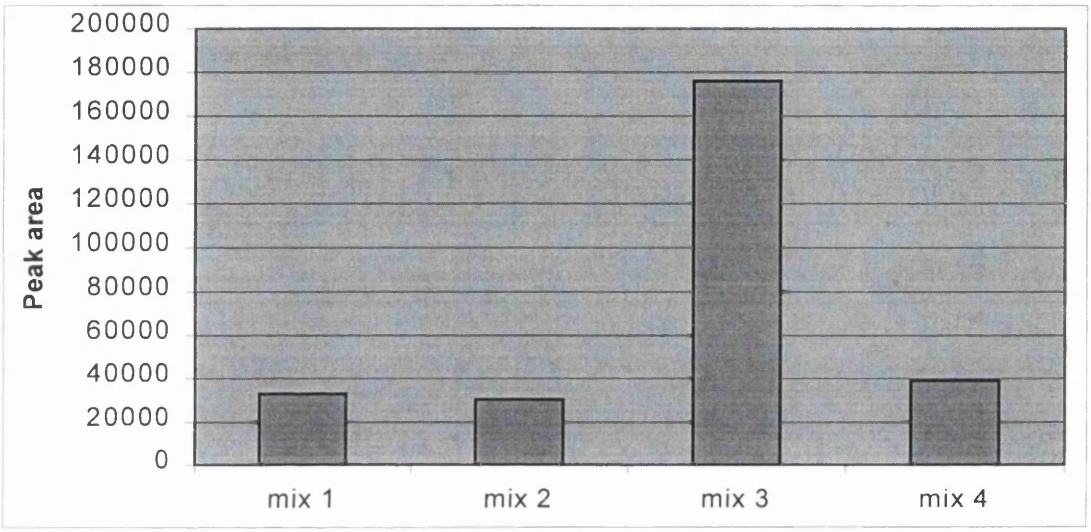


Figure 6.2 Effect of pH and addition of salt on extraction of fenthion from blood. Mix 1= (0.5 ml blood +0.5 ml water), Mix 2= (0.5 ml blood+0.5 ml water and 100 ul 0.1M HCL, Mix 3= (0.5 ml blood+0.5 ml water+ 100 ul 0.1M HCL + 100 ul saturated salt, Mix 4= (0.5 ml blood+0.5 ml water and 100 ul of saturated salt).

6.3.3. Validation

6.3.3.1.Calibration curve

The method was linear in the range 50-400 ng/0.5 ml of blood. The correlation coefficient was 0.999 (n=3 at each concentration).

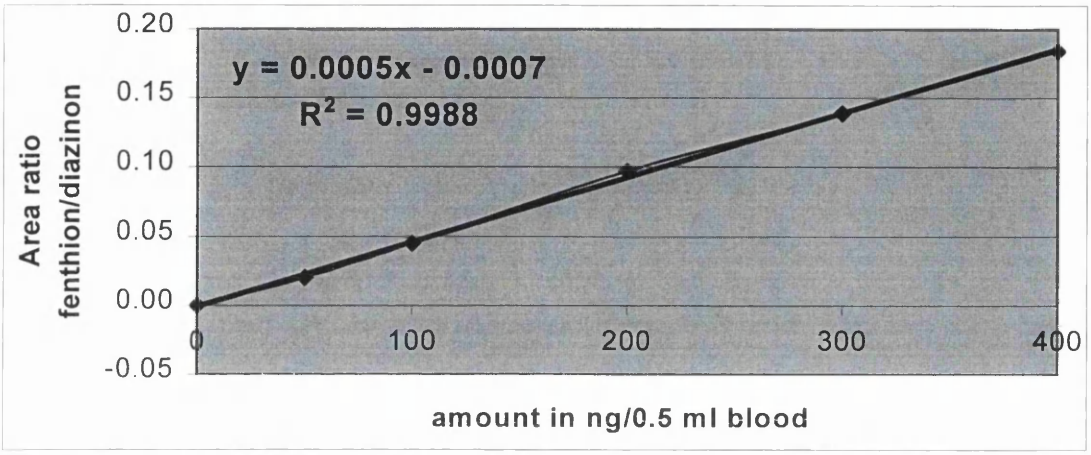


Figure 6.3. Calibration curve for fenthion.

6.3.3.2.Limit of detection, precision and recovery

The detectable limit was found to be 50 ng/0.5 ml blood (n=5). The precision of the method was measured based on several extractions at concentrations of 50 ng and 200 ng/0.5 ml blood. The relative standard deviation was 4.7% and 9.9% respectively. The between-day coefficient of variation deviation was on average 3.6% (n=3 days) (Table 6.1)

Table 6.1 Limit of Detection, Precision and Recovery by GC-NPD

Limit of detection	50 ng/ml of blood	
Precision :	Relative standard Deviation (RSD%)	
50 ng/0.5 ml blood 200 ng/0.5 ml blood	4.7% (intra day) 9.9% (intra day)	3.6% (between day)
Recovery at 200 ng/0.5 ml blood	2.8%±0.6%	

Table 6.2 Limit of detection and principal fragment ions recorded by GC-MS in full scan mode .

Analyte	Mass fragments (m/z)	LOD (per 0.5 ml blood)
Fenthion	278, 125, 109, 169	100 ng (FS)
Diazinon (IS)	179, 137, 152, 304	60 ng (FS)

The technique was applied to actual cases after screening in stomach content by TLC and UV procedure to ascertain the presence of the pesticide.

6.3.3.3.Application to real cases

The Forensic Laboratory in Mauritius as well as the Department of Chemistry, Kuala Lumpur, Malaysia submitted samples for analysis. Specimens were sent by courier and through the post, with care being taken to observe WHO recommendations on packaging.

Case 1.

This was a 48 year old male driver who ingested a poisonous substance on 5th September 2000 at about 13.00 hrs. He was taken to hospital where he was given treatment. No details of the hospital treatment are available. He subsequently died at 0200 hrs the following day, 6th September. The time interval between ingestion and death was therefore about 13 hours. An autopsy was carried out later that day, 6th September, at 1000hrs. Samples of blood and stomach content were removed and kept refrigerated at -4°C. Toxicological analysis by TLC and UV spectroscopy after liquid-liquid extraction revealed the presence of Fenthion in stomach contents (data not included). Analysis of blood by TLC was negative but analysis by headspace solid phase microextraction indicated it was positive for Fenthion. The concentration was found to be 101.4ng/0.5 ml blood (equivalent to 202.8 ng/ml).

Case 2.

A female aged 29 years, working as a rubber tapper, was found dead at home on 12th September 1998 at 1645 hrs. Nothing was found at the scene to indicate the cause of death. At autopsy a smell indicative of an organophosphate pesticide was detected.

Stomach contents and blood were taken for toxicological analyses. Initial screening by liquid-liquid extraction followed by TLC and UV revealed the presence of fenthion in the stomach contents. The blood was extracted using HS-SPME and analysed by the method described above. The analyses indicated a concentration of 321.4 ng fenthion/0.5 ml blood (equivalent to 642.8 ng/ml).

6.4. Conclusion

The method was successfully applied to cases related to pesticide poisoning without any difficulties. It has been employed for a wide variety of analyses from biological fluids and matrices [112]. It has shown low detection limits and excellent characteristics for quantitative analysis. Especially in the headspace mode, SPME offers the potential for very clean analyses with little to no interference from non-volatile compounds. The technique was simple and did not involve the use of any solvent. Environmental friendly techniques such as HS-SPME should be explored for use in other routine analysis.

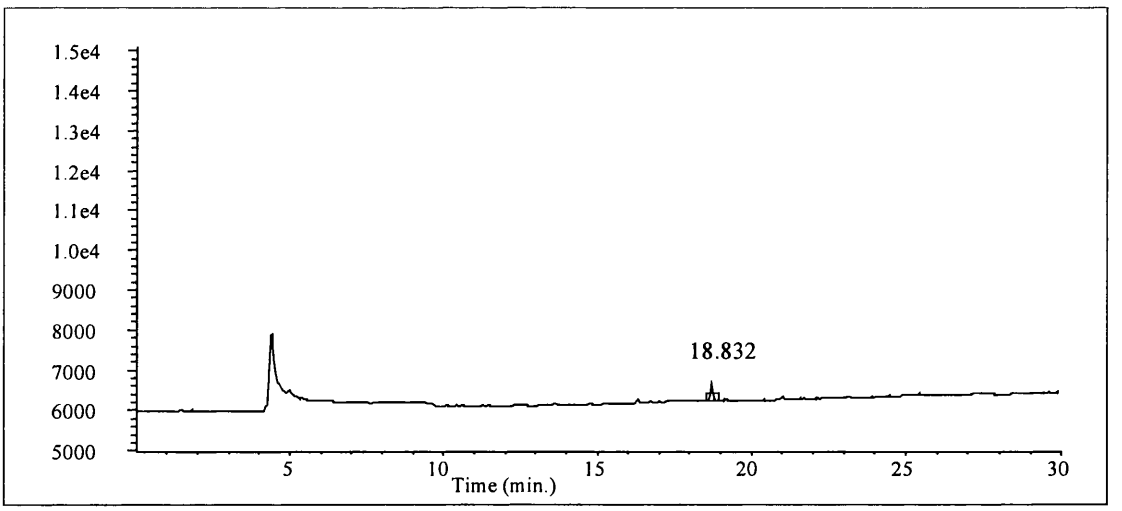


Figure 6.4 Blank blood (HSSPME-GC-NPD)

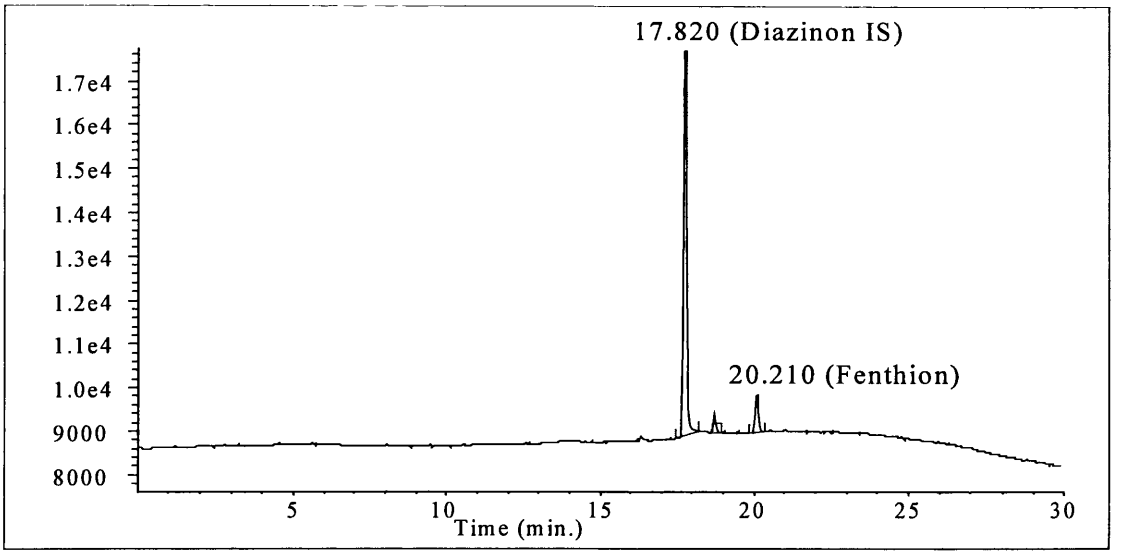


Figure 6.5 HSSPME GC-NPD of blood containing Fenthion (Case 1).

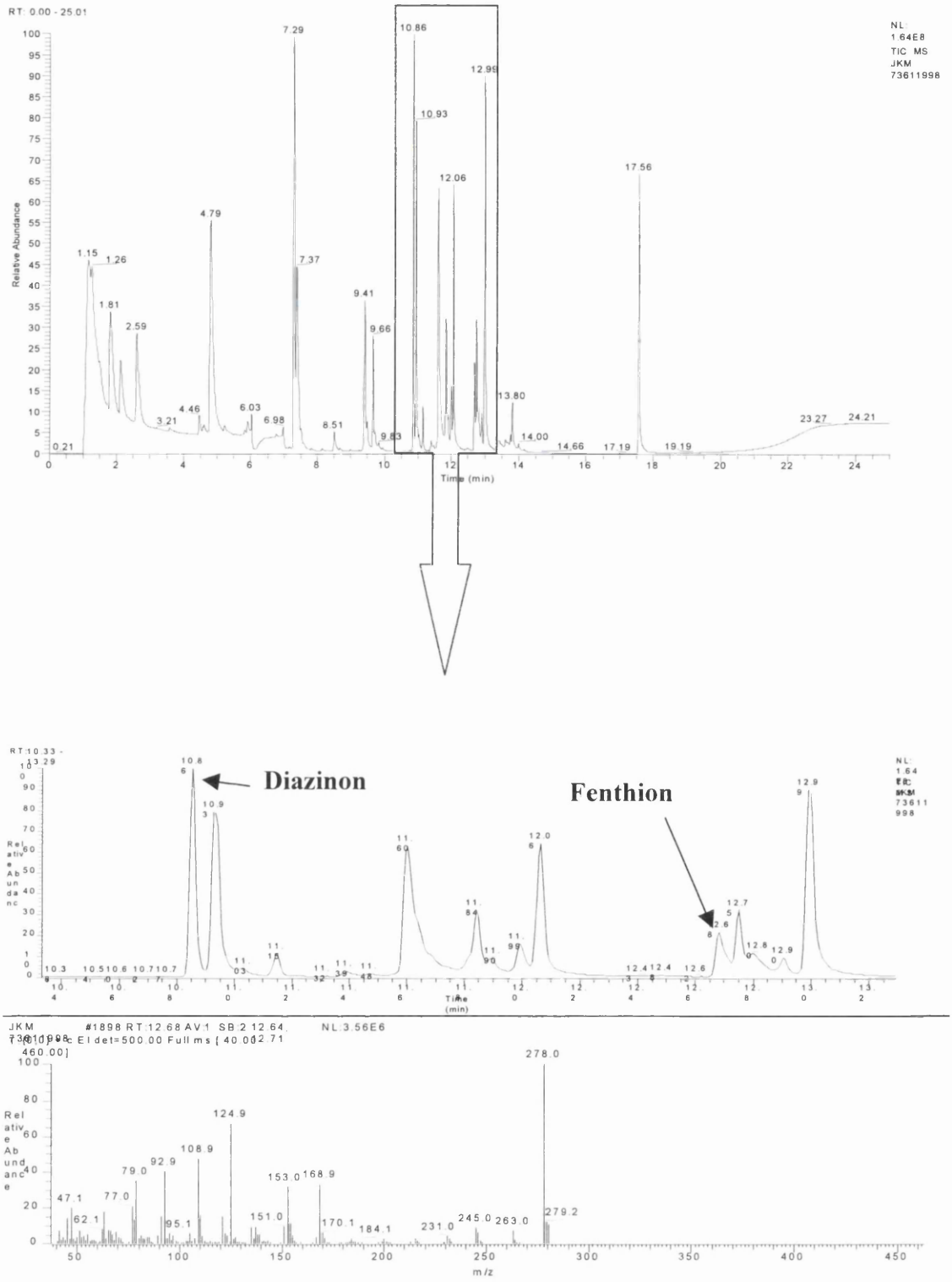


Figure 6.6 Total Ion Chromatogram in Full Scan mode of blood sample and representative mass spectrum of fenthion.

7. ANALYSIS OF THE ORGANOPHOSPHATE CHLORPYRIFOS IN BLOOD AND URINE BY HEAD SPACE SOLID PHASE MICROEXTRACTION (HS-SPME)

7.1. Introduction

Deliberate self-harm is a major problem in the developing world, responsible for around 600,000 deaths in 1990 [151]. Pesticides are the most important poisons used throughout the tropics, being both common and associated with a high mortality rate. Organophosphate pesticides are widely used in agriculture and domestically for pest control. Determination in human body fluids has generally carried out by applying solvent extraction procedures and subsequent cleanup steps if necessary [56, 58, 60, 61, 63, 65], or solid phase extraction [57, 59]. This situation derives from the high complexity of the sample matrices involved, containing both polar and non-polar components, especially problematic being the presence of the fat and protein fractions of the matrices. Development of adequate SPME procedures for organophosphate determination should allow us to achieve a reduction in sample manipulation, even eliminating the need for sample cleanup.

Chlorpyrifos is a well known organophosphate pesticide besides the better known malathion which is largely used as a liquid formulation in controlling pests like white grub and *holotrichi consanguine blanch*, which generally affect the ground nut crop. The importance of this group is reflected by the fact that chlorpyrifos has been the world's largest selling insecticide (in tonnage terms) for the last ten years [113]. However, when chlorpyrifos is applied to plants or mixed with soil, it will produce hazardous effects to the environment, especially to the ozone layer in the atmosphere [114].

This section deals with the analysis of chlorpyrifos in whole blood and urine by the headspace SPME technique.

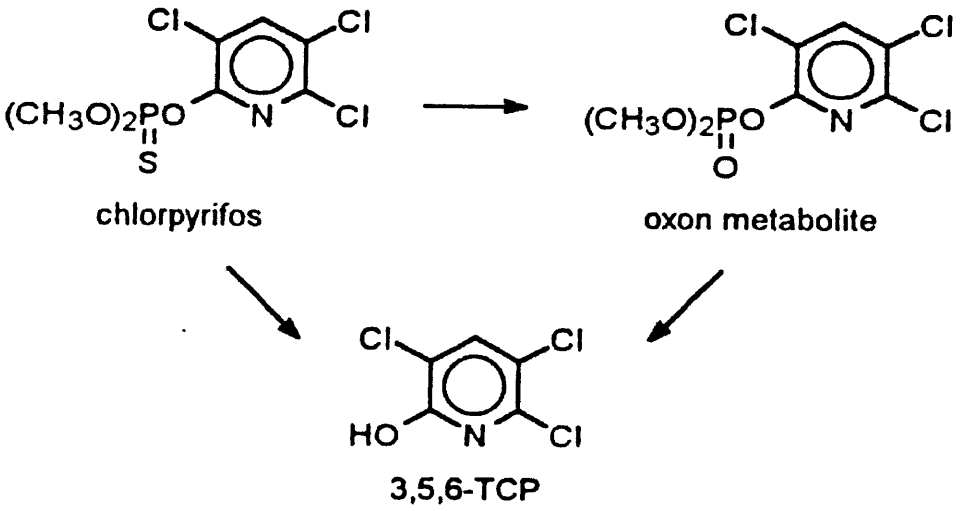


Figure 7.1 Metabolic pathway of chlorpyrifos

7.2. Materials and Methods

7.2.1. Reagent

Chlorpyrifos and Diazinon were purchased from Promochem Ltd, Hertz, England. A stock solution (1 mg/ml) was prepared by dissolving an appropriate amount in methanol. A working standard containing 10 ng/μL was prepared by diluting the stock solution. Hydrochloric acid and sodium chloride were of Analar grade. Blank blood for preparation of blood standards used time-expired red blood cells from the blood transfusion service.

7.2.2. Apparatus

A manual assembly was used for SPME with a replaceable extraction fibre coated with 100 μm polydimethylsiloxane (from Supelco, UK). Amber headspace vials (4.0 ml volume) were used with screw septum caps fitted with PTFE/Silicone Septa. A Corning hot plate (setting from 25-550 °C)/stirrer (60-1100 rpm) was used with SPME sampling stand (holding 8 vials) and PTFE covered magnetic stirrers (10 x 3 mm). All these items were purchased from Supelco Sigma-Aldrich Com. Ltd.

7.2.3. Gas Chromatography with Nitrogen-Phosphorus and Mass Spectrometric Detection

Gas Chromatography used a Hewlett-Packard Model HP 5890 Series II instrument with a Nitrogen Phosphorus Detector and equipped with an HP-1 capillary column (30 m x 0.53 mm x 0.88 μm phase thickness). Column conditions were: 100°C for 2 minutes, programmed from 100°C to 280°C at 10°C/minute. The temperature of the injection port was set at 250°C and detector at 280°C. The splitless injection mode was used for the first 5 minutes.

GC-MS was carried out using a Thermo-Finnigan Trace GC-MS instrument fitted with an HP-5 X-link 5%Ph, 95% methyl silicone column (30 m x 0.32 mm x 0.25 μm film thickness). The column temperature was set at 100°C for 2 minutes and then programmed from 100°C to 300°C at 10°C/minute, which was then held for 3 minutes. The temperature of the injection port was set at 250°C and the interface was also at 250°C. The ionisation energy was 70eV in the EI+ mode. Fragment ions used for selected ion monitoring were m/z 97, 197, 199, 314 for Chlorpyrifos and m/z 179, 137, 152, 304 for Diazinon.

7.2.4. Headspace SPME Set-up

The polydimethylsiloxane-coated fibre for SPME was pre-treated in the GC injection port at 250°C overnight prior to use to remove contaminants from fibre. It was subsequently tested for carry-over by injecting the fibre after every run.

7.2.5. Optimising the absorption and desorption conditions

7.2.5.1.Desorption conditions

The SPME fibre was exposed to 1.0-ml aliquots of blood containing chlorpyrifos at a concentration of 100 ng/ml for 20 minutes and was then injected into the gas chromatograph and left for different time intervals (1 minute, 2 minutes, 3 minutes, 4

minutes, and 5 minutes). The peak height was recorded and chromatograms were checked for carry-over and for the presence of interfering peaks.

7.2.5.2.Extraction temperature and extraction time profile

A comparison was made of extraction temperature (set at 80°C and 90°C) *versus* absorption time profile. The fibre was exposed to the sample headspace at various absorption times (ranging from 5-70 minutes) with and without stirring. Conditioning time to attain the desired temperature was also measured.

Blood (1 ml) containing 100 ng chlorpyrifos was placed in a 4-ml vial. A 100μm polydimethylsiloxane (PDMS) fibre was exposed to the headspace for different times (Figure 4.5).

7.2.5.3.Additives and pH Adjustment

Four sets of 5 vials were set up. Each vial contained 0.5-ml blood spiked with 100 ng of chlorpyrifos. The first set was diluted with 0.5-ml distilled water. The second set was diluted with 0.5-ml 0.1M HCL. The third set was diluted with 0.5-ml of saturated saline, and the forth set was diluted with 0.5-ml water, 100 μL 0.1M HCL and 100 μL saturated sodium chloride.

7.2.5.4.Standard Calibration curve

In a set of 4.0-ml vials with magnetic stirrers were placed 0.5-ml aliquots of blood containing chlorpyrifos at concentrations in the range 10-80 ng/0.5 ml. Diazinon (IS) at 60 ng/0.5ml was spiked into all samples. For urine the concentration range was from 5-60 ng/0.5 ml and the internal standard diazinon was added at 10 ng/0.5 ml. The vials were placed in a vial receptacle and placed over a heated hot plate/stirrer at 90°C at the maximum stirrer speed. After heating for 10 minutes, the septum-piercing needle of the SPME was passed through the septum (Chapter 4, Figure 4.5). The pre-

treated fibre was pushed out from the needle and exposed in the headspace for 30 minutes to allow the adsorption of the compound. The fibre was withdrawn into the needle and pulled out from the vial. It was then inserted into the injection port of the capillary gas chromatograph and the fibre was exposed for 5 minutes to ensure complete desorption of the compound. This method was adopted after determining the steps mentioned above.

7.3. Results and Discussion

7.3.1. Optimising desorption time

Desorption was complete after 3 minutes but it was preferable to leave the fibre in the injector for 5 minutes in order to eliminate memory effects.

7.3.2. Optimising the extraction conditions.

It was found that the time taken for the sample to reach the desired temperature of 90°C was 5.0 minutes ± 1.0 minute. A longer time was taken for stabilisation (10 minutes).

The extraction time profile in Figure 7.2 shows the equilibrium time was reached most probably after 60 minutes. This time is too long for practical purposes, thus a 30 minute time limit was taken as extraction time. Provided a constant extracting time, temperature and stirring rate are used, the method precision will not present any problems.

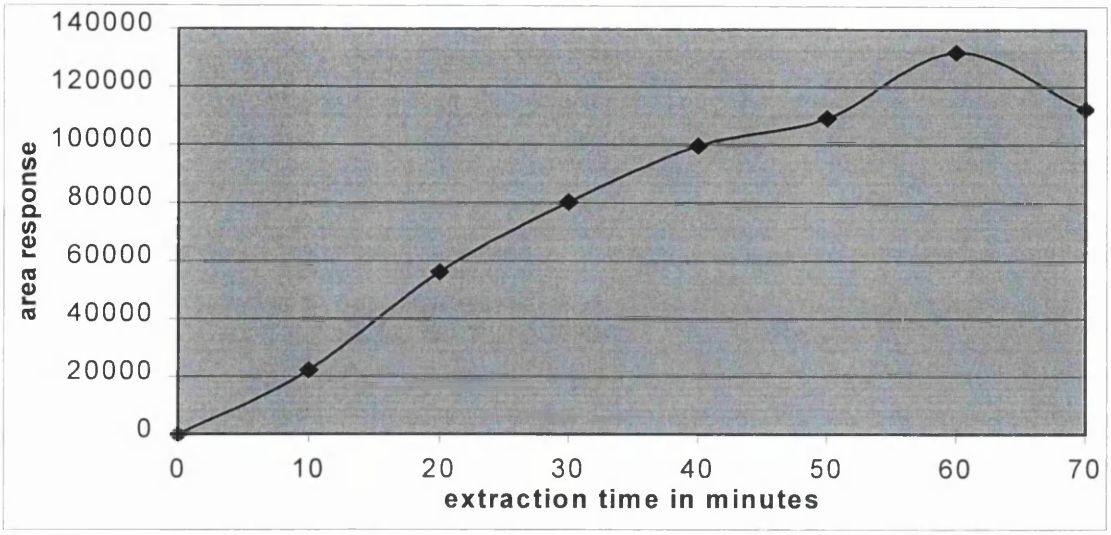


Figure 7.2 Extraction time profile for chlorpyrifos

The effect of pH adjustment and salt addition can be seen in Figure 7.3. Even though the pH adjustment alone will increase the response in terms of GC peak area, it was decided to add salt since it tends to give better precision. Precision was found to be around 11.2% (when only pH adjustment was used) and 6.2% (when both salt addition and pH adjustment were used). Recoveries were very different: 3.4%, with pH adjustment and salt addition, compared to 5.3%, when only pH adjustment was used. The evaluation was carried out at a concentration level of 50 ng chlorpyrifos/ 0.5 ml blood.

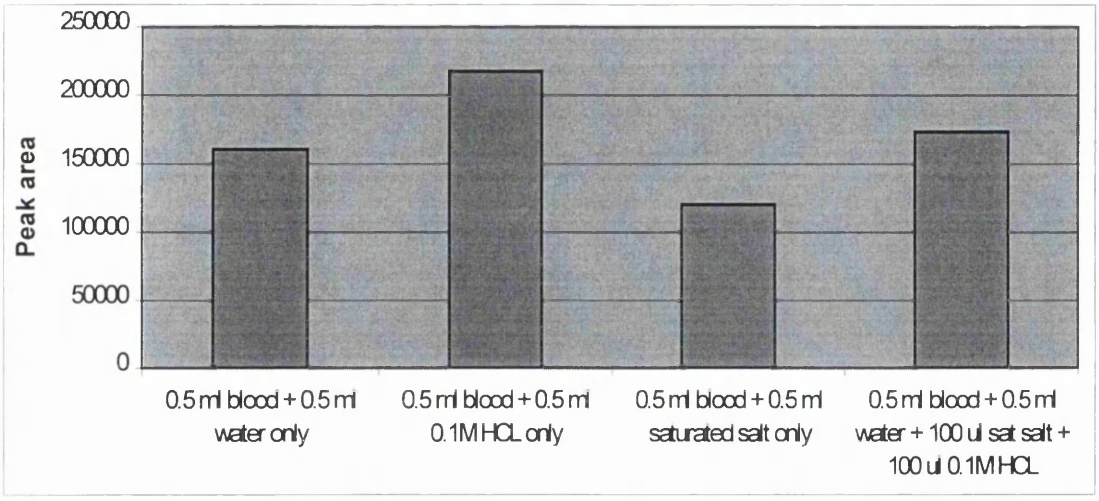


Figure 7.3 Effect of pH adjustment and salt addition.

7.3.3. Limit of detection, precision study and calibration curve

The limits of detection in blood and urine using GC-NPD were determined to be 10 ng and 5 ng/ 0.5 mL respectively. Series of standards at eight concentration levels were obtained by spiking blood with chlorpyrifos in the concentration range 10 to 80 ng/0.5 ml. Each level was run in triplicate. A calibration graph was plotted, which had a correlation coefficient of 0.995 (Figure 7.4).

For urine, standards at 7 concentration levels were obtained by spiking blank urine to give solutions in the range 5-60 ng/0.5 ml. Each level was run in triplicate as for blood. The correlation coefficient was 0.988 (Figure 7.5).

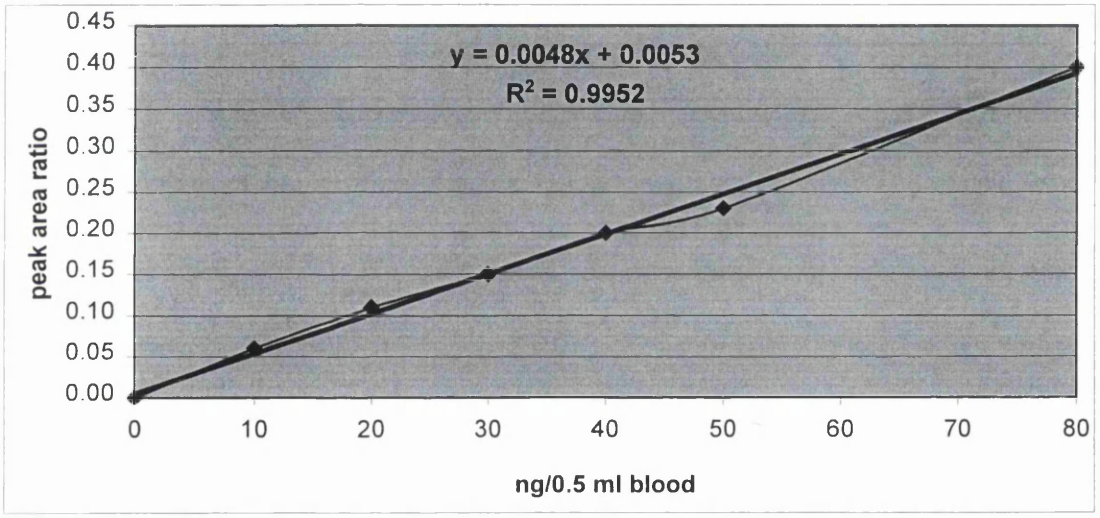


Figure 7.4 Calibration graphs for Chlorpyrifos in blood (IS diazinon 60 ng/0.5 ml)

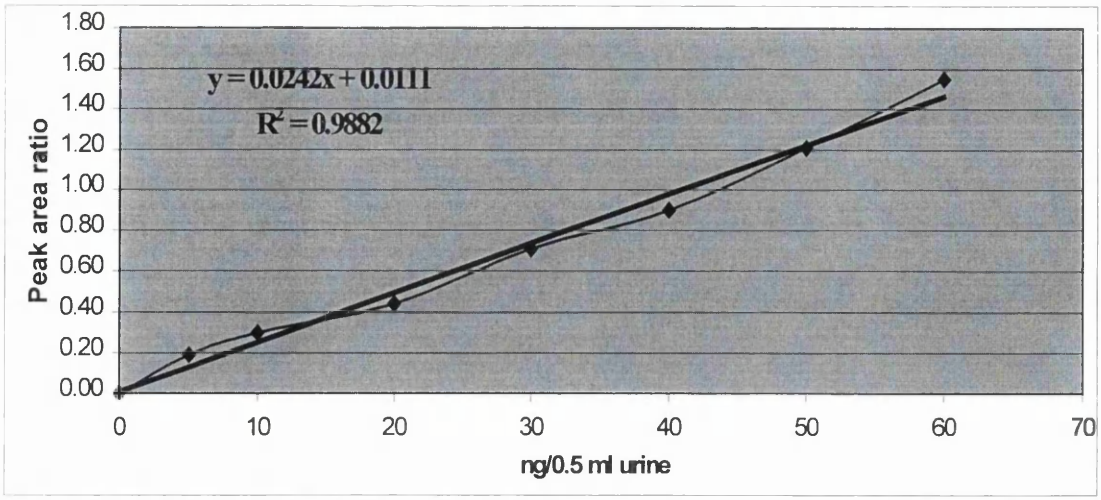


Figure 7.5 Calibration graphs for chlorpyrifos in urine (IS diazinon 10 ng/0.5 ml)

7.3.4. Application to a real case

The method was applied to a case of suspected pesticide poisoning in a 37 year old male found dead at home. The police investigation indicated that there was no foul play. The fatality was taken to the hospital mortuary and the post-mortem was carried out 3 hours after admission. The method successfully identified the pesticide as chlorpyrifos and quantitative analysis gave a concentration of 21.8 ng/0.5 ml blood. The concentration in urine was found to be 21.0 ng/0.5 ml.

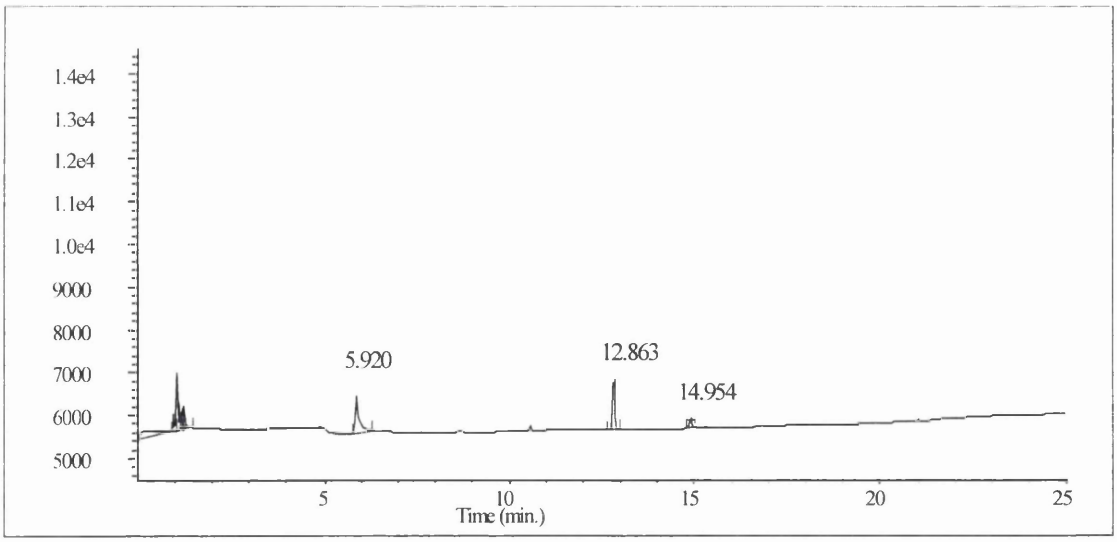


Figure 7.6 Blank Blood

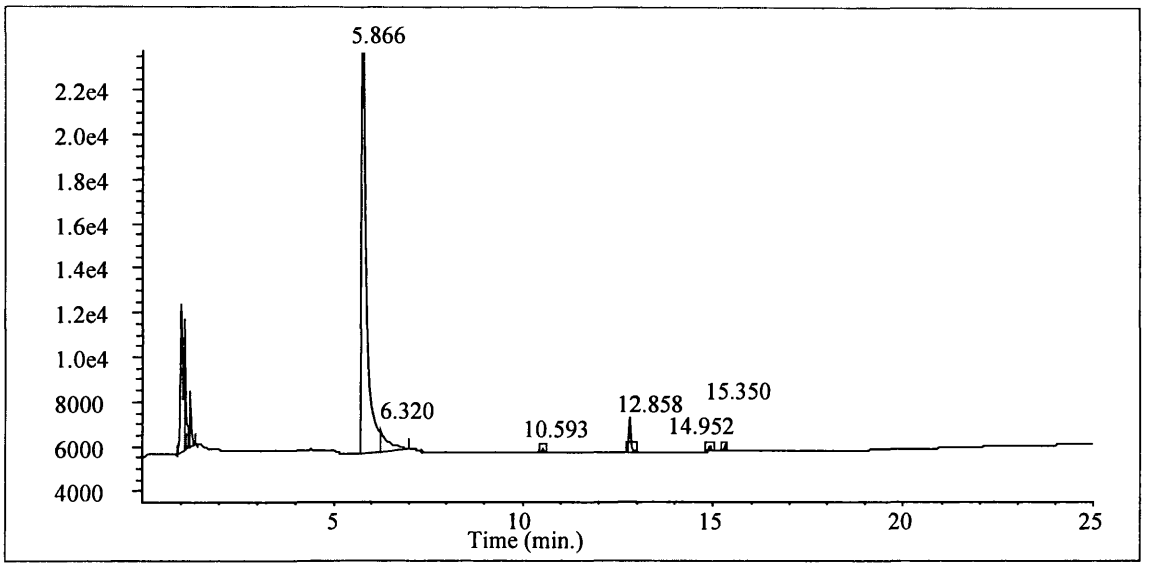


Figure 7.7 Blank Urine

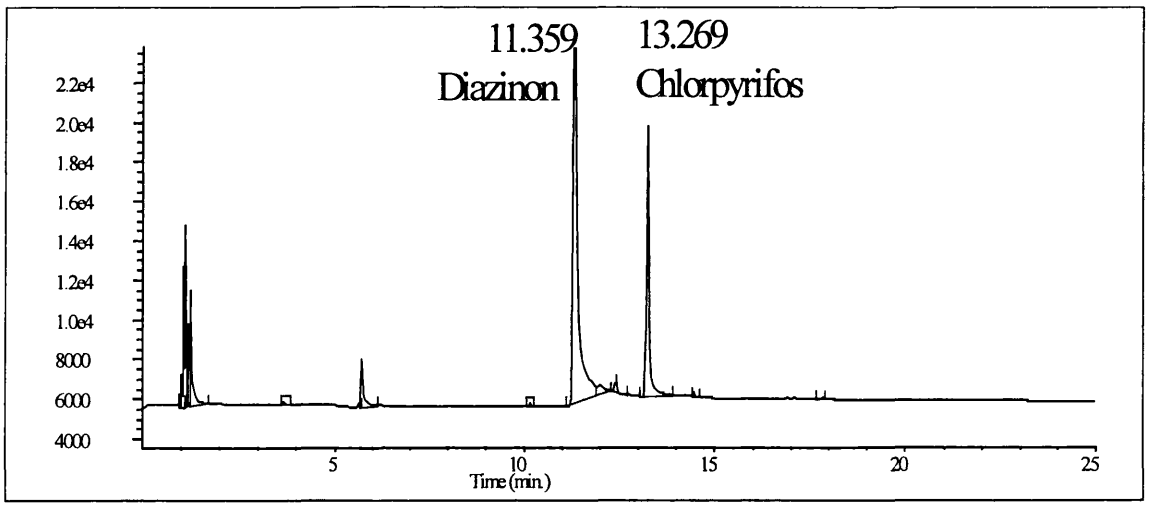


Figure 7.8 Case JKM 1999-00 (urine)

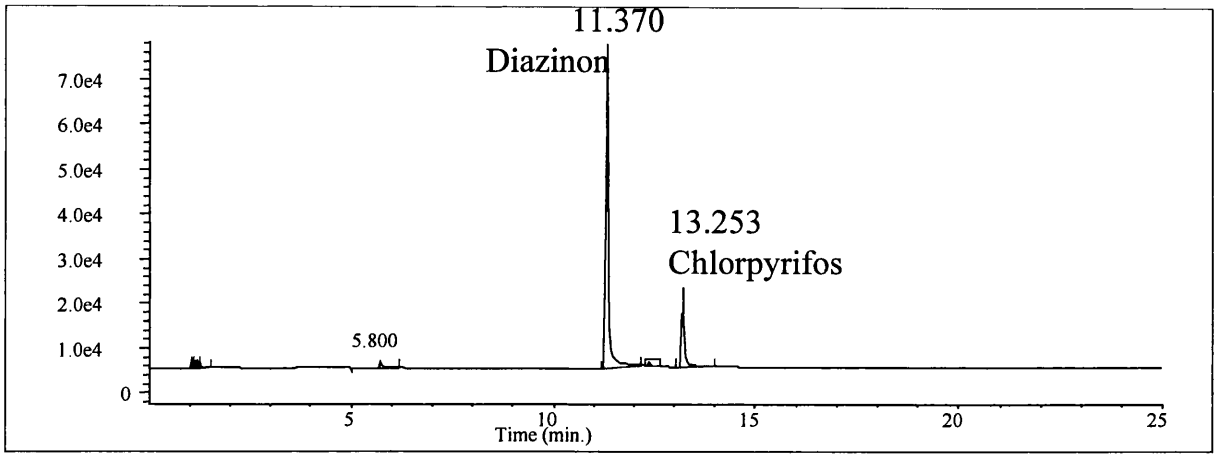


Figure 7.9 Case JKM 1999-00 (blood)

A confirmatory analysis was carried out for the blood specimen using GC-MS in full scan mode (Figure 7.10) and using a computer library search (NIST library) for hits and fit factors. Selected ion monitoring was also utilised to measure the pesticide concentration (Figure 7.11). Both techniques gave a detectable level of 10 ng/0.5 ml blood. The confirmation analysis was carried out with acidification after diluting the blood with water. No addition of salt was necessary.

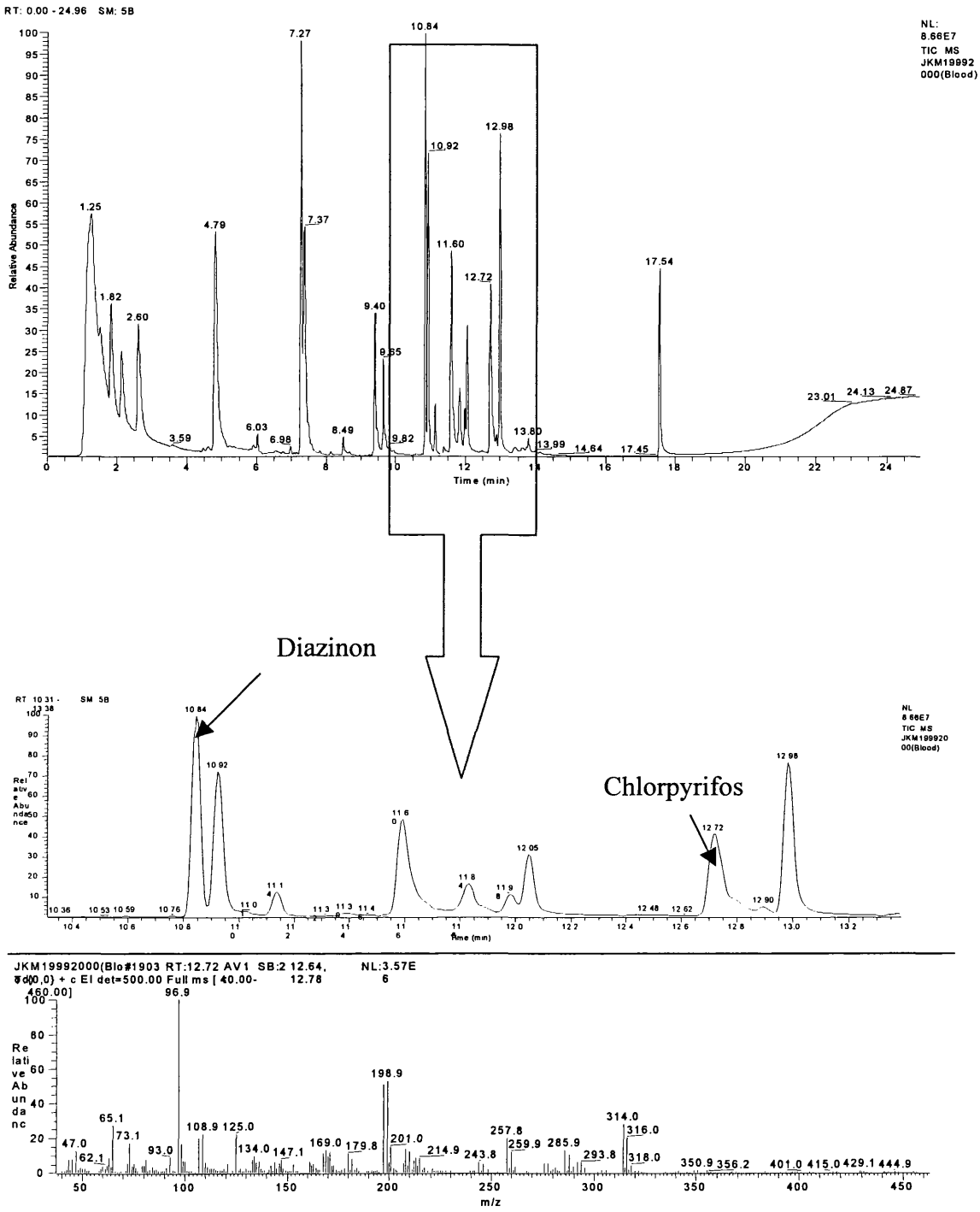


Figure 7.10 Total Ion Chromatogram created from data collected in full scan mode for case JKM 1999-2000 (Blood) with representative m/z spectra

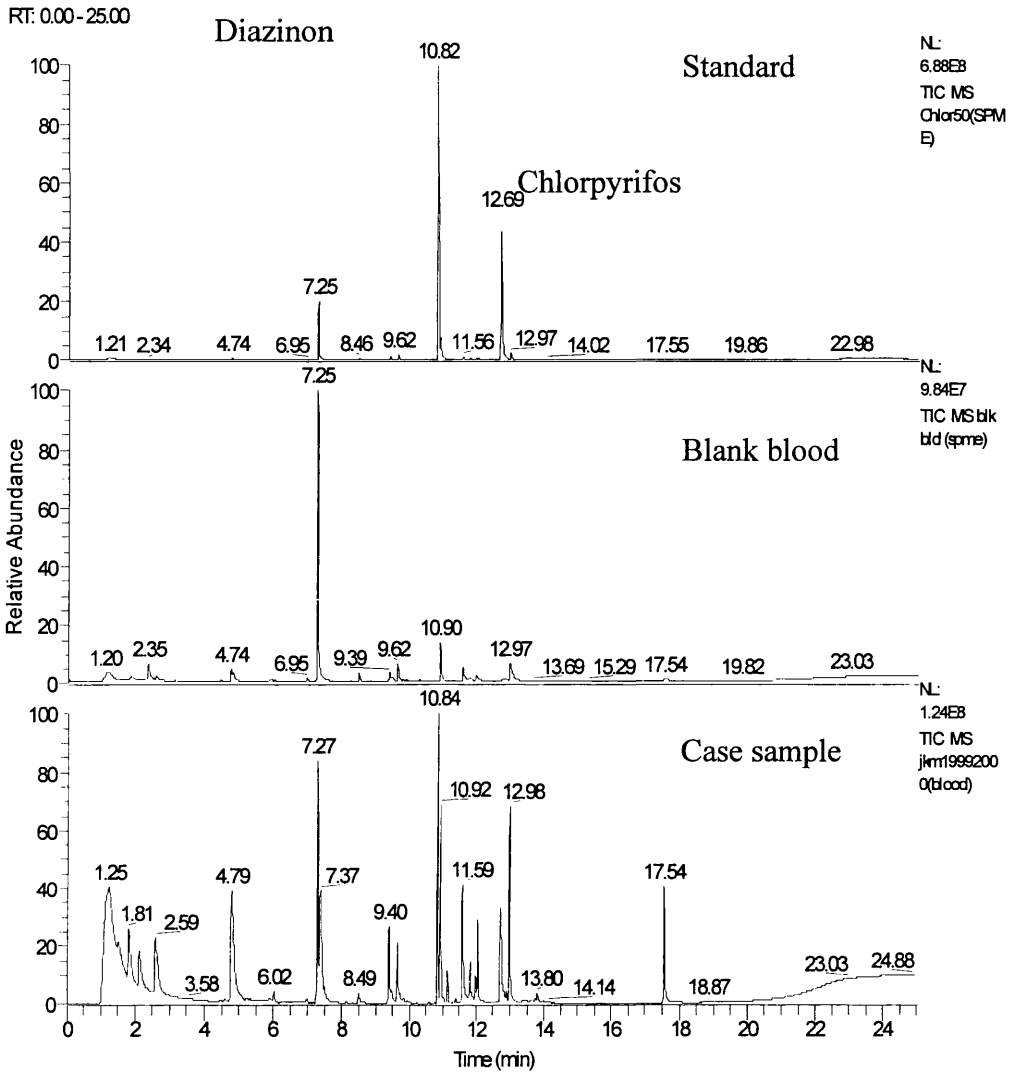


Figure 7.11 Total ion chromatograms obtained under SIM mode, obtained by summing the ion chromatograms for m/z 179,137, 152, 304 for Diazinon and m/z 97, 197, 199, 314 for Chlorpyrifos.

The metabolism of chlorpyrifos has been studied in human blood. Analyses revealed that in humans, chlorpyrifos remained stable in blood for at least 180 minutes [60] and from our own study it was found to be stable for approximately 72 hours (data not included). Fumio Moriya [56] found chlorpyrifos-methyl to be stable for up to 72 hours in blood. Chlorpyrifos was readily metabolised to TCP and conjugates of TCP, which were then primarily excreted *via* the urine. Using this HS-SPME technique it was not possible to analyse either the oxon or the metabolite TCP without derivatisation.

7.4. Conclusion

The applicability of SPME for the determination of chlorpyrifos in urine and whole blood has been explored using HS-SPME/GC-NPD and GC-MS for their analysis. It was shown that SPME could be a valuable tool in pesticide analysis of organophosphates in samples of human fluids.

8. SOLID PHASE EXTRACTION

8.1. Introduction

Solid phase extraction (SPE) for liquid samples became a widely used laboratory technique following the introduction in the 1980's of disposable sorbent cartridges containing porous particles sized to allow sample processing by gentle suction . A typical solid phase extraction cartridge consists of a short column (generally an open syringe barrel) containing a sorbent with a nominal particle size of 50-60 μm, packed between porous metal or plastic frits. In the last decade, many reports on analytical methods for organophosphate pesticides were published in various fields.

Environmental contamination caused by agricultural activities has increased constantly over the last few years. A wide variety of pesticides reach the soil in the agricultural area where they are applied, but they may be carried off soils and into surface waters by leaching as consequence of water displacement in soil environment. Water from small loughs was analysed by Jimenez et al [115]. ODS cartridges from Varian followed by florisil packed columns as clean up were used. The recoveries were improved by about 10-20%. Extracts were analysed by gas chromatography with nitrogen phosphorus detection or high performance liquid-chromatography with diode array detection. Ruiz M.J. and co-workers [22] published a procedure for determining various pesticides in the leachates from soil. The method uses 47-mm disks of octyl-bonded silica. The analysis was carried out by gas chromatography with nitrogen phosphorus and electron capture detectors. Recoveries varied from 28-98% based on the type of extraction solvent and the ionic strength of the sample. Other researchers have also published methods based on SPE with GC, HPLC or TLC for several pesticides in a variety of matrices including soil, water and food [116-124].

In the fields of toxicology and biomedical applications, procedures for analysing organophosphates using C_{18} Bond Elut columns, for example, were introduced. Serum samples were directly applied to the Bond Elut columns followed by washing with 2 ml of water. Collection of analytes of interest was done by eluting with 1 ml of n-hexane-ethyl acetate (75-25, v/v) and evaporating to dryness. Following reconstitution in 10 μl acetone, the extracts were applied to HPTLC plates, developed

in three solvent systems and visualised using UV radiation [53]. Another sample preparation system for detecting multiple residues was proposed by a team of Japanese lead by Shigeki Tomojiri in 1997 [43]. Thirteen (13) organophosphorus pesticides could be simultaneously analysed after samples of whole blood were extracted with Bond Elut Certify cartridges and after serial washing with phosphate buffer (pH 6) and 1.0 M acetic acid, the analytes were eluted using dichloromethane. Recoveries for most organophosphates were greater than 86% and precision was within a level of 10%. Detection used GC-NPD. E.Lacassie et al. proposed a method for multiresidue analysis of around 29 organophopshorus pesticides using Oasis HLB cartridges and identification by GC-MS in EI-SIM mode [125]. The 2-ml blood specimen was first treated with acetonitrile and the supernatant was then deposited on an Oasis HLB cartridge after concentrating the extract. The cartridge was washed with deionised water and eluted after drying (20 min) with 3 ml of ethyl acetate. For serum the specimen was directly applied on to the cartridges. Recoveries ranging from 40% to 108% were obtained. The limit of detection ranged from 5 to 25 ng/mL and the limit of quantification ranged from 10 to 50 ng/mL of blood and serum.

Several other reports regarding the analysis of organophosphates in blood have been published in forensic toxicology [30, 31, 32, 90] and clinical toxicology [91,97]. Many of them attempted to use various solid-phase extractions. However, not as many studies have been developed as in drugs. In this section of the present work, the emphasis has been in using an established method [43] with minor adjustment and applying it to actual cases. Also, a study on parameters effecting the extraction was also carried out. The SPE method was then compared to the method that was already developed previously based on headspace solid phase microextraction (HS-SPME).

8.2. Introduction to the principles of SPE

Solid phase extraction utilises the same analyte/sorbent interactions that are exploited in the powerful separation technique of high performance liquid chromatography (HPLC). The extraction cartridges are packed with a variety of surface-modified bonded silica sorbents, which selectively retain specific classes of chemical compounds from within a given matrix. As an example, the strong cation exchanger

(HCX) can be used to retain the cationic drug, amphetamine, from urine [126]. The more specific the interaction between the sorbent and analyte, the cleaner the final extract.

Bonded silica sorbents are in many ways the ideal materials for chromatographic isolation, primarily due to the number of different functional groups that can be readily bonded to the silica surface. The names and functional groups of the bonded silica sorbents can be obtained from manufacturers' guidance manuals. In addition, bonded silicas are rigid supports that do not shrink or swell, possess very large surface areas due to porosity, are stable under a wide range of aqueous and organic solvent conditions and form a clean, non-leachable substrate upon which the bonded functional groups are attached.

In SPE, a liquid is passed over a solid or "sorbent" that is packed in medical-grade polypropylene cartridge or embedded in a disk. As a result of very strong interactive forces between the analytes and the sorbent, the analytes are retained on the sorbent. Later, the sorbent is washed with a small volume of a solvent that has the ability to disrupt the interactions between the analytes and the sorbent. The result is that the analytes are concentrated in a relatively small volume of clean solvent and are therefore ready for use in GC, LC, UV spectroscopy or immunoassay without any additional sample work-up apart from evaporation. In addition, extraneous compounds are usually removed. The sorbent normally consists of a silica substrate bonded to organosilane compounds. The functional groups on the organosilane moiety determine the selectivity of a particular sorbent. A typical SPE cartridge is shown in Figure 8.1 and the extraction process is summarised in Figure 8.2.

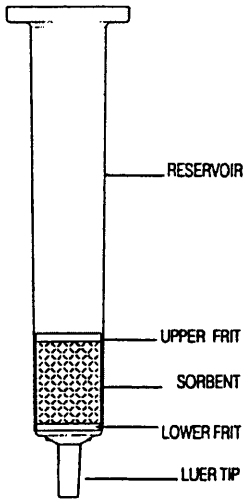


Figure 8.1 Schematic diagram of a solid-phase extraction cartridge.

A variation on the extraction cartridge is the disk where the sorbent (on a polymer or silica substrate) is embedded in a "web" of Teflon™-based fibrils. The sorbent particles are smaller than those in cartridges (8 μm in diameter rather than 40 μm). The short sample path and small particle size allow efficient trapping of analytes with a relatively high flow rate through the sorbent as compared to the cartridges. The disks are primarily used to reduce the analysis time when handling large volumes of aqueous environmental samples. Disks are available in several different diameters with the larger diameters allowing faster flow rates. The solid-phase extraction process is quantitative. It has been used to prepare samples for biological, environmental and industrial applications.

8.3. Steps in Solid Phase Extraction

The most common goals of an extraction protocol are to extract, clean-up, concentrate, and solvent exchange (eg, aqueous to organic) prior to analysis. Solid phase extraction achieves these goals in four simple steps. They are:

- Conditioning: Preparing the cartridge for reproducible interaction with the sample matrix by solvating the sorbent bed. This is done by passing a volume of an appropriate solvent through the cartridge, followed by a volume of a liquid similar

in nature to the sample matrix. A common example of cartridge conditioning would be to pass methanol, followed by water or buffer, through a C18 cartridge prior to extraction of an aqueous sample matrix.

- Retention: Applying the sample to the conditioned cartridge results in the analyte, and perhaps other matrix components, being retained on the sorbent surface due to one or more specific chemical interactions (e.g., Van der Waals or "non-polar" interactions between the hydrocarbon chain of an analyte and the hydrocarbon chain of a C18 bonded phase). It should be pointed out that matrix contaminants might pass through the cartridge unretained, hence cleaning up the sample even at the retention or loading step.
- Rinsing: Passing solvents through the cartridge rinses away additional interfering compounds while leaving the analyte undisturbed within the sorbent bed. A common rinse solvent for a non-polar extraction on a C18 sorbent would be water.
- Elution: Passing an appropriate solvent through the cartridge, which is specifically chosen to disrupt the analyte-sorbent interactions, resulting in selective elution of the analyte. To use a non-polar extraction example again, an organic solvent such as methanol would be a sufficiently strong solvent to disrupt the interaction between most non-polar analytes and a C18 bonded phase.

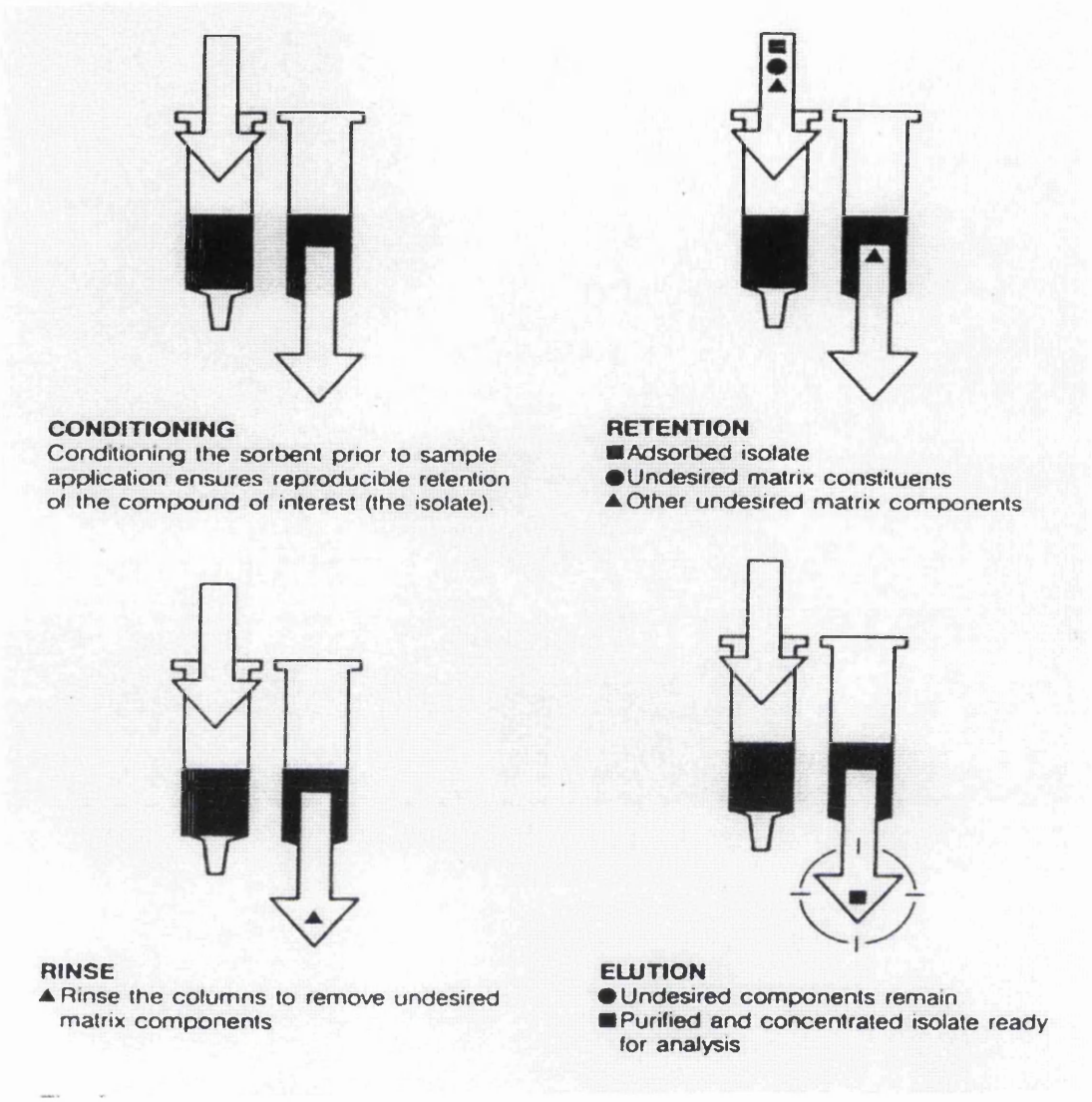


Figure 8.2 Principles of solid phase extraction.

8.4. Method Development

8.4.1. Selection of the SPE Cartridge and Eluting Solvent

When developing a SPE method, a number of factors must be considered:

- A sorbent should be chosen with selectivity characteristics such that the analytes will be retained and most of the interferences will not be retained.
- The sorbent capacity should be sufficient to retain all the analytes.
- The elution solvent should be capable of recovering virtually all the analyte from the sorbent in as small a volume as possible while leaving any interfering

compounds on the sorbent. A rule of thumb is that the elution volume should be two to five times the bed volume of the cartridge [127].

8.4.2. Standardising the SPE Procedure

Procedure includes:

- Conditioning the sorbent
- Retention
- Rinse

8.4.3. Optimising the wash and Elution Steps

The eluting solvent must have the ability to disrupt the interactions between the sorbent and the analytes. Often, mixtures of solvent are most effective. Usually two to five bed volumes of solvent are used [127]. To optimise the wash steps, a solvent should be identified that does not elute the analytes. A likely wash solvent is similar to the eluting solvent but not strong enough to break/disrupt the sorbent-analyte bonds.

8.4.4. Special considerations

When the sample extract is to be analysed by GC, special considerations apply:

- The solvent should be compatible with the GC detector;
- Electron-Capture Detectors (ECDs) should not be used with halogenated solvents;
- Nitrogen-Phosphorus Detector (NPD) should not be used with nitrogen-containing solvents such as acetonitrile; halogenated solvents are also not recommended;
- Buffers and other solvents containing non-volatile materials are not suitable for GC injection;
- The solvent should be compatible with the GC column phase; for example, a polar solvent such as water or methanol is not compatible with a non-polar phase such as dimethyl silicone;
- Some compounds must be derivatised prior to GC analysis. Examples are metabolites of some insecticides and herbicides that contain carboxylic acid

functional groups. If derivatisation is necessary, it is desirable to elute the compound in a dry and inert solvent such as hexane.

8.4.5. Strategies for Solid Phase Extraction

There are two simple strategies for sample preparation. By choosing a cartridge sorbent and sample solvent to cause the component(s) of interest to be:

- Unretained while matrix interferences are absorbed.
- Retained while matrix interferences pass through unretained.

The first strategy is usually chosen when the desired sample component is present in a high concentration. When the analyte of interest is present at low levels or multiple components of wide differing polarities need to be isolated, the second strategy is generally employed. The second strategy may also be used for trace enrichment of extremely low-level compounds and concentration of dilute samples. With either strategy, there are three different chromatographic modes to choose from.

- Normal phase
- Reversed phase
- Ion-exchange

There are many different types of sorbents for each mode, and the selection of strategy, mode, sorbent, and elution solvents will depend upon the specific sample mixture and goal of separation.

9. METHOD DEVELOPMENT AND OPTIMISATION OF CONDITIONS FOR SOLID PHASE EXTRACTION OF ORGANOPHOSPHATE PESTICIDES

9.1. Experimental

9.1.1. Reagents and Materials

Eleven pesticides standard were obtained from Promochem Ltd (UK).

Malathion	Chlorpyrifos	Diazinon	Ethion	Fenthion
Fenitrothion	Coumaphos	Methyl Parathion	Profenofos	Prothiofos
Quinalphos				

Residue analysis grade solvents - ethyl acetate, methanol, acetone, acetonitrile and n-hexane were supplied by BS & S (Scotland) and ODS C18 and Bond Elute Certify cartridges were obtained from Varian (UK).

9.1.2. GC system

Gas chromatography was carried out using a Hewlett-Packard Model 5890 Series II instrument fitted with a nitrogen phosphorus detector, a 0.75 mm splitless insert and an HP1 capillary column (30 m x 0.53 mm and 0.88 μm phase thickness). The column temperature was set at 100°C for 2 minutes and then programmed from 100°C to 300°C at 10°C/minute (held for 3 minutes). The temperature of the injection port was set at 250°C and the detector at 250°C.

GC-MS was carried out using a Thermo-Finnigan Trace GC linked to a Trace MS, with an HP-5 X-link column (5%Ph, 95% Me Silicone, 30 m x 0.32 mm x 0.25 μm film thickness). The column temperature was set at 100°C for 2 minutes and then programmed from 100°C to 300°C at 10°C/minute (held for 3 minutes). The temperature of the injection port was set at 250°C and interface also at 250°C. The ionisation energy was 70eV in the EI+ mode. Full scans were collected over the mass range m/z 50 to 460.

9.1.3. Study of extraction on Bond Elut Certify (BEC) cartridges

A study of SPE of pesticides on BEC and ODS C18 cartridges was carried out using a vacuum workstation supplied by Varian. The first type of cartridge contains mixed-mode bonded silica, which consists of hydrophobic and cation exchange functional groups whereas the ODS C18 phase consists of hydrophobic functional groups only. A set of 5 cartridges of each type was conditioned by successive elution of 2 ml of methanol followed by 2 ml of deionised water: methanol (ratio 1:1) and finally with 2 ml of deionised water by means of gentle vacuum, to avoid drying-out during the procedure. Then 1 ml of blank blood diluted with 2 ml of deionised water was percolated through the cartridge at 2 ml/min. The cartridges were washed with 2 ml of deionised water and later dried under vacuum. The cartridges were then eluted with 2 ml solvent, which was collected and evaporated to dryness with nitrogen. The residue was reconstituted with 50 µl methanol and 1 µL was injected into the GC-NPD instrument. The chromatograms for both types of cartridge were compared to see which gave cleaner extraction.

Ethyl acetate, dichloromethane (DCM), acetonitrile, n-hexane and methanol were used as eluents to collect the extract, which was assayed in 1 ml of blood spiked with 100 ng of each pesticide prior to extraction. Those solvents were used in the study as eluents because theoretically they had adequate polarity and solvent strength to achieve the complete elution of pesticides retained on the cartridges.

The influence of an equilibrium (or soaking) time of 2 minutes between the solvent and stationary phase before eluting the cartridge, and of the solvent volume eluted (2, 3 and 4 ml) on the recovery was also studied, carrying out the elution with the solvent previously selected.

Diluting the specimen will reduce the matrix effect but it also dilutes the analyte of interest as well as potentially decreasing the recovery due to an increase in solubility of the analyte(s) in the aqueous phase.

Also, a study was made on the effect and influence of pH adjustment (addition of 0.1 M HCl and 0.1M NaOH) and ionic strength (addition of saturated NaCl) on the extraction process.

9.1.4. Procedure proposed for blood analysis

Extraction was performed on BEC conditioned as described in Section 9.2. A blood sample volume of 1 ml, diluted with 2 ml. of deionised water and 100 µL 0.1M HCl was percolated through the cartridge at 2 ml/min, which was then washed with 2 ml. of deionised water and dried under vacuum. The analytes were eluted with 3 ml of ethyl acetate. The eluate was evaporated to dryness under a stream of nitrogen. The residue was finally redissolved in 50 µl of ethyl acetate and 2 µl were injected into the GC-NPD instrument.

9.2. Results and Discussion

9.2.1. Extraction on ODS C18 and BEC Cartridges

Figure 9.1 and Figure 9.2 show chromatograms for extracts of whole blood (blanks) made with BEC and ODS cartridges respectively. It was found that there was no significant difference between BEC and ODS C18 with respect to cleaner extracts. BEC was chosen for subsequent work since the presence of cationic exchange groups would be advantageous for extraction of polar metabolites in future.

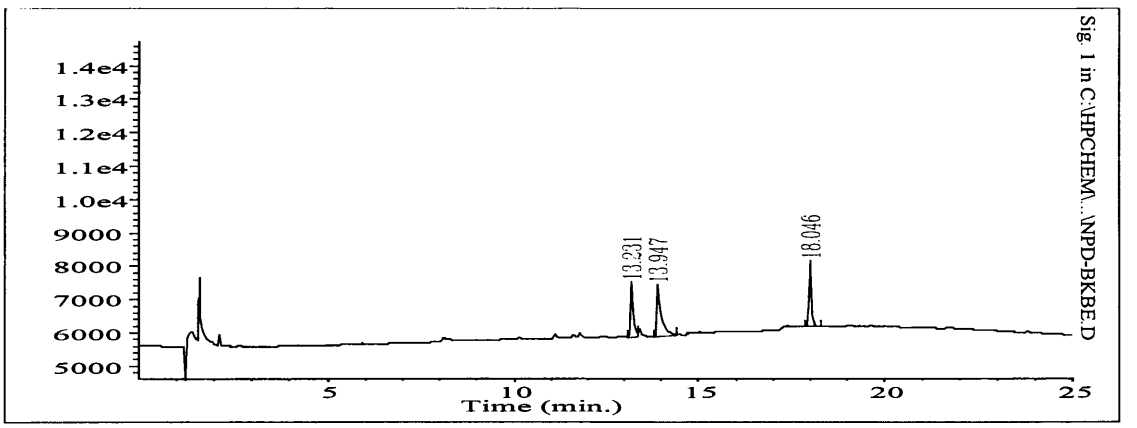


Figure 9.1 Chromatogram of blank blood after BEC cleanup

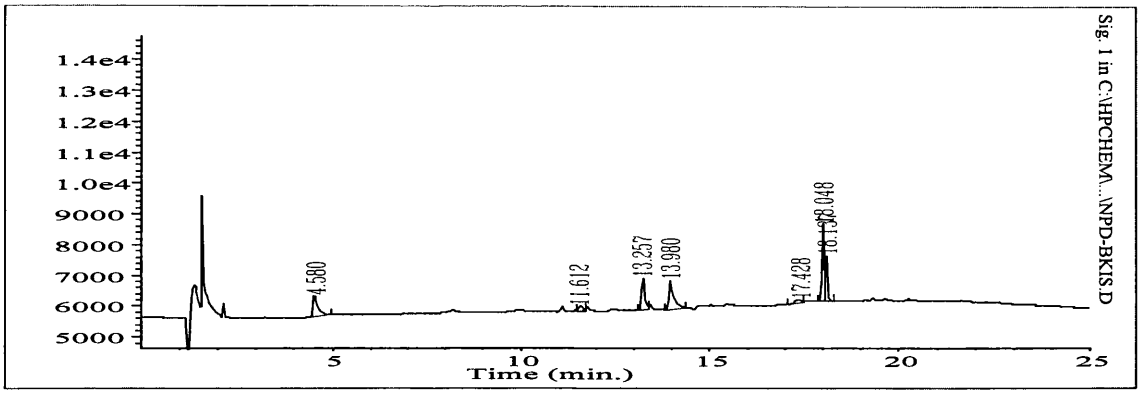


Figure 9.2 Chromatogram of blank blood after ODS C18 cleanup.

9.2.2. Volume and type of organic solvent used as eluent

The first parameter studied was the eluting solvent to determine the best solvent for elution based on recovery. Table 9.1 shows that the average recoveries for, n-hexane, acetonitrile, ethyl acetate, dichloromethane and methanol were 24.0%, 94.0%, 94.6%, 67.2% and 81.1% respectively. Ethyl acetate was chosen due to the high recoveries obtained with it and also because the other solvents tend to be quite harmful to the environment especially n-hexane, acetonitrile and dichloromethane.

The influence of the equilibrium time (cartridge soaked for 2 minutes prior to elution) did show an overall improvement in recoveries (Table 9.2). However, the effect of eluent volume did not show a marked improvement in recoveries and the volume of eluent used in subsequent work was set at 3 ml.

9.2.3. Effects of aqueous solution volume on recoveries from BEC

Since the structures and chemical properties of pesticides vary greatly, the aqueous solution volume is an important parameter to optimise in the extraction procedure. From Table 9.3 it can be seen that the recovery of most pesticides decreases as the volume of aqueous solution increases. The increased amount of water will promote better solvation of the pesticides, leading to breakthrough on the cartridge and a lower recovery.

Initial washing decreases the recovery on the majority of pesticides but subsequently this effect was negligible after washing a few times (4-10 ml). Thus, in subsequent work washing was kept to a minimum (Table 9.4).

9.2.4. Influence of pH and ionic strength adjustment

Changing the pH affects the overall percentage recovery (Table 9.5). An increase in recovery was observed when the pH was decreased except for diazinon and fenitrothion. Both are considered to be more stable in the higher pH region. The same criteria should be observed when the ionic strength of the specimen was adjusted by adding salt, in this case sodium chloride. Adjustment of salt content at one end can reduce the solubility of organic compounds in water, thus increasing extraction efficiency, but it can also suppress the release of pesticides from the erythrocyte membrane by trapping lipophilic compounds in membranous vesicles e.g. malathion. Also the presence of a high molarity of salt could disrupt the ion exchange retention mechanism on the cartridge phase.

Thus changing the pH and salt content should be minimised. Ionic strength adjustment was not necessary in this extraction technique

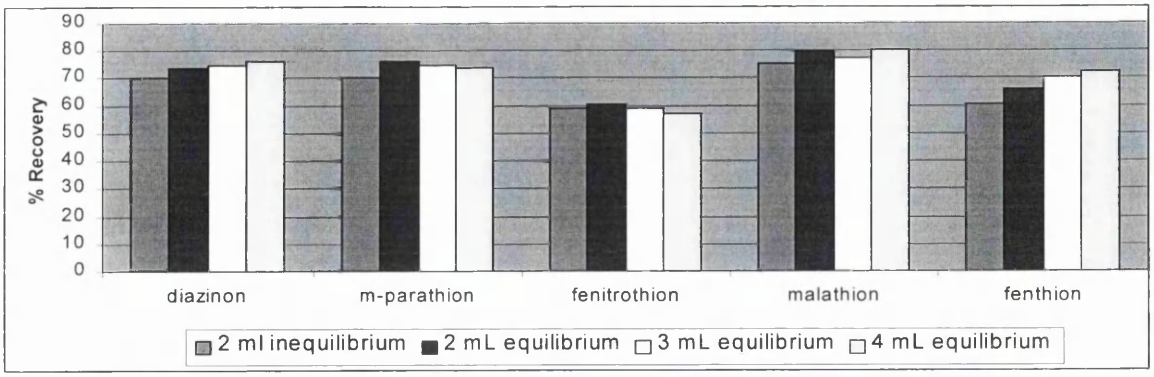
Despite the absence of clean-up procedure prior to analysis, the technique allowed good selectivity for the eleven pesticides evaluated here, owing to the use of a selective nitrogen detector. Endogenous components and potential interferences were not observed using the nitrogen detector. This SPE procedure is well suited for non-polar and semi-polar compounds such as most of the organophosphates and should be applicable to urine and other biological matrices such as vitreous humor, saliva and cerebrospinal fluid. The major setback encountered was sample clogging of the cartridge if the blood was not homogeneous (free from clots and haemolysed). This can be time consuming and the result found is of doubtful accuracy. However, this problem can be reduced by vortexing the sample prior to applying it onto the cartridge.

Table 9.1 Recovery (%) of pesticides from 1 ml blood spiked with 100 ng of each pesticide from BEC eluted with 2 ml of different solvents (n=5).

Solvent / Pesticide	n-hexane	acetonitrile	ethyl acetate	dichloro-methane	methanol
Chlorpyrifos	29.2	82.8	103.0	62.4	73.0
Diazinon	8.5	53.2	83.6	67.2	8.8
Ethion	20.1	106.2	105.2	80.0	108.0
Fenitrothion	41.4	103.5	105.8	62.7	75.1
Fenthion	36.8	117.1	62.0	79.8	87.7
Malathion	13.2	101.2	107.8	72.0	80.2
Methyl Parathion	8.8	107.3	102.1	61.6	99.6
Coumaphos	17.6	79.3	80.2	54.2	77.9
Profenofos	50.3	63.4	75.2	59.1	78.7
Prothiofos	30.6	84.8	84.0	67.6	68.5
Quinalphos	7.4	105.2	103.8	72.4	101.5

Table 9.2 Recovery (%) of pesticides from 1 ml of blood spiked with 100 ng of each pesticide from BEC eluted with different volumes of ethyl acetate and different equilibrium times (n=5).

Pesticide	Equilibrium time = 0	Equilibrium time = 2 minute		
	2 ml	2 ml	3ml	4 ml
Chlorpyrifos	86.3	90.8	91.1	92.1
Diazinon	70.1	73.7	74.5	76.0
Ethion	84.6	86.1	83.9	79.7
Fenitrothion	58.7	60.5	58.7	56.9
Fenthion	60.4	65.9	70.3	72.1
Malathion	75.3	79.5	77.2	80.2
Methyl Parathion	70.2	76.3	74.5	73.7
Coumaphos	62.4	68.2	69.8	67.2
Profenofos	76.3	79.1	74.7	75.1
Prothiofos	67.3	76.0	79.9	75.9
Quinalphos	81.2	84.9	81.2	87.4



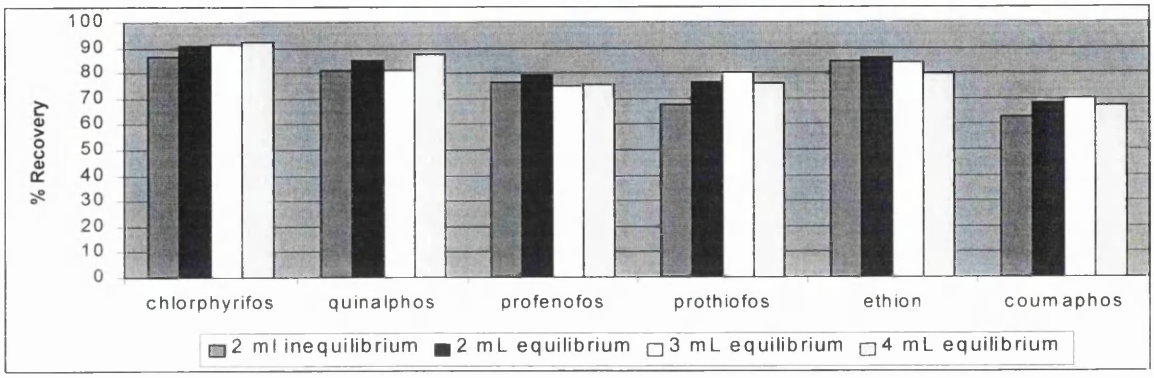
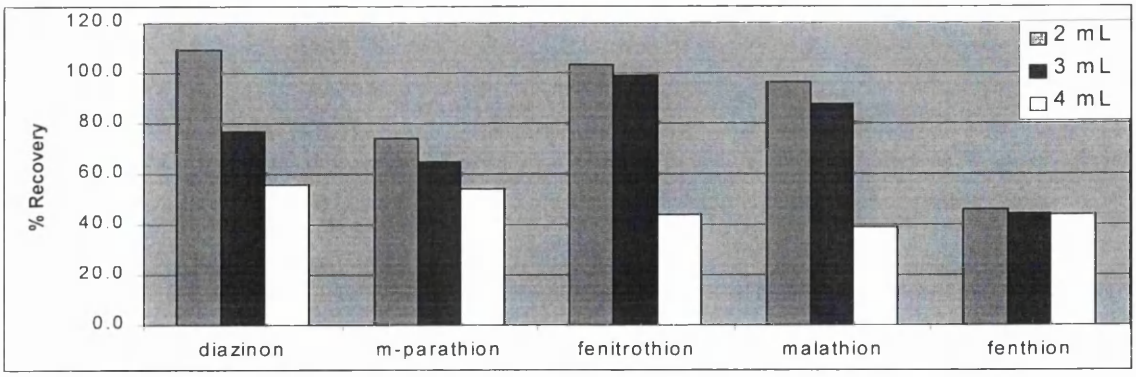


Table 9.3 Recovery (%) of 100 ng pesticides spiked into 1 ml blood and diluted with different volumes of deionised water (n=5).

Deionised Water / Pesticide	2 ml Recovery	3 ml	4 ml
Chlorpyrifos	79.0	62.5	31.0
Diazinon	109.0	76.7	55.7
Ethion	101.9	62.7	77.1
Fenitrothion	103.2	98.9	44.0
Fenthion	46.1	44.4	43.9
Malathion	96.2	87.8	38.9
Methyl Parathion	74.1	64.7	53.9
Coumaphos	53.1	47.9	31.2
Profenofos	60.0	57.2	55.3
Prothiofos	56.7	54.1	46.1
Quinalphos	84.6	77.7	46.5



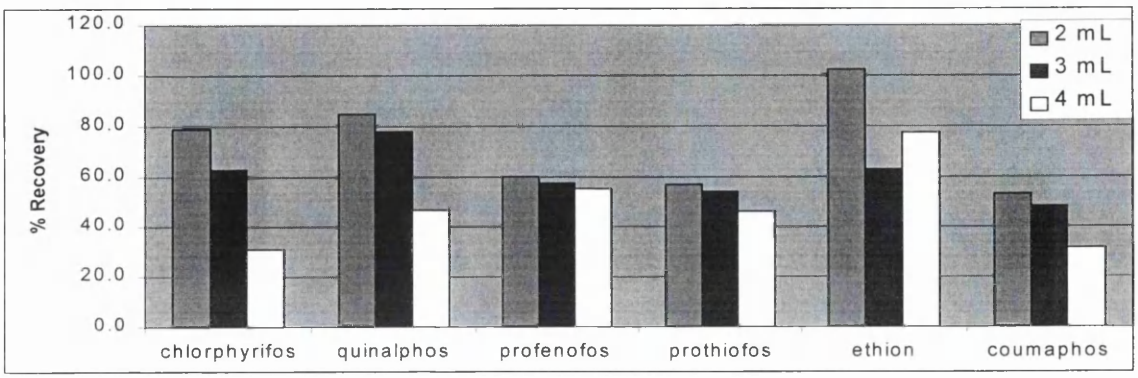
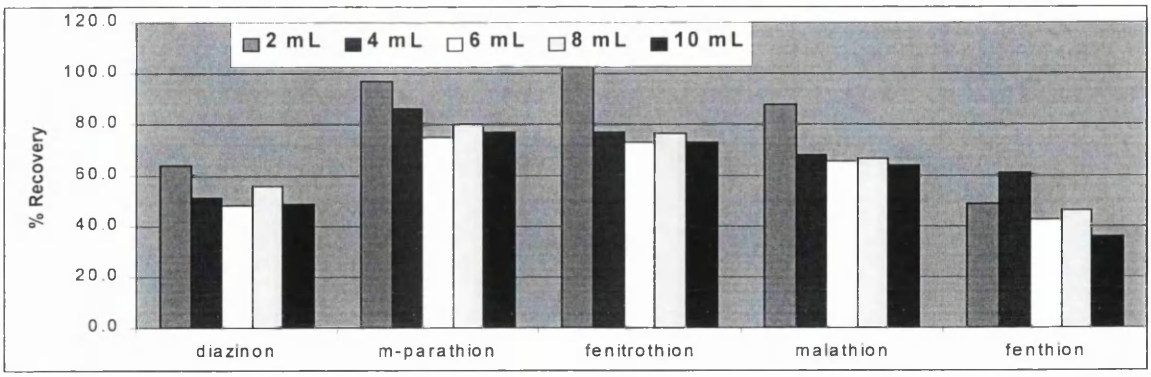


Table 9.4 Recovery (%) of 100 ng pesticides spiked into 1 ml blood diluted with 2ml deionised water, after washing the sorbent with different volumes of deionised water (n=5).

deionised Water / Pesticide	2 ml	4 ml	6 ml	8 ml	10 ml
Chlorpyrifos	95.5	76.7	69.6	69.4	73.9
Diazinon	63.6	51.3	47.8	55.9	48.3
Ethion	105.2	88.3	81.6	74.3	91.7
Fenitrothion	102.8	77.1	72.9	76.7	73.1
Fenthion	48.6	60.8	42.2	46.0	35.8
Malathion	87.8	68.0	65.2	66.5	63.6
Methyl Parathion	97.0	86.4	75.3	80.4	77.2
Coumaphos	59.7	61.4	57.1	56.4	59.3
Profenofos	55.9	59.1	60.9	53.7	54.6
Prothiofos	60.7	58.5	57.0	56.3	56.9
Quinalphos	90.0	78.6	76.7	73.9	74.6



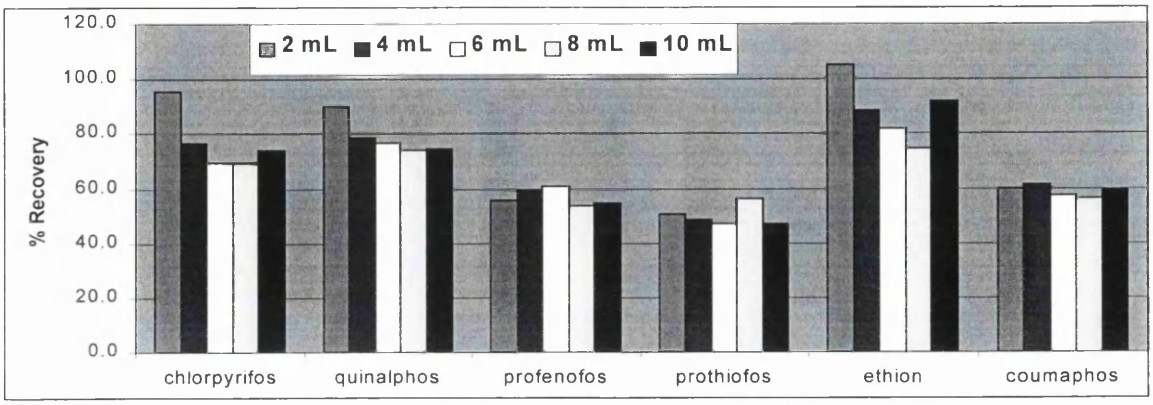
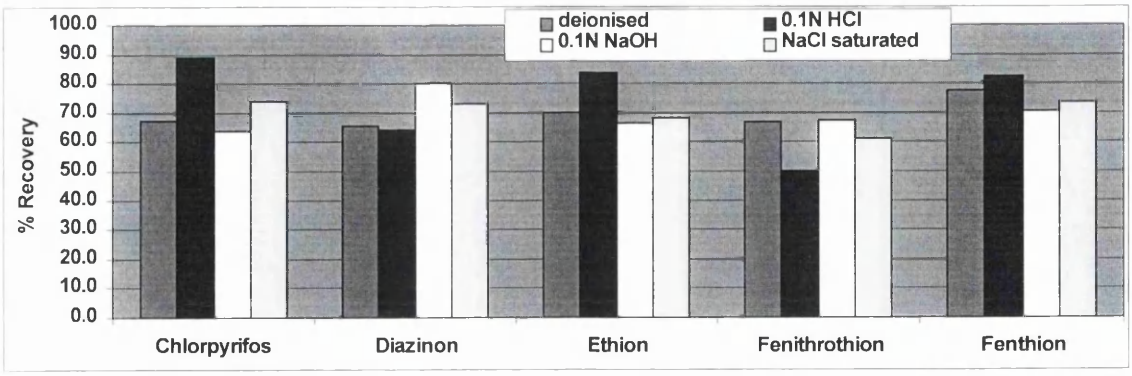
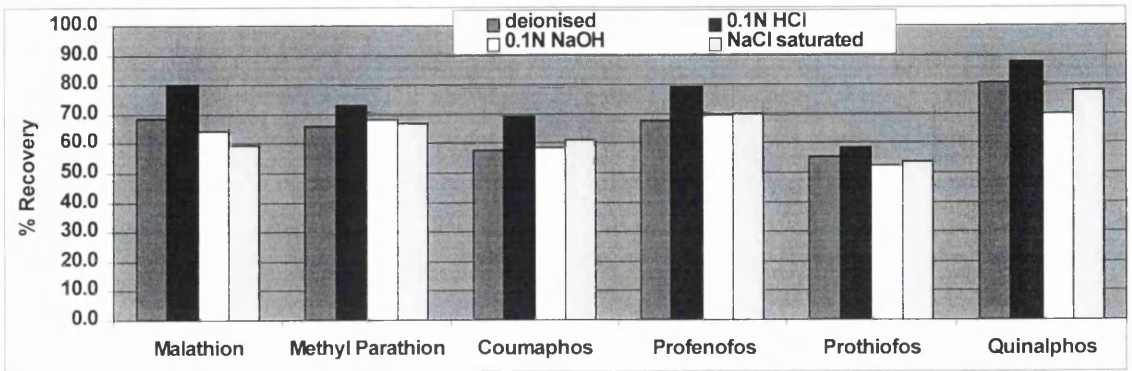


Table 9.5 Recovery (%) of 100 ng pesticides spiked into 1 ml blood after pH and ionic strength adjustment (n=5).

Pesticide	100 µl of 0.1M HCl Recovery %	100 µl of 0.1M NaOH Recovery %	100 µl of Satd. NaCl Recovery %
Chlorpyrifos	89.0	63.9	74.1
Diazinon	64.3	79.9	69.4
Ethion	79.7	66.4	65.3
Fenitrothion	50.2	67.3	51.1
Fenthion	79.8	73.5	73.6
Malathion	80.2	64.2	59.5
Methyl Parathion	73.2	68.2	66.9
Coumaphos	67.2	58.3	60.9
Profenofos	75.1	69.4	70.1
Prothiofos	55.9	52.1	49.2
Quinalphos	77.9	70.1	63.2





9.2.5. Procedure Evaluation

Table 9.6 shows the recoveries at the detection limit for the eleven OP's and Table 9.7 lists the precision achieved in the application of the proposed procedure to blood spiked with 50 ng of each pesticide, using diazinon (60 ng) as Internal Standard. The recoveries were above 50% with RSD for precision ranging from 1.9% to 10.7%.

Table 9.8 gives the recovery from urine at the minimum detectable level based on the technique used for blood. The minimum detectable level was found to be in the range of 1-5 ng/ml. Precision study at 50 ng/ml urine gave RSD ranging 1.2-8.4% (Table 9.9)

Table 9.6 Recoveries of pesticides (%) at detection limit from 1 ml blood spiked with different amount of pesticide (n=3).

Pesticide	10 ng Recovery	20 ng Recovery	30 ng Recovery	40 ng Recovery	50 ng Recovery
Chlorpyrifos	80.2	77.9	89.6	80.4	83.8
Diazinon	77.3	69.5	78.1	80.3	73.7
Ethion	84.3	78.4	79.9	83.8	90.1
Fenitrothion	Nd	56.3	49.8	54.9	60.5
Fenthion	Nd	Nd	73.4	80.1	81.9
Malathion	Nd	Nd	Nd	57.6	70.4
Methyl Parathion	Nd	Nd	60.3	59.1	66.3
Coumaphos	Nd	Nd	nd	nd	58.2
Profenofos	Nd	Nd	69.4	80.1	79.1
Prothiofos	Nd	Nd	73.4	82.8	76.0
Quinalphos	80.1	78.1	72.4	81.3	84.9

Nd = Not detected

Table 9.7 Recovery (%) and precision (RSD) of 50 ng pesticide spiked into 1 ml blood by the proposed procedure (n=5) using diazinon (60 ng) as internal standard.

Pesticide	Recovery (RSD) Day 1	Recovery (RSD) Day 3	Recovery (RSD) Day 5
Chlorpyrifos	90.2 (3.0)	85.1 (2.6)	82.5 (1.9)
Ethion	85.6 (6.7)	82.1 (2.9)	81.1 (3.1)
Fenitrothion	59.6 (5.2)	59.1 (4.7)	58.0 (3.1)
Fenthion	80.4 (3.5)	76.3 (5.8)	80.4 (3.5)
Malathion	73.4 (6.4)	76.4 (2.5)	73.4 (6.4)
Methyl Parathion	68.6 (5.5)	68.2 (4.1)	68.6 (5.5)
Coumaphos	60.3 (7.9)	58.9 (2.9)	60.3 (7.9)
Profenofos	77.4 (6.4)	73.4 (3.8)	79.6 (2.9)
Prothiofos	67.5 (10.7)	58.7 (9.4)	55.4 (3.2)
Quinalphos	82.0 (5.1)	80.9 (4.2)	79.3 (2.1)

Table 9.8. Recovery at detectable limit (%) of pesticide from 1 ml urine spiked with different amount of pesticide (n=3)

Pesticide	1 ng Recovery	2 ng Recovery	3 ng Recovery	4 ng Recovery	5 ng Recovery
Chlorpyrifos	82.3	80.3	81.6	83.5	82.5
Diazinon	77.9	80.4	81.2	81.9	80.7
Ethion	Nd	Nd	79.5	77.5	78.2
Fenitrothion	Nd	Nd	82.8	83.1	82.7
Fenthion	Nd	Nd	81.1	81.3	80.4
Malathion	Nd	Nd	nd	80.3	83.9
ethyl Parathion	Nd	Nd	79.2	79.4	79.9
Coumaphos	Nd	Nd	nd	nd	69.4
Profenofos	Nd	Nd	71.1	72.7	71.5
Prothiofos	Nd	Nd	82.1	80.3	78.9
Quinalphos	83.9	84.1	83.7	82.7	83.2

Nd: not detected

Table 9.9 Recovery (%) and precision (RSD) of 50 ng pesticide spiked into 1 ml urine by the proposed procedure (n=5) using diazinon (60 ng) as internal standard.

Pesticide	Recovery (RSD) Day 1	Recovery (RSD) Day 3	Recovery (RSD) Day 5
Chlorpyrifos	82.5 (2.3)	82.3 (3.7)	81.9 (2.9)
Ethion	78.2 (3,1)	79.3 (2.8)	77.8 (5.7)
Fenithrothion	82.7 (4.5)	80.3 (4.3)	82.1 (6.2)
Fenthion	80.4 (3.8)	76.3 (5.0)	82.1 (2.1)
Malathion	83.9 (3.9)	83.4 (2.8)	80.2 (5.9)
Methyl Parathion	79.9 (4.1)	78.9 (3.1)	73.7 (6.7)
Coumaphos	76.1 (1.9)	74.7 (4.0)	77.2 (7.2)
Profenofos	79.9 (2.1)	77.8 (3.1)	77.9 (3.2)
Prothiofos	78.9 (2.7)	78.3 93.2)	77.9 (8.4)
Quinalphos	83.2 (1.2)	80.5 (2.8)	77.8 (2.5)

9.2.6. Analysis of case samples

Application to five blood specimens submitted by the Department of Chemistry, Malaysia and Forensic Laboratory in Mauritius revealed the presence of malathion (2 samples), fenthion (2 samples) and chlorpyrifos (1 sample). Figure 9.3 shows the calibration curves constructed using standards for the respective pesticides found in positive cases and Table 9.10 gives their respective coefficients of variation. The procedure for extraction was as stated above with diazinon (60 ng) as an Internal Standard. Table 9.11 shows the results of analysis of the case specimens and concentrations in blood in ng/ml.

Table 9.10 Calibration curves for organophosphorus pesticides extracted from whole blood by SPE

Type of Pesticide	Calibration curve	Correlation Coefficient (R^2)	Concentration Range (ng/ml)
Malathion	y = 0.0019x - 0.0766	0.9895	50-250
Fenthion	y = 0.0138x - 0.536	0.9900	50-250
Chlorpyrifos	y = 0.018x - 0.447	0.9895	50-250

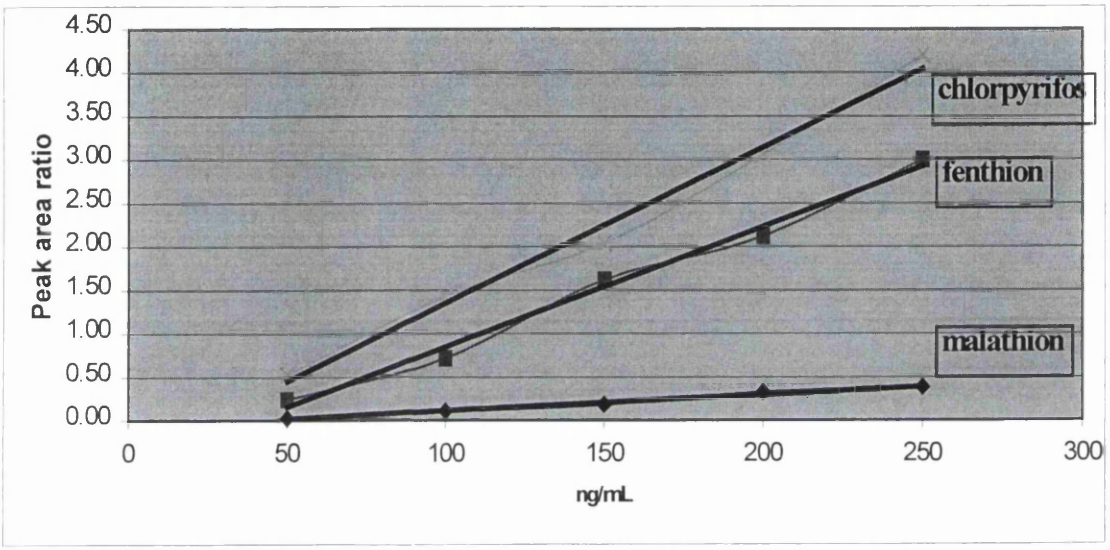


Figure 9.3 Calibration curves for pesticides found in positive cases using GC-NPD

Table 9.11 Results obtained from analysis of actual case samples submitted by the Chemistry Department, Malaysia and Forensic Laboratory, Mauritius

Case Number	Pesticide detected	Concentration ng/ml blood
T2393	Chlorpyrifos	110.9
T2790	Fenthion	257.0
T1374	Fenthion	50.4
T6392	Malathion	114.0
T7560	Malathion	187.7

The identity of these compounds was confirmed by analysis of the extracts by GC-MS in the full scan mode. All of these samples were also analysed by the HS-SPME method developed previously.

Table 9.12 Preferred Fragments for Identification and Limit of Detection in Full Scan mode

Compound	Mass fragments (m/z)				LOD (ng/mL)
Chlorpyrifos	314	286	197	97	50
Diazinon (IS)	137	179	304	152	50
Fenitrothion	260	125	277	109	100
Fenthion	169	153	278	109	50
Malathion	173	158	125	98	100

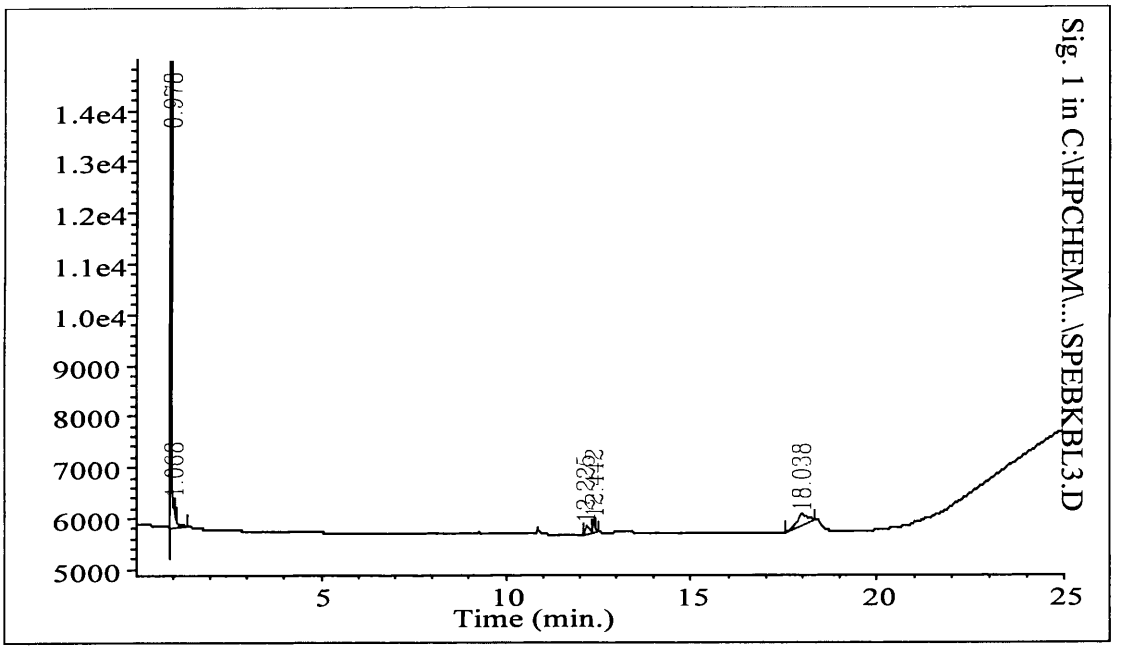


Figure 9.4 Blank blood extracted by SPE and analysed by GC-NPD.

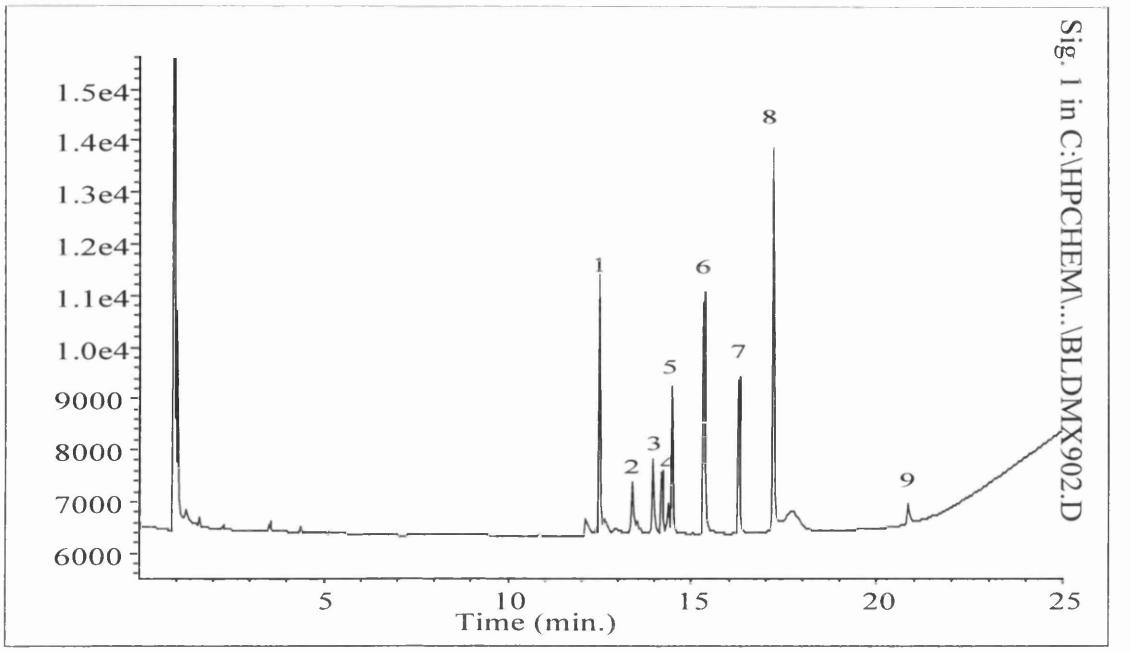


Figure 9.5 Chromatogram of organophosphates spiked in blood at 50 ng/ml using GC-NPD; 1= diazinon, 2= methylparathion, 3= fenitrothion, 4= malathion, 5= fenthion and chlorpyrifos (almost same RT), 6= quinalphos, 7= prothiofos & profenofos, 8= ethion, 9= coumaphos

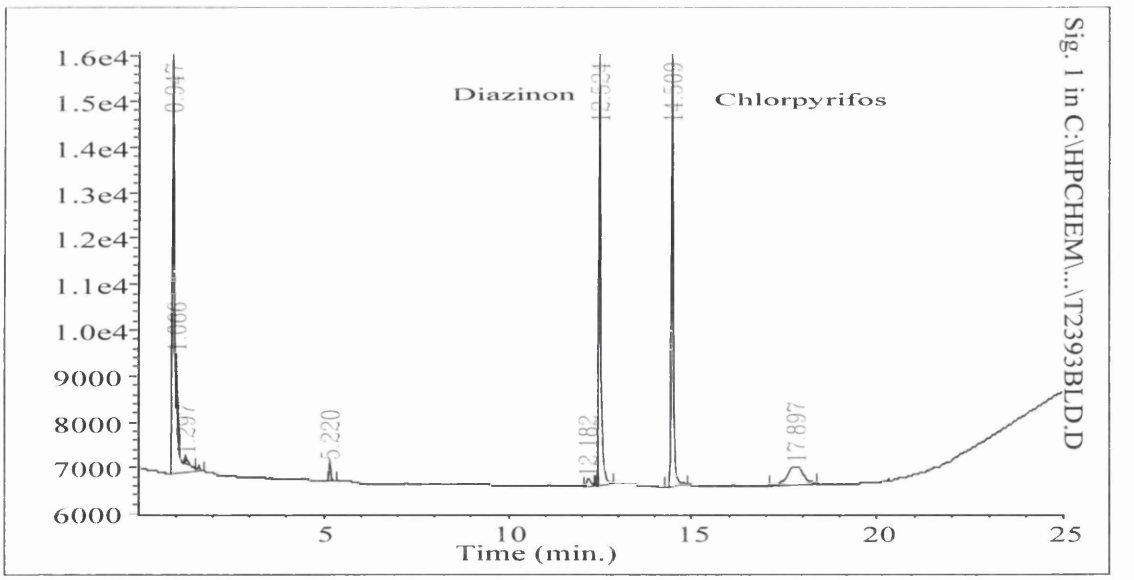


Figure 9.6 Positive case T2393BLD extracted by SPE and analysed by GC-NPD.

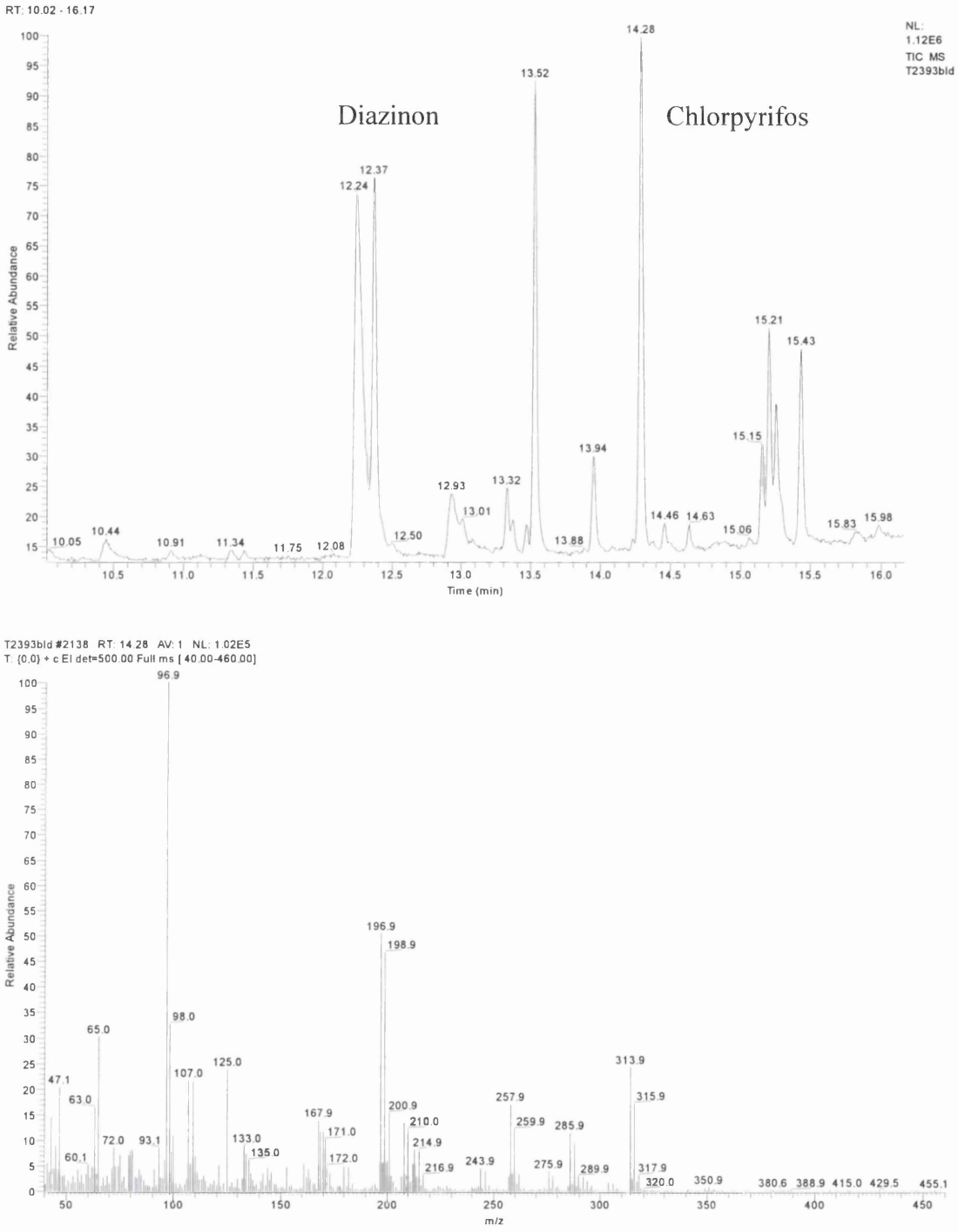


Figure 9.7 Total ion current chromatogram (generated from full scan data) for a autopsy whole blood specimen in a fatality due to an OP pesticide (T2393/Chlorpyrifos).

9.4. Conclusion

The combined use of SPE followed by GC-NPD and GC-MS makes possible the direct, reliable, efficient and economical determination of pesticides in blood samples. The optimisation of the experimental variables that affect the extraction and elution processes is advisable in order to ensure high recoveries as a consequence of the wide variability of properties of the pesticides, which are potentially present. Hence, the present method developed involved a selective SPE procedure with satisfactory recoveries and specific GC-NPD determination. This method is suitable for the routine toxicological analysis of biological fluids containing residual amounts of pesticides. The limits of detection were occasionally insufficient for monitoring residues in the non-exposed case, but nevertheless they were appropriate for analysis of the higher levels encountered in acute poisoning cases.

10. SAMPLE PREPARATION FOR GAS CHROMATOGRAPHY WITH SOLID PHASE EXTRACTION AND SOLID-PHASE MICROEXTRACTION: THE COMPARISON

10.1. Introduction

The successful extraction of drugs from biological fluids and matrices presents several challenges. Biological materials, including, blood, urine, saliva and hair, are much more complex than many others. They often contain proteins, salts, acids, bases and numerous organic compounds with similar chemistry to the analytes of interest. Also, within classes of pesticides such as organophosphates, a variety of acid-base behaviours and functional groups can be present, which strongly affect solubility or affinity to a sorbent. Thus, general extraction methods for biological matrices have either been complex, if selectivity is desired, or straightforward, but not selective, leaving the analyst with complicated separations following the extraction. At the present time, sample preparation is the area where a great deal of work needs to be done [127]. For biological matrices, selective analysis for pesticides in a variety of matrices is often performed at present by liquid-liquid extraction [58, 60, 61, 63, 64] or solid phase extraction [43, 57, 62, 125]. Liquid-liquid extraction (LLE), the traditional method of treatment of samples prior to GC injection, is no longer acceptable for many reasons: a typical LLE procedure requires several steps, making the clean-up process tedious and difficult; the highly purified solvents that are required are expensive to purchase and to dispose of; many of these solvents are suspected of endangering the health of laboratory workers. Several alternative methods, which reduce or eliminate the use of solvents, are now being used to prepare samples for GC analysis. These include solid phase extraction (SPE) and solid phase microextraction (SPME).

This section deals with a comparison of SPE and SPME.

10.2. Experimental

Chemicals, Equipment and Extraction Procedure as stated previously in Chapters 5 and 9.

10.3. Results

10.3.1. Limit of Detection, Precision and Recovery

These are summarised for the two methods in Table 10.1.

Table 10.1 Limits of Detection, Recoveries and Precision for organophosphate pesticides in blood analysed by GC-NPD.

Pesticide	LOD (ng/mL)		% Recovery		RSD Precision %	
	SPE	HSSPME	SPE	HSSPME	SPE	HSSPME
Chlorpyrifos	10	20	82.5	3.4	1.9	6.5
Diazinon	10	2	83.4	5.8	2.5	4.8
Ethion	10	20	81.1	4.1	3.1	4.2
Fenitrothion	20	30	58.0	0.9	3.1	14.0
Fenthion	30	10	80.4	2.7	3.5	3.8
Malathion	40	100	73.4	0.4	6.4	4.4
Methyl Parathion	30	80	68.6	0.5	5.5	10.0
Coumaphos	50	60	60.3	0.7	7.9	7.3
Profenofos	30	100	79.6	0.5	2.9	8.1
Prothiofos	30	60	55.4	0.3	3.2	3.5
Quinalphos	10	80	79.3	0.4	2.1	6.1
Overall	10-50	2-100	Average 72.9 %	Average 1.8 %	1.9-7.9	3.5-14.0

Table 10.2 Comparison of quantitative analytical results obtained for the same samples by the two methods of extraction.

Case No.	Pesticide	SPE (ng/mL)	HS-SPME (ng/mL)
T2393	Chlorpyrifos	110.9	89.5
T2790	Fenthion	257.0	230.4
T1374	Fenthion	50.4	35.2
T6392	Malathion	114.0	94.3
T7560	Malathion	187.7	115.2

10.4. Discussion

Solid phase microextraction is often considered as another form of solid-phase extraction or micro-SPE. There are significant differences between the methods however.

Solid phase extraction is essentially a three-step process. A sample is initially passed through the sorbent bed, and analytes present in the sample are exhaustively extracted from the sample matrix to the solid sorbent. In a second step, unwanted analytes are selectively desorbed from the solid sorbent by washing with a solution capable of desorbing unwanted analytes, but leaving desired analytes retained on the sorbent. In the final step the wash solution is changed for one able to desorb analytes of interest. The resulting eluent may then be concentrated by evaporation, to the desired volume.

Solid phase microextraction, in this case use of the headspace, takes advantage of equilibrium extraction and selective sorption from the matrix onto the fibre coating. In the first step, the fibre coating is exposed to the sample headspace (vapour in a confined volume) and analytes with a high affinity for the sorbent are selectively extracted. In the second step, everything extracted by the fibre is desorbed into the analytical instrument. No intermediate clean-up step is normally implemented. Micro-SPE is related to SPE as it is a total extraction method, but utilises a reduced sample and sorbent volume.

A degree of selectivity is required for any sample preparation method. It is impractical to introduce all compounds present in a sample to an analytical instrument. The method developed must eliminate compounds incompatible with the instrument including matrix components. It is also desirable to remove as many of the unwanted compounds as possible, to make the resulting data interpretation as clean and simple as possible. Thus, with selective extraction, sample preparation is simplified and typically results in significant gains in time and precision.

Selectivity is therefore important when choosing a fibre coating. High capacity, even for a range of analytes, is more important for solid-phase extraction, where prevention of break-through is a significant concern. Because break-through is not an issue to be addressed in an equilibrium extraction method such as SPME, more emphasis may be placed on sorbent selectivity or analytical procedure.

Another significant difference, in that SPE sorbents, because of the large volume of sorbent required relative to SPME, have a potential to retain non-adsorbed components in the void volume. It is difficult to design a wash regime that removes unwanted compounds completely, without impacting on retention of the analyte(s) of interest. In this way there is a possibility that unwanted compounds may remain, either adsorbed, or present as non-adsorbed analytes in the bulk of the sorbent. Because of the geometry of the SPME device, and the modes of extraction used, unwanted analytes are not normally present in the sorbent at the time of desorption.

SPME devices have an open-bed structure, relative to SPE devices, where the extraction medium is packed into a cartridge-like container. In SPME the surface of the extraction phase is itself accessible for analysis. This is less valid with in-tube SPME, although limited surface characterisation has been performed with the capillaries as well. Therefore, with SPME it is possible to perform convenient spectroscopic analysis of surface adsorbed components and not only extracted chemical species, but also the composition of collected aerosols or particulates. This can have an important advantage in speciation and characterisation of natural systems.

Laborious operations such as conditioning, washing, elution and solvent evaporation are needed. Aspects of involving significant volumes of organic solvents in SPE cannot be neglected with regards to environmental pollution. Problems involving clotting, channelling and percolation are typical of SPE and are encountered in every-day laboratory work.

The advantages claimed for SPME were that it is solventless, easy to handle, needs little equipment, is a fast method (in terms of sample preparation), gives good linearity and gives quite high sensitivity. However, taking into account the method developed during this work, it is obvious that SPME can display these advantages only in some areas of biomedical analysis. In SPME especially, the headspace problem encountered would be the target analytes, which need to volatile or semi-volatile. The extraction is quite slow in comparison to SPE. Extraction times considerably lower than t_e must be used because of practical requirements and limitations. Thus, the recovery was very low and the sensitivity was critical for some analytes. Table 10.1 showed recovery (~2 %) compared to (~73%) and precision (3.5-14.0%) compared to (1.9-7.9%) for SPME to SPE respectively. Careful consideration must be given, especially for precision (involving extraction at equilibrium and non-equilibrium condition) in a manual procedure. But this can be overcome by automation.

Given the reasons above, the results in Table 10.2 show that SPE and HS-SPME are quite comparable to one another but HS-SPME gave a lower extraction value.

10.5. Conclusion

Both SPE and SPME are helping laboratories to achieve the goals of minimising the use of solvents for sample preparation and freeing workers from the tedium of sample clean up. SPE can replace liquid-liquid extraction in many instances; SPME can be used instead of liquid-liquid extraction and/or static headspace analysis. For dirty samples, it may be of value to combine SPME and SPE. When a SPE procedure requires the elution of analytes into a buffer, rather than extracting into an organic solvent prior to injection into a GC, it would be convenient to use a SPME fibre to

concentrate the analytes. Table 10.3 summarises some of the main features of SPE and SPME.

Table 10.3 Overall Summary of SPE and SPME (direct and headspace)

	SPE	SPME
Sample matrices	Liquids - organic or aqueous	Aqueous liquids, solids, gases
Sample pre-treatment	Blood, tissue, and some environmental samples; usually require pre-treatment to remove solids or proteins; many liquid sample do not	For determining volatiles, sample pre-treatment usually not required. Analytes not amenable to headspace may require some matrix cleanup
Analytes	Semi-volatiles and slightly volatile compounds; recovery a problem with volatiles	Volatiles and semi-volatiles
Use of organic solvents	Minimal - much less than with liquid-liquid extraction	Almost None
Recovery of analytes	Generally recoveries close to 100% expected	Equilibrium method - quantification usually by comparing to spiked blank matrix or standard additions.
Automation	Limited on-line with GC	Online technique available for GC and HPLC
Government regulatory agency approval	Several approved methods	Relatively new technique (no approved method at the present time)

11. DEGRADATION OF MALATHION IN VITRO: HS-SPME AS TOOL FOR EXTRACTION

11.1. Introduction

Measurement of intact organophosphorous pesticides in blood and urine has often been performed to confirm exposure in acute or chronic poisoning cases. Cases involving poisoning by OP's either intentionally or accidentally are quite frequently encountered in Malaysia [14]. Many OP's will undergo changes when they are exposed to the biological environment. If the process is fast and drastic (chemically labile pesticides), then problems will be encountered during the analysis. The structure and composition of the component of interest will change. On many occasions, problem arise in identifying the parent compound due to the rapid biodegradation process. Moreover, because some OP's are unstable, and with few exceptions, they can only be detected in biological specimen for a few hours after exposure. Thus, it is important to stabilise them in the *in vitro* environment so as to stop or reduce the rate of degradation of the analytes of interest.

Most of the phosphorus-containing insecticides are derivatives of phosphoric and thiophosphoric acids and can be represented by the general formula shown in Figure 2.1 in Chapter 2. Depending on the configuration of the oxygen or sulphur atoms, six main groups of organophosphorous compounds can be distinguished (Chapter 2, Table 2.1,). Since these compounds act through the inhibition of acetylcholinesterase (AChE), the determination of AChE activity in red blood cells and pseudocholinesterase (PChE) activity in serum or plasma have been the most reliable and widely used biological indicators of human exposure to organophosphate insecticides. However, these biomarkers have limited significance in monitoring human exposure to organophosphate herbicides since these compounds are structurally different from the insecticides and their cholinesterase inhibiting power is usually weak [128, 129]. Carbamates also inhibit AChE. There is no doubt that by AChE analysis can prove poisoning by OP's (this serves as a diagnostic test in acute poisoning cases) as mentioned above but this is not conclusive in identifying the parent compound of interest, which is important in the forensic context.

Determination of metabolites in urine is the most practical and widely used method to estimate the internal dose of OP's. The main metabolic reaction, common to all OP's, is hydrolysis of the ester bond, with the production of alkylphosphate derivatives and chemical residues specific for each compound. Metabolism of most OP compounds yields alkylphosphates or alkyl-(di)-thiophosphates as terminal products. Alkylphosphate metabolites are the result of the hydrolysis of the P-X bond in the OP molecule. This reaction is catalysed by the enzyme commonly known as A-esterase or phosphoryl-phosphatase, which is present in several mammalian tissues such as liver, plasma and intestine.

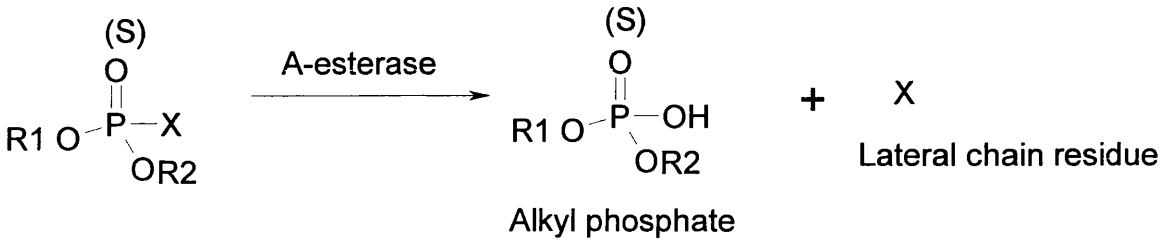


Figure 11.1 Hydrolysis of organophosphate compounds

Undoubtedly alkylphosphate determination in urine for OP exposure has been used widely but these metabolites are common to several OP's and this indicator is not specific but is generally used to assess exposure to a group of parent compounds (Table 11.1). Moreover a rather complicated and complex sample preparation is required for the analysis including the following steps: extraction of the metabolites, conversion into volatile derivatives using potent lachrymatory materials such as pentafluorobenzylbromide (PFBBr) [130, 131] or carcinogenic diazoalkanes [132, 133, 134].

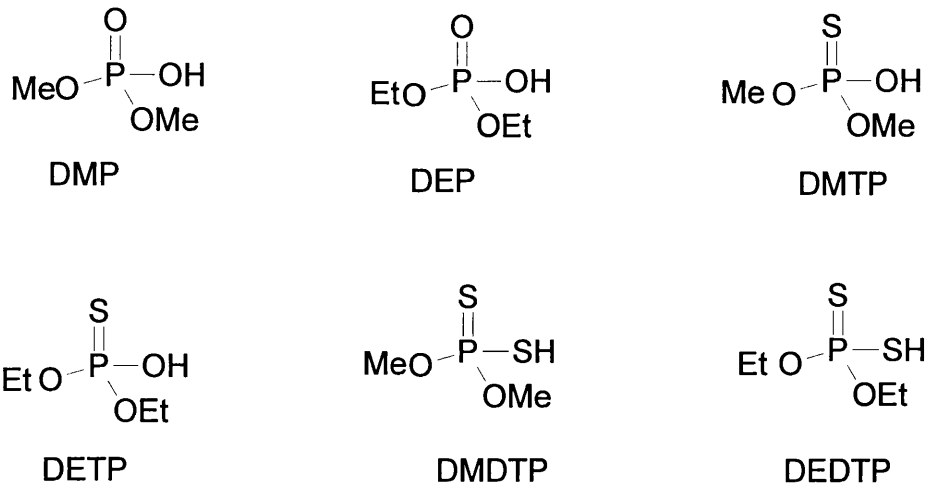


Figure 11.2 Chemical structure of the six dialkylphosphate metabolites

Table 11.1 Alkylphosphates detectable in urine as metabolites of organophosphate pesticides.

Metabolite	Main parent compounds
Monomethylphosphate (MMP)	Malathion
Dimethylphosphate (DMP)	Dichlorvos, dimethoate, malathion, monocrotophos
Diethylphosphate (DEP)	Diazinon, parathion, quinalphos, disulfoton
Dimethylthiophosphate (DMTP)	Azinphos-methyl, dimethoate, malathion fenitrothion,
Diethylthiophosphate (DETP)	Diazinon, parathion, quinalphos
Diethylphosphorothiolate (DEPTh)	Disulfoton, phorate
Dimethyldithiophosphate (DMDTP)	Azinphos-methyl, dimethoate, malathion
Diethyldithiophosphate(DEDTP)	Disulfoton, phorate
Phenylphosphoric acid (PPA)	EPN, Leptophos

Due to the aspects mentioned above, when biological fluids containing poisons and chemicals are to be analysed many conditions have to be taken into consideration in order to minimise the effect of degradation. Studies by Indeerjeet Kaur et.al. [135] showed that the degradation of malathion and methyl-parathion in water and soil is strongly influenced by temperature and pH and that the rate is much slower in water

than in soil. On most occasions, when samples are not analysed soon after collection, an enzyme inhibitor such as sodium fluoride (NaF) is frequently used to reduce the effect of paraoxogenase (esterase) activity that contributes to the main metabolic hydrolysis pathway at the time of sampling [56]. The question arises whether this type of inhibitor might help in maintaining a suitable environment for the stability of OP's or whether it will act as catalyst to speed up the chemical degradation process. It is apparent from the above discussion that the decay of these pesticides in blood/urine, in which the major component is water, is affected by a number of variable parameters.

This Chapter deals with an evaluation of the parameters that affect the decay of the pesticides of interest. and also whether it is possible to overcome these problems and find alternative ways for stabilising the samples and thus detect the parent compounds. The factors considered were as follows:

- Examination of the effect of pH adjustment and storage temperature;
- Examination of the effect of addition of preservative such as NaF/oxalate, EDTA/K, Lithium/Heparin;
- Storage on an SPME fibre as an alternative.

11.2. Materials and Methods

11.2.1. Reagents, standards and equipment

All pesticide standards of interest were obtained from Promochem Ltd (UK). The stock solutions were prepared by dissolving 25 mg of each neat material in 25 ml methanol. The working solutions were diluted with methanol.

Other reagents are of AR grade. Solutions of 0.1M HCl and 0.1M NaOH were made by diluting and dissolving the appropriately amount with deionised water. Saturated salt solution was prepared by dissolving sodium chloride in deionised water until no more salt could be dissolved.

A manual assembly for SPME with replaceable extraction fibre, coated with 100 μm polydimethylsiloxane was obtained from Supelco UK.

Vacutainers consisting of prepared NaF/oxalate (2 ml), EDTA/K_2 (10 ml) and Heparin/Li (10 ml) were from Teklab Ltd.(UK).

Amber headspace vials (4.0 ml volume) were used with screw septum caps fitted with PTFE/Silicone Septa. A Corning hot plate (setting from 25-550 °C)/stirrer (60-1100 rpm) was used with SPME sampling stand (holding 8 vials) and PTFE covered magnetic stirrers (10 x 3 mm). All these items were purchased from Supelco Sigma-Aldrich Com. Ltd.

11.2.2. GC-NPD and Mass Spectrometry

Gas Chromatography used a Hewlett-Packard Model 5890 Series II chromatograph equipped with a nitrogen-phosphorus detector, and an HP-1 capillary column (30 m x 0.53 mm i.d. x 0.88 μm phase thickness). Oven/Column conditions were: 100°C for 2 minutes, rising to 300°C at 10°C/minute and held for 3 minutes. The temperature of the injection port was set at 250°C and detector at 280°C. Splitless injection mode was used for the first 5.0 minutes.

GC-MS was carried out using a Thermo-Finnigan Trace GC linked to a Trace MS, with an HP-5 X-link column (5% Ph, 95% Me Silicone, 30 m x 0.32 mm x 0.25 μm film thickness). The column temperature was set at 100°C for 2 minutes and then programmed from 100°C to 300°C at 10°C/minute (held for 3 minutes). The temperature of the injection port was set at 250°C and interface at 250°C.
The ionisation energy was 70eV in the EI+ mode.

11.2.3. Extraction conditions

In a 4.0-ml vial with magnetic stirrer, 0.5-ml of water/buffer/blood containing the OP's of interest was added to 0.5 ml deionised water. The vials were placed in a vial receptacle and placed over a heated hot plate/stirrer at 90°C at the maximum stirrer

speed. After heating for 10 minutes, the septum-piercing needle of the SPME syringe was passed through the septum (Figure 4.5). The pre-treated fibre was pushed out from the needle and exposed in the headspace for 30 minutes to allow the adsorption of the compound. The fibre was withdrawn into the needle and pulled out from the vial. It was then inserted into the injection port of the capillary gas chromatograph and the fibre was exposed for 5 minute to ensure complete desorption of the analytes.

11.3. Results.

11.3.1. Method for persistence studies

Laboratory experiments were conducted using deionised water and human blood. Known amounts of deionised water and blood as such, buffered to the required pH were placed in capped universal bottles and spiked to make up a concentration of 0.4μg/ml of the respective pesticide. The samples were extracted using the SPME procedure specified above. The decay profile of the pesticides was followed for three days under different sample conditions.

11.3.1.1. Effect of temperature on the rate of decay.

The effect of storage temperatures 0-4 °C and 20-25 °C on the decay of malathion in deionised water at pH 7.4 was shown in Figure 11.3. It was found that the rate of decay was slightly faster at the higher temperature.

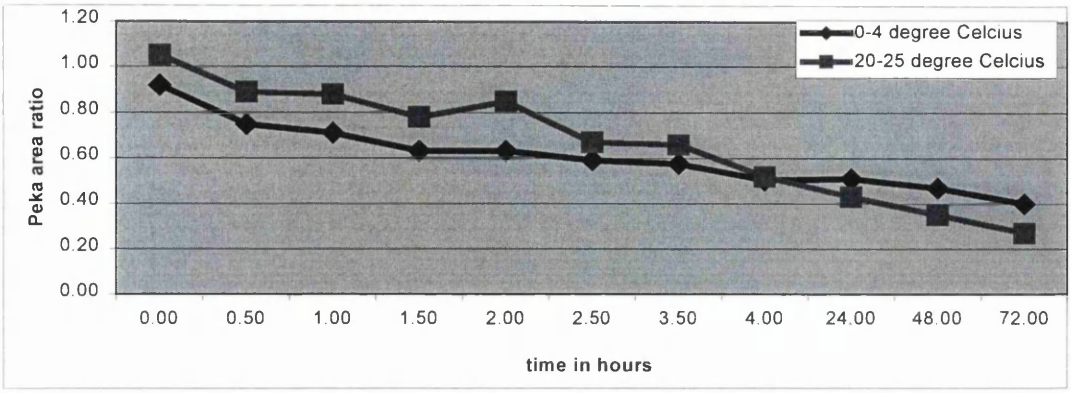


Figure 11.3 Decay of malathion in water at pH 7.4 at two different temperatures

11.3.1.2. Effect of pH on the decay

The deionised water was adjusted to the required pH with 0.1M HCl or 0.1M NaOH. The effect of the pH on degeneration of pesticide in water (pH 5-6 and pH 7-8) at 20-25 °C was shown in Fig.11.4. It is apparent that the decay was faster at the higher pH. The faster hydrolysis kinetics of malathion in alkaline medium takes place at the C-S bond whereas in acidic condition it takes place at the P-S bond [136]. The resulting compounds formed due to rupture of the C-S bond is stabilised by resonance and that explains the faster decay in an alkaline medium.

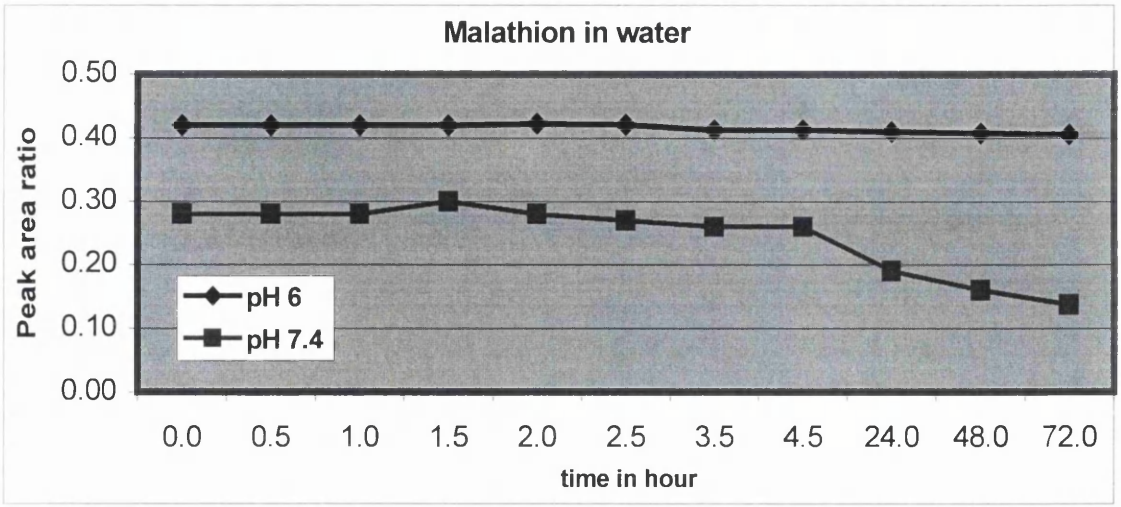


Figure 11.4 Decay of malathion in water at 20-25°C at different pH values.

11.3.1.3. Effect of adding preservative

Sodium fluoride/oxalate, heparin/Litium and EDTA/K are mostly used as preservatives to maintain the stability of specimens, especially blood and urine. These preservatives were used in conjunction with deionised water (pH 7.4) to determine their effect chemically on the pesticide. The effects are shown in Figure 11.5 and Figure 11.6.

The rate of decay increased when malathion was placed in the water solution containing NaF/oxalate as the preservative and stabilised if placed in heparin/Li or EDTA/K. The pH of the deionised water changed from neutral to basic (around pH 8) in NaF and decreased or remained unchanged when EDTA/K or Heparin/Li was added (around pH 5-6 and pH 7 respectively).

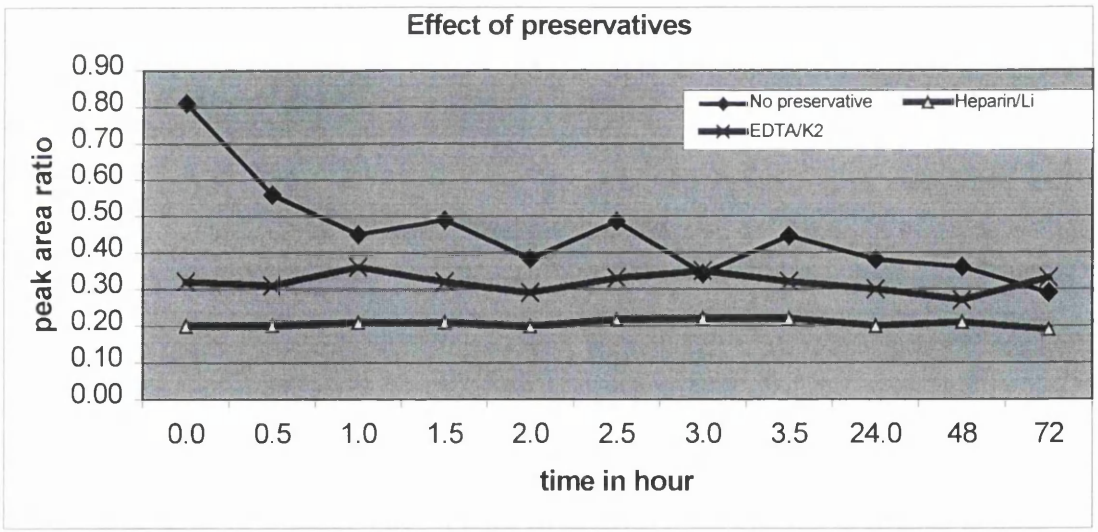


Figure 11.5 Decay of malathion in water containing different preservatives

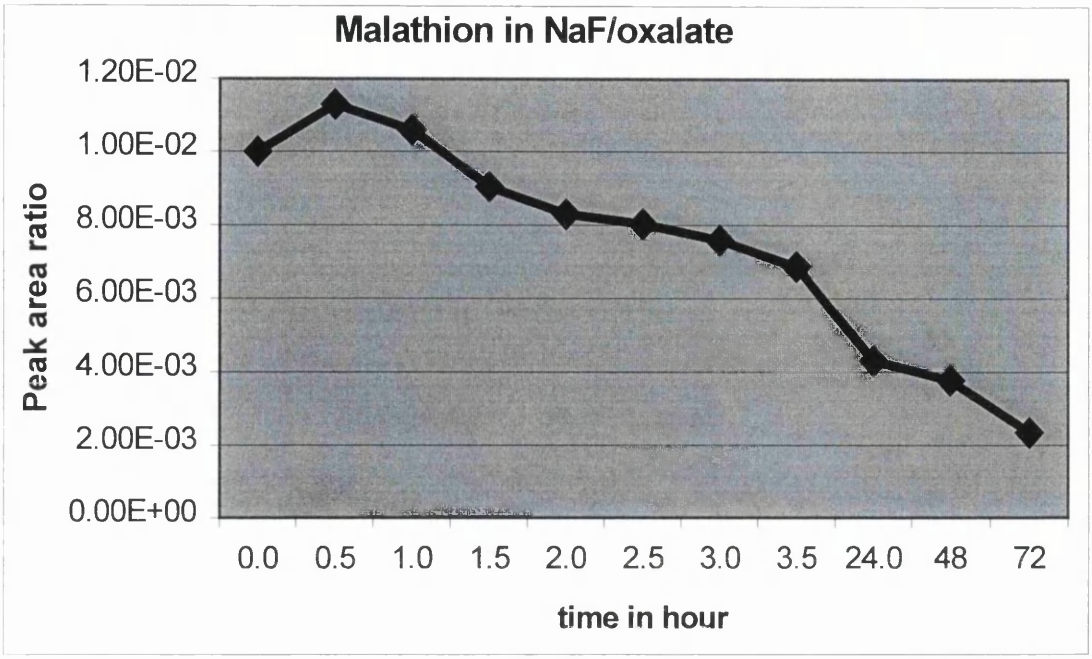


Figure 11.6 Decay of malathion in water containing NaF.

11.3.2. Study on the stability of OP's in blood during storage in freezing (0°C-4°C) and ambient temperature (20-25°C) without preservative

Stability of the OP malathion in blood was studied by spiking 20 ml of fresh blood to make up a concentration of 10 ppm. The sample was split into 2 halves. One half was placed in several 4 ml vials (0.5 ml blood/vial) and left in the fridge (0-4°C) and the other half was place in a wrapped vacutainer and left at room temperature (20-25°C). No preservative was added. The specimens were analysed initially every half-hour and then over a period of days (11 days).

The figures 11.7 showed that the hydrolysis for malathion was much faster at room temperature compared than at 0-4 °C.

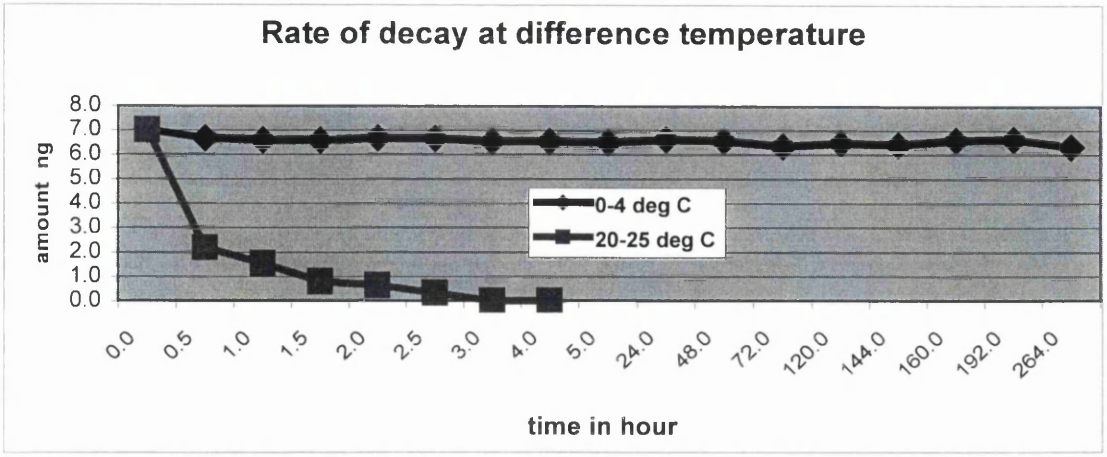


Figure 11.7 Rate of decay of malathion in blood at different temperatures.

11.3.3. Effect of preservative sodium fluoride on chemical degradation in blood

The effect of NaF as a preservative in blood was also studied. Malathion was spiked into sample of blood containing NaF/oxalate to give a concentration of 10 μg/ml. Figure 11.8 showed that NaF increased the rate of decay due to hydrolysis of the OP.

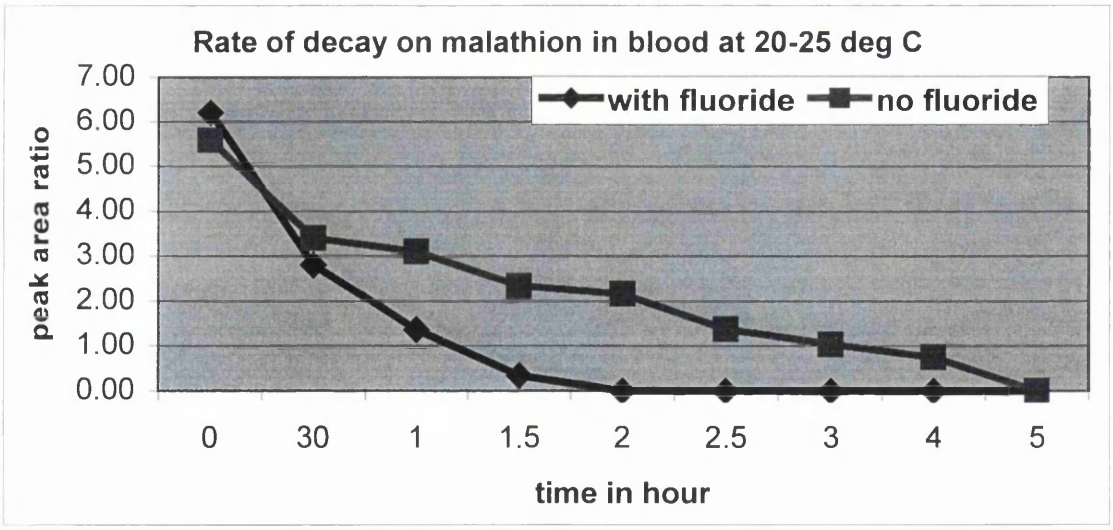


Figure 11.8 Decay of malathion in blood having NaF as preservative at room temperature, around 20-25 °C.

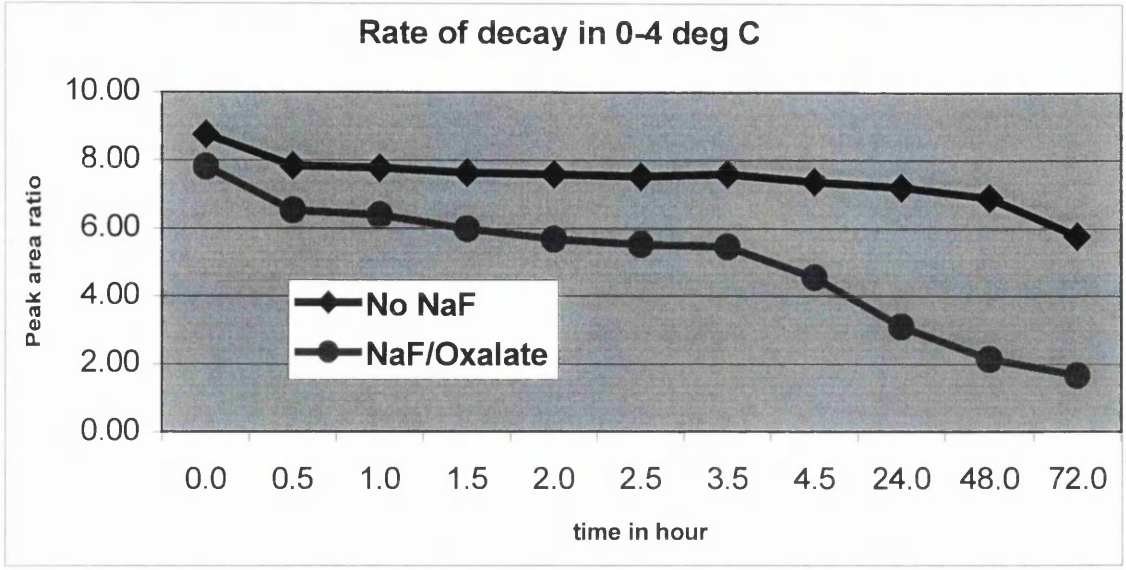


Figure 11.9 Decay of malathion in blood with and without preservative NaF at 0-4°C.

11.3.4. Effect of storage on an SPME fibre

The effect in response of compounds was evaluated when they remained on the 100 um PDMS fibre for a period of time before desorption into the GC. Samples of blood were spiked with malathion at 200 ng/0.5 ml and extracted using the method stated in Chapter 5 Section 5.1.3. The periods studied were from 0-240 hours (equivalent to 10 days). Each experiment was repeated three times and the mean response calculated. During this time, the syringe was left after extraction at room temperature (20-25 °C) and was placed suspended with the fibre pierced through the septum of an empty vial to prevent it from contamination by impurities in air. Figure 11.10 shows the effect of storage time on the response for malathion.

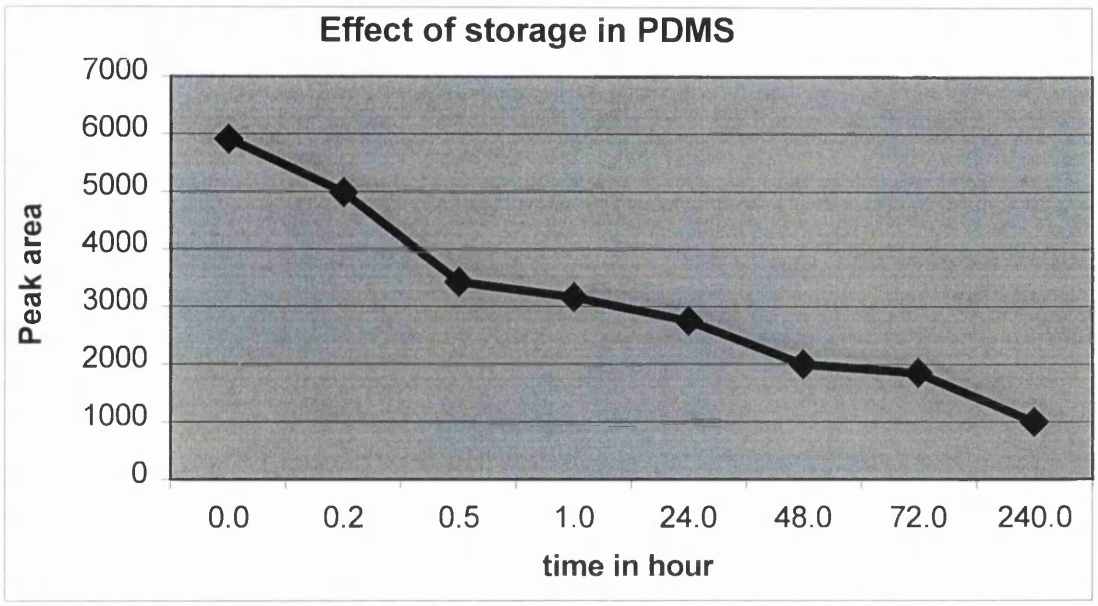


Figure 11.10 Effect of storing on a PDMS 100 μm fibre after extraction prior to desorption in the GC.

11.4. Discussion

Upon collection of a blood sample, the enzymatic activity that leads to the hydrolysis of the parent compound to the metabolites which are excreted in urine was halted with the addition of an acidified salt solution. Although salt was added as an aid in the extraction of the analytes, the role of the acid was to inactivate the enzymes. Most enzymes have a characteristic pH at which their activity is maximal; the activity declines above or below this (pH 5-7). Thus from the result above, the stability of the analytes can be maintained if the pH can be maintained acidic.

Another aspect with regard to the use of preservative is that the pH of the sample will increase or decrease depending on its type. EDTA/K decreases the pH whereas NaF/oxalate increases the pH. This explains the increased rate of degradation of malathion in blood when NaF is used as a preservative. The increase in alkalinity increases the rate of degradation. Using SPME fibre for storage before desorption may be an alternative for maintaining the integrity of the parent compound so as to identify it latter. An interesting alternative, which warrants further investigation in future, was recently presented at the TIAFT meeting in Prague 2001, involving the storage of

blood spots on filter paper. This storage medium was found to inhibit decomposition of cocaine during storage.

11.5. Conclusion

Parameters such as pH, temperature and preservative used, play an important part in regulating the condition of the sample. The rate of decay can be minimised by keeping the sample in a cooler environment such as a freezer before transporting and analysing the sample. Sometimes it is not possible to reduce the effects of hydrolysis without proper preservatives, but some preservative could also act to increase the rate. The conclusion is that it is better not to use preservative but to have the specimen analysed as soon as possible. Another alternative is to extract the specimen on-site (at the mortuary) and then transferr the fibre to the lab for analysis.

12. SCREENING OF POST-MORTEM BLOOD IN PESTICIDE POISONING CASES BY HEADSPACE SOLID PHASE MICROEXTRACTION.

12.1. Introduction

In Malaysia, as in many other countries in the Asian continent, pesticides are one of the most significant causes of fatal and non-fatal poisoning [21-23], exceeding those due to other substances such as prescribed or illicit drugs, which are more prominent in Europe and the United States [24-25].

These poisoning cases form an important part of the work of Forensic Toxicology in Malaysia [14] and it is essential that an appropriate and adequate range of analytical procedures should be available for the detection and measurement of these substances in biological specimens. There is a need for robust, facile and inexpensive methods of analysis that can be used even in laboratories that do not have GC-MS or LC-MS available.

In this study, 25 specimens of post-mortem blood, collected at autopsy in Malaysia, were examined using HS-SPME as a routine screening procedure for pesticides after method development. They were tested during a two-week return visit to the Chemistry Department laboratory in Malaysia. The method described earlier was used with slight alterations due to the use of a twin detector gas chromatograph and different types of column.

12.2. Materials and Methods

12.2.1. Reagents

Reagents and standards were of AR grade and were obtained from suppliers mentioned before.

12.2.2. Apparatus

A manual assembly for SPME with replaceable extraction fibre, coated with 100 μm polydimethylsiloxane was obtained from Supelco UK. Amber headspace vials (4.0 ml volume) were used with screw septum caps fitted with PTFE/Silicone Septa. A Corning hot plate (setting from 25-550 °C)/stirrer (60-1100 rpm) was used with SPME sampling stand (holding 8 vials) and PTFE covered magnetic stirrers (10 x 3 mm). All these items were purchased from Supelco Sigma-Aldrich Com. Ltd.

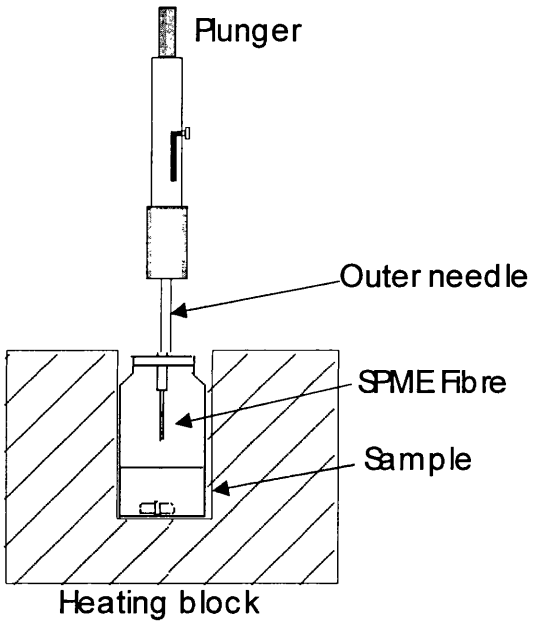


Figure 12.1 Headspace SPME system

12.2.3. Gas Chromatography NPD dual Column.

Gas Chromatography was carried out using a Hewlett-Packard Model HP 5892 Series II Plus chromatograph equipped with twin nitrogen-phosphorus detectors. The oven was fitted with two columns: (a) RTX01, 15 m x 0.32 mm with 0.25 μm phase thickness and (b) DB1301, 15 m x 0.25 mm with 0.25μm phase thickness. The GC was programmed from 120°C (held for 0.5 minute) to 300°C at 20°C/minute. The injection port temperature was set at 250°C and detector at 280°C. Splitless injection mode was used with a purge time of 5.0 minutes.

12.2.4. Extraction Technique

Spiked blood standards and case blood samples, volume 0.5 ml, were diluted with 0.5 ml distilled water followed by addition of 100 μL of 0.1M HCL and 100 μL saturated sodium chloride solution. The extraction sequence consisted of 10 minutes sample conditioning followed by 30 minutes SPME fibre exposure to the sample headspace at a temperature of 90°C. Samples were agitated continuously during the exposure period using miniature magnetic stirrers.

12.2.5. Linearity / Calibration Curves

Calibration standards in the range 100 ng to 500 ng malathion per 0.5-ml blank whole blood were prepared. The Internal Standard (Diazinon) was spiked at an amount of 10 ng/sample.

12.3. Results

The limit of detection was found to be 50 ng malathion per 0.5-ml blood. No interferences were found in the analysis. Figures 12.2 and Figure 12.3 show representative chromatograms obtained with blank blood containing the internal standard, diazinon, and with a blood specimen that was positive for malathion. Confirmation was obtained using a second column (RTX01) run in parallel with column DB1301, having another NP Detector (Figure 12.4).

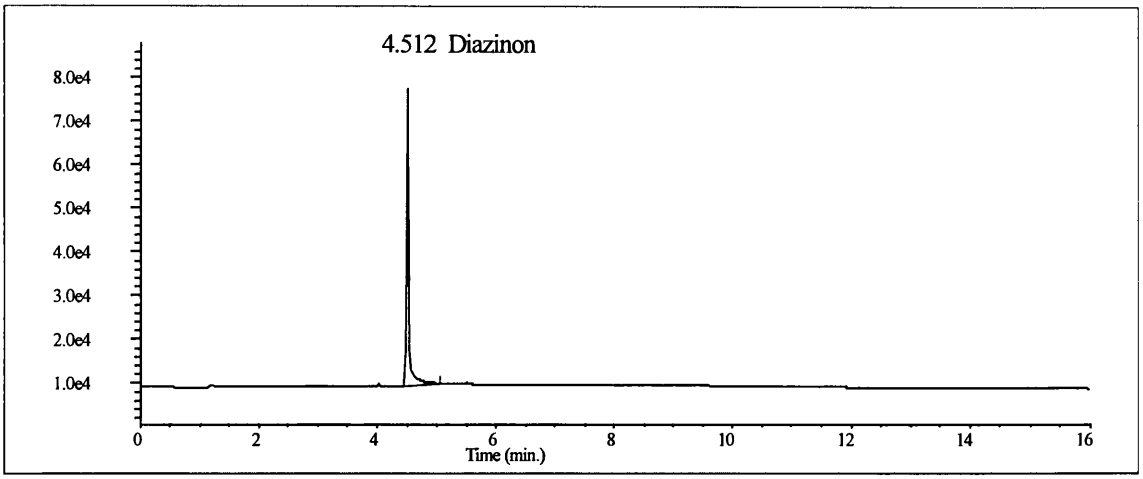


Figure 12.2 HS-SPME chromatogram (column DB1301) of a blank blood sample containing the internal standard, diazinon.

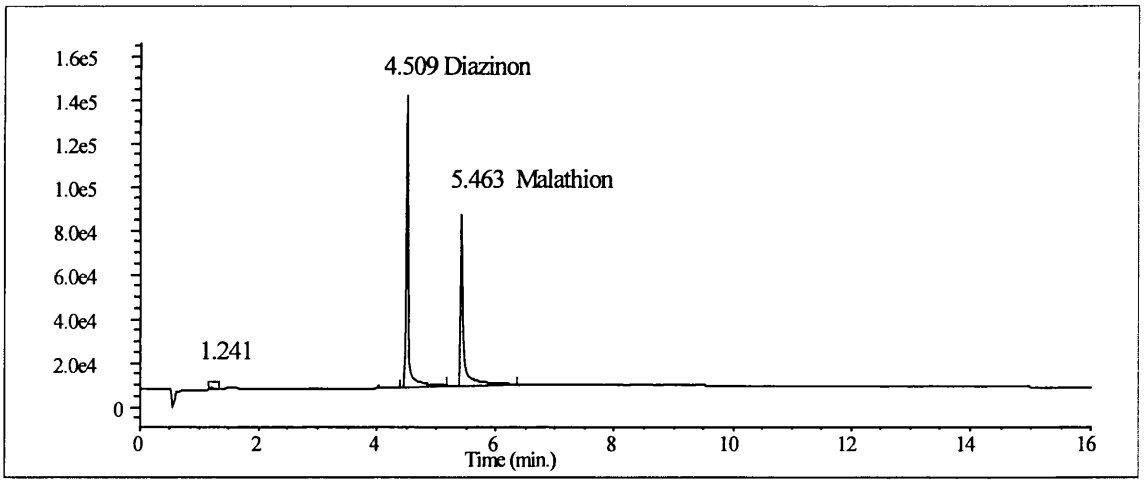


Figure 12.3 HS-SPME chromatogram (column DB1301) of a sample which is positive for malathion

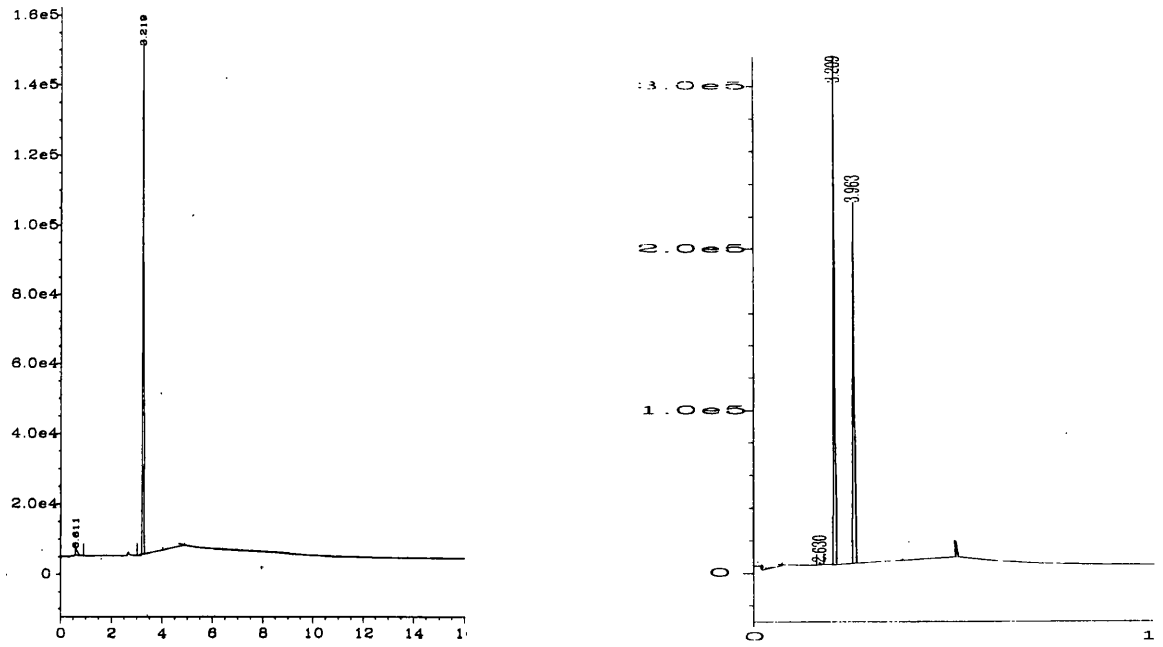


Figure 12.4 Confirmation by the second column (RTX01) running in parallel with DB1301. On the left, blank blood-containing diazinon as internal standard (retention time 3.2 minutes). On the right, positive sample with malathion at 3.9 minutes.

The method was linear in the range 100 ng to 500 ng per 0.5 ml blood (n=3) and the linear correlation coefficient (r^2) was 0.995 (Figure 5). Recovery of malathion at a concentration of 100 ng per 0.5 ml blood was 0.35% and coefficient of variation 5.2% (n=10). Precision measurements under repeatable conditions for blood samples (n = 10) of concentration 100 ng/0.5 ml gave a coefficient of variation of 5.4%.

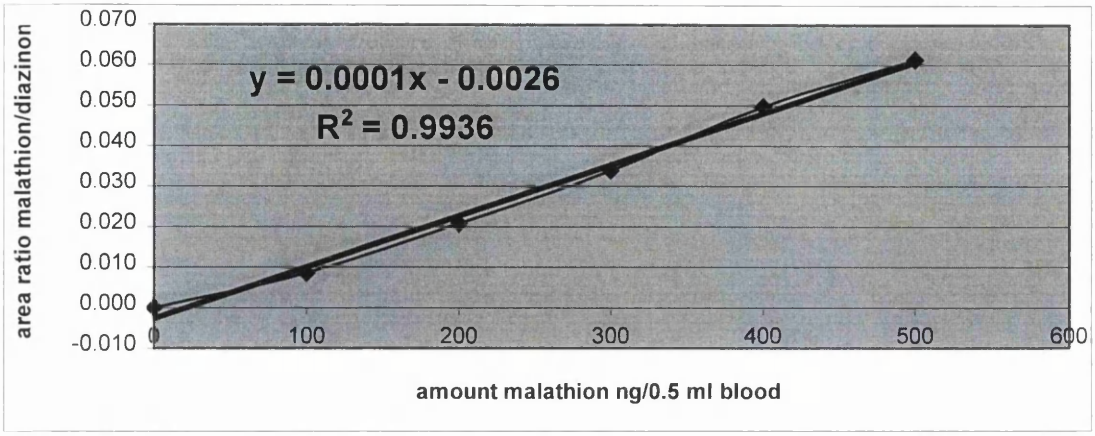


Figure 12.5 Calibration curve for HS-SPME analysis of malathion in blood using column DB1301.

Analysis of 25 specimens of blood obtained at autopsy in Malaysia found three positive poisoning cases involving malathion, which are described below. In addition, the stomach contents were analyzed using a liquid-liquid extraction method with diethyl ether as extracting solvent. Malathion was found to be present in all three cases.

Case 1: A unemployed Indian man, age 44, alcoholic, was found to have stomach contents positive for malathion. The blood malathion concentration was 135 ng/0.5 ml (270 ng/ml)

Case 2: A Filipino maid, female, ages 27, was found dead. The stomach contents were positive for malathion. The blood malathion concentration was 104 ng/ml (in this case, 1 ml blood was used).

Case 3: Indian female, unemployed, age 18 took her own life by drinking malathion. The stomach contents were found to be positive for malathion and the blood malathion concentration was 6025 ng/0.5 ml (12050 ng/ml).

Urine was not available in any of the positive cases

12.4. Discussion

Organophosphorus pesticide screening is a routine procedure in the Department of Chemistry (Toxicological Unit) in Malaysia. A simple, robust, sensitive, solvent free and easy to handle technique was needed, especially for sample extraction. SPME seems to offer an alternative procedure for sample preparation compared to the normal technique of liquid-liquid extraction using hexane or diethyl ether after deproteination. In the present study, the use of HS-SPME was introduced. The technique was introduced following the method development sequence stated in Chapter 4 with minor adjustment such as column types. The technique was found to be quite easy to handle and not much sample preparation was required. The method can be used as a screening procedure, especially for volatile and semivolatile pesticides

12.5. Conclusions

The HS-SPME method was simpler and more rapid than the conventional liquid-liquid extraction method. The method showed good linearity and excellent results for quantitative measurements, even though the SPME extraction method was based on an equilibrium process. Headspace SPME is suitable as an alternative extraction technique, especially for the analysis of pesticides.

13. DETERMINATION OF BENZODIAZEPINES (FREE FORM) IN URINE BY SOLID PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY WITH NITROGEN PHOSPHORUS DETECTION.

13.1. Introduction

Benzodiazepines are widely used as anticonvulsant, hypnotic, anxiolytic and muscle relaxant drugs and, while they are important in treating a variety of medical disorders, they are also subject to abuse by young illicit drug users, often in large doses causing profound behavioural effects. Their continued abuse can lead to dependence. Benzodiazepines may also cause or contribute to sudden death if misused [137]. Since benzodiazepines are widely seen in clinical as well as forensic cases in many parts of the world including Malaysia, their measurement in biological specimens is widely practised [138].

A sample preparation technique is often necessary to extract organic compounds of interest from the matrix and several have been developed. Liquid-liquid extraction (LLE) methods have been published. Solvents used to extract benzodiazepines include diethyl ether, toluene, dichloromethane, butyl chloride, and mixtures of these solvents (also including others not mentioned here). LLE is mostly conducted under slightly alkaline conditions involving the use of sodium carbonate, phosphate, borate or tetraborate. Dilute sodium hydroxide, near neutral or unbuffered conditions have also been described.

Solid phase extraction (SPE) is now the most commonly used technique. The methodological approaches, which have been published, include the use of Extrelut and octadecylsilane-bonded cartridges (C_{18}). Other methods include the use of C_2 and mixed phase Bond-Elut Certify cartridges. These were also commonly used, particularly for GC applications. However, these methods, especially LLE, are time consuming, require complicated procedures and are difficult to automate. Large amounts of organic solvents may be used, except in SPE which is usually considered to require smaller amounts of solvents.

Solid phase microextraction (SPME) has been recently developed [139], which is fast, solvent-free (environmental friendly) and shows excellent performance. Suzuki and his group first reported utilising direct insertion (DI)-SPME-GC-FID for analysis of 13 benzodiazepines in urine [140]. Very recently the same group reported a modification of the method employing hydrolysis of benzodiazepines to form benzophenones prior to extraction [141]. Krogh et al. [142] used another approach to improve extraction recovery through a solvent modified scheme. It employed the modification of a PA fibre by sorption of 1-octanol before its direct immersion in blood plasma samples. Luo et al [143] optimised the extraction of five benzodiazepines from aqueous solutions and biological fluids. They stated that the extraction of oxazepam and lorazepam from unmodified urine and serum samples results in much lower extraction yields than those obtained from aqueous solutions, which shows that biological matrices interfere with the sorption process. SPME coupled to HPLC had been successfully applied to the analysis of benzodiazepines [144, 145].

In this section is described the feasibility of a robust but sensitive analysis of unconjugated benzodiazepines in human urine using manually injected SPME-GC-NPD. Parameters affecting the extraction were also studied. The method was applied to the detection and quantification of analytes in urine specimens from road traffic offence and/or post mortem cases.

13.2. Experimental

13.2.1. Materials and reagents

The holder and the assembly of the SPME device for manual sampling were purchased from Supelco U.K. Ltd. The fibres used were 1.0 cm long, coated with 85 μm polyacrylate (PA) and 100 μm polydimethylsiloxane (PDMS) and were also obtained from Supelco. New fibres were conditioned in the injection port at 300°C (for PA) or 250°C (for PDMS) prior to use.

Diazepam, oxazepam, desmethyldiazepam (DMD), temazepam and prazepam were obtained from Sigma Chemical Co. Methanol, sodium hydroxide, sodium chloride

(analytical grade) were from Merck. Solutions of 1.0M NaOH, 0.1M NaOH and saturated salt (sodium chloride) solution were prepared with deionised water. Individual methanolic stock solutions containing 1.00 mg/ml of the drugs were prepared. Working solutions of concentrations 0.1 and 0.01 mg/ml were subsequently prepared for all standards. Blank human urine was spiked with diazepam and desmethyldiazepam at concentrations in the range 0.01-0.1μg/ml and 0.1-0.5 μg/ml for preparation of calibration curves. Calibration curves for temazepam and oxazepam in the range of 0.1-0.5 μg/ml were also prepared. Quality control samples for measurement of precision under repeatability conditions containing 0.1 μg/ml diazepam, desmethyldiazepam, temazepam and oxazepam were also prepared. Drug-free urine was analysed as a negative control in all assays.

13.2.2. Instrumentation set-up

GC-NPD analysis was performed with a Hewlett-Packard Model 5890 Series II chromatograph equipped with an HP-1 capillary column (30 m x 0.53 mm i.d., phase thickness 0.88 μm). The injection port (operated in split/splitless mode) was set to 250°C and the purge time to 5.0 minutes. The column temperature was initially held at 100° C for 2 min, then increased to 300° C at 10° C/min and held for 3 minutes.

13.2.3. SPME Screening Design

In a 4-ml vial, 2ml of urine spiked with a mixture of standards (100 ng of each benzodiazepine, including the internal standard prazepam) were diluted with 2 ml of deionised water. The solution was then subjected to different conditions so as to optimised the extraction. Parameters such as fibre/coating type, temperature, pH and salt adjustment were studied in the screening design. Sample vials were heated in a heating block with magnetic stirrer /agitation. The SPME fibre was fully immersed and left in contact with the sample for the selected time. As soon as the extraction was finished, the fibre was retracted and then transferred to the GC injector where the analytes were thermally desorbed into the GC system.

13.3. Results and Discussion

13.3.1. Evaluation of the fibre coating

PDMS and PA fibres were immersed directly into the urine samples exposed to a temperature of 90°C for 30 minutes. To extract polar/non polar compounds from an aqueous matrix (e.g. urine) using polar (PA) or non-polar (PDMS) coated SPME fibres, the affinity of the fibre with the analytes must be stronger than that of the matrix. PDMS and PA have the properties of liquids and solids respectively. Analytes diffuse more rapidly in PDMS than in PA, therefore achieving lower absorption equilibrium times. On the other hand, PDMS is non-polar compared to PA, which is more polar, and as a result the affinity for the selected benzodiazepines, which are amphoteric and relatively polar compounds [146], should be stronger using the PA fibre. The results suggested that the PA coating gave better recoveries of benzodiazepines than the PDMS coating (Figure 13.1) and this type of fibre was used for subsequent work in the investigation. Problems do occur with urine specimens from putrefied cadavers due to suspended gel-like substances. It is better to wash the fibre with water after every extraction with this type of specimen.

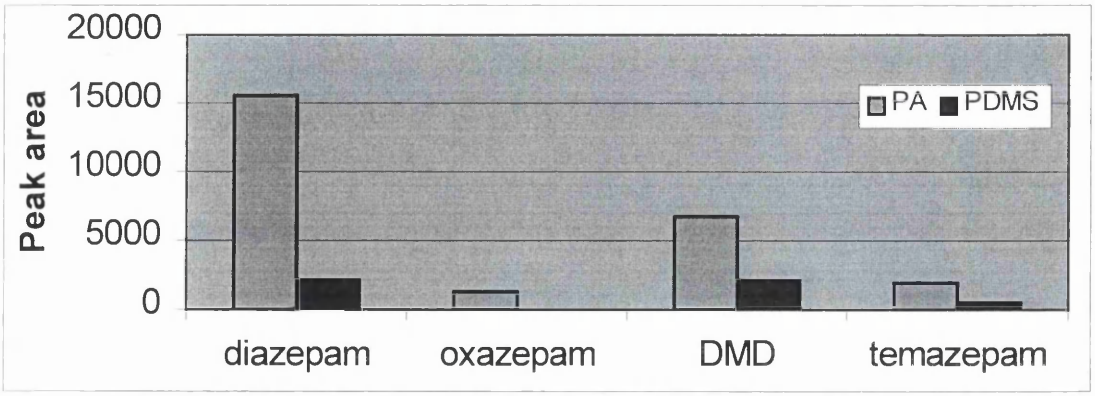


Figure 13.1 Extraction of benzodiazepines by directs insertion SPME in urine with PA and PDMS fibers.

13.3.2. Optimisation of the SPME conditions

The effect of sample temperature on the extraction is known to be an important parameter in the extraction process [103]. Plots for relative peak areas versus temperature are shown in Figure 13.2. Temperatures higher than 60°C resulted in decreased efficiency for the extraction of diazepam, oxazepam and temazepam. As the extraction is controlled by mass transfer from the sample matrix to the fiber coating, the mass transfer resistance is reduced by increasing the temperature as well as agitation by the magnetic stirrer. Also, at temperatures higher than 60°C, benzodiazepines are more likely to decompose. The extraction temperature was set at 60°C for subsequent analyses.

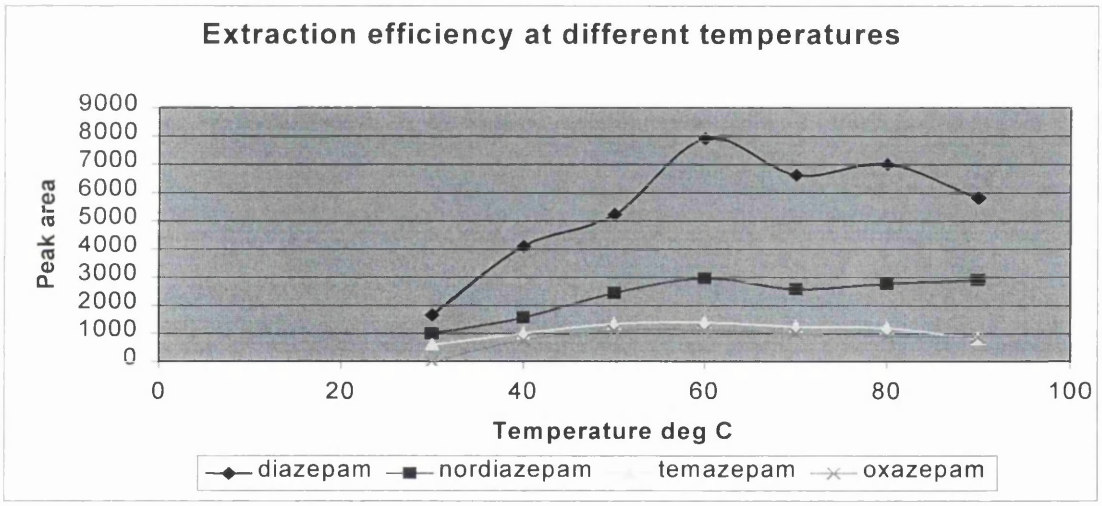


Figure 13.2 Effect of sample temperature on extraction efficiency

Extraction by SPME is based on equilibrium between the sample matrix and the fibre rather than attempting to achieve total recovery of the analytes. Mass transfer from the sample matrix to the fibre coating controls the speed of the extraction. In SPME sampling, the mass transfer rate is determined by means of the diffusion of analytes into the coating, if the sample matrix is perfectly agitated. The time to reach equilibrium depends on other factors, including the distribution constant between the sample and the coating, and is usually up to several hours for analytes such as the benzodiazepines [146]. Figure 13.3 below shows an example of time profiles for the extraction of benzodiazepines. For most drugs, the equilibrium is not attained even

after one hour. Due to practical time factor constraints, 30 minutes was taken as a reasonable time for extraction.

The addition of salt to the sample matrix in principle decreases the solubility of the target analytes, thus enhancing the extraction efficiency. Sensitivity can be significantly increased for polar analystes. From the evaluation, (Figure 13.4) it appeared that there is a significant increase in extraction efficiency for diazepam but not for oxazepam, DMD, and temazepam. Nevertheless, saturated salt solution (100 µl) was introduced into the subsequent analyses.

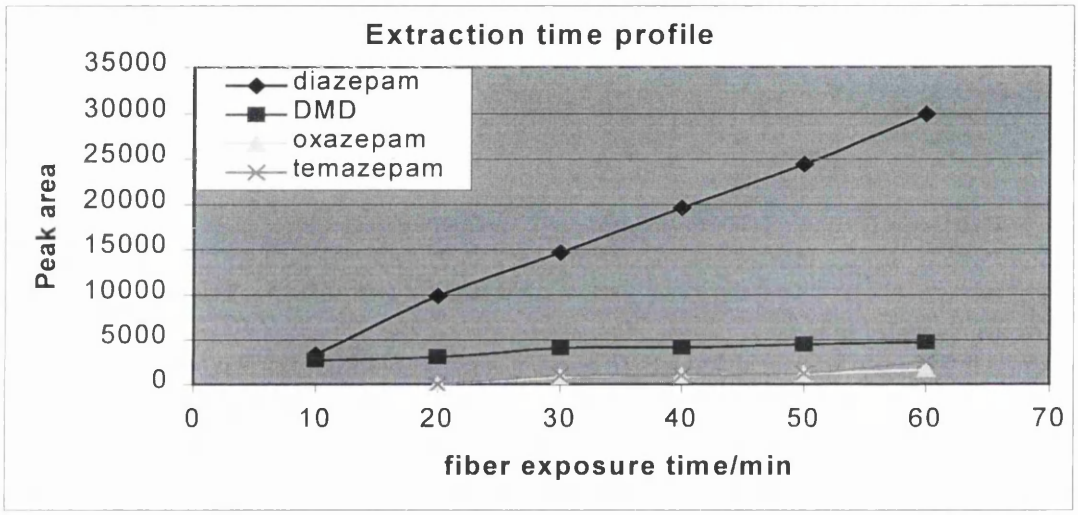


Figure 13.3 Effect of immersion time on extraction efficiency.

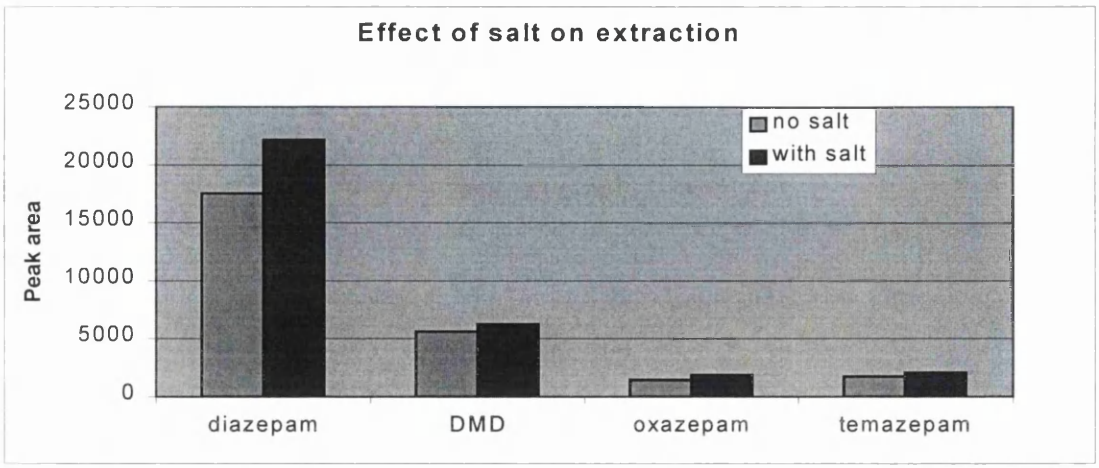


Figure 13.4 The effect of adding 100 µl of saturated sodium chloride solution on the extraction efficiency of benzodiazepines

It has been shown that adjusting the pH of the matrix solution will alter the dissociation constant (K) for dissociable species, assuming that only the undissociated form of acids and bases can be extracted by the coating [148]. The effect of the matrix pH on the extraction of benzodiazepines was examined at pH 6.0 ± 0.5 and pH 8.0 ±0.5 (Figure 13.5). These were chosen because most benzodiazepines are weak bases and are undissociated at pH values above pH 7, resulting in higher extraction efficiencies. From the chart it was shown that the extraction efficiency increased at pH 6.0±0.5 especially for DMD, temazepam and oxazepam

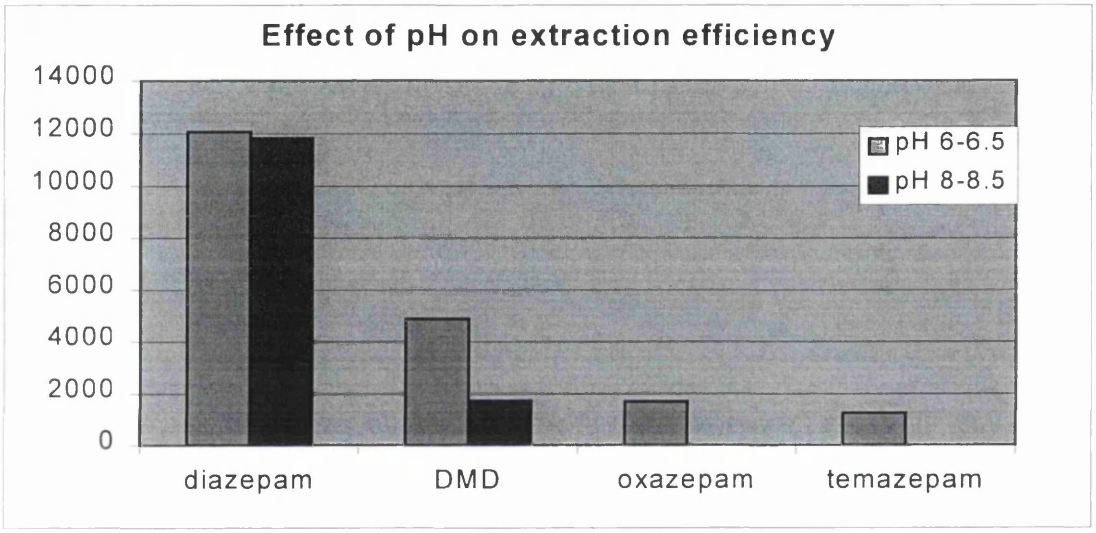


Figure 13.5 Effect of sample pH on extraction efficiency.

13.3.2. Proposed technique

Urine (2 ml), distilled water (2 ml), internal standard prazepam (10 µL, 10 µg/ml), saturated sodium chloride solution (100 µl) and sodium hydroxide solution (0.1M, 100 µL) are mixed in a 4 ml amber screw-top septum vial with a PTFE/silicone septum. The specimen is conditioned with stirring for 10 minutes on a hotplate/stirrer at 60°C. The needle probe holding the SPME fibre is pushed through the septum and the fibre is immersed in the urine specimen for 30 minutes at a temperature of 60°C. After this time, the fibre is transferred to the gas chromatograph inlet operated in the split/splitless mode at 250°C. The purge valve is opened after 5.0 minutes

13.3.3. Validation

The analysis of precision was based on analysing urine samples containing 100 ng benzodiazepines /2 ml urine sample (n=5) under repeatability conditions. The relative standard deviations for all the analytes were less than 14.0%. The linearity of the method was tested by quantitative measurements and peak areas of the analytes in standard solutions were measured and calibration curves were constructed. The method was found to be linear over the concentration ranges tested (Figure 13.6-Figure 13.8). Linear correlation coefficients (r^2) in the range 0.980-0.994 were obtained for all analytes. The detection limits for all analytes were in the range of 5-50 ng/ml based on 2 ml of urine sample. Recoveries were calculated by measuring the peak area of each analytes (at 100 ng/2ml urine) without internal standard and compared again authentic standard injected at 10 ng. The results were as in Table 13.1 and Table 13.2.

Table 13.1 Linear Calibration Range, Limits of Detection and Relative Standard Deviation

Analytes/Drugs	Calibration range (ng/2ml urine)	Limit of detection (ng/2ml urine)	RSD or CV (%)
Diazepam	0-100 100-500	10	9.2
Desmethyldiazepam	0-100 100-500	10	10.4
Temazepam	100-500	50	12.3
Oxazepam	100-500	100	9.9

Table 13.2 Recoveries and precisions at concentration 100 ng/2ml urine

Analytes/Drugs	Recoveries % (CV%)	Precision (CV%)
Diazepam	7.3 (7.7)	6.7
Desmethyldiazepam	4.1 (7.4)	13.9
Temazepam	2.5 (6.0)	10.0
Oxazepam	0.2 (8.8)	5.0

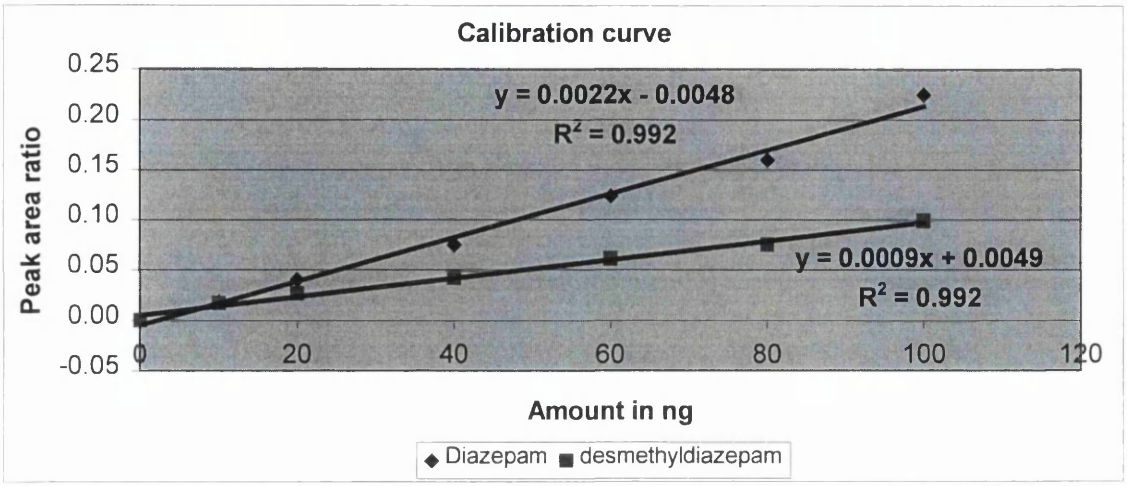


Figure 13.6 Calibration curve (1) for diazepam and desmethyldiazepam with prazepam as IS.

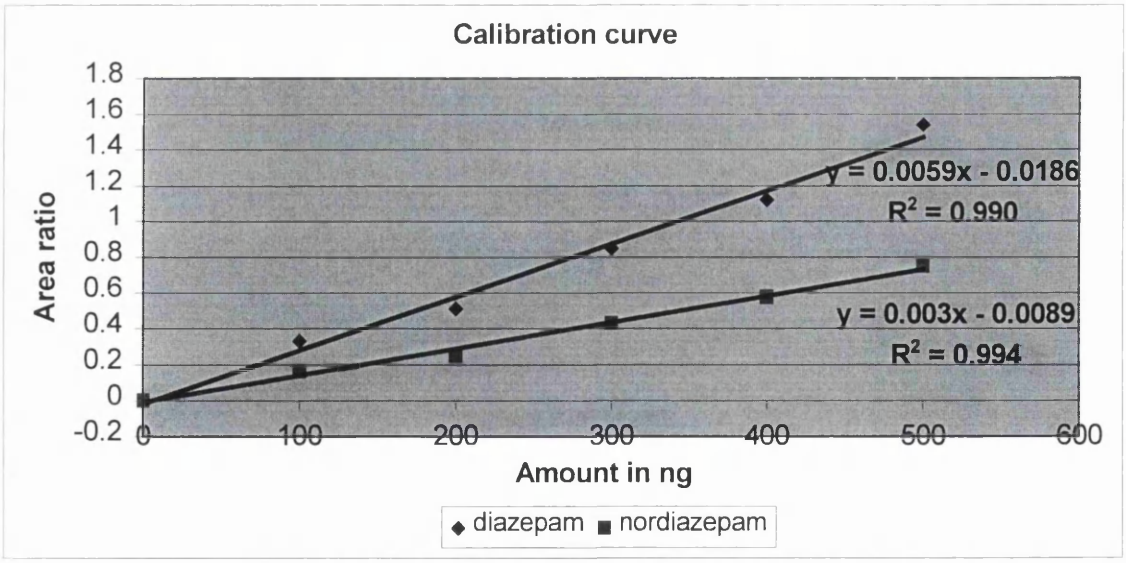


Figure 13.7 Calibration curve (2) for diazepam and desmethyldiazepam with prazepam as IS.

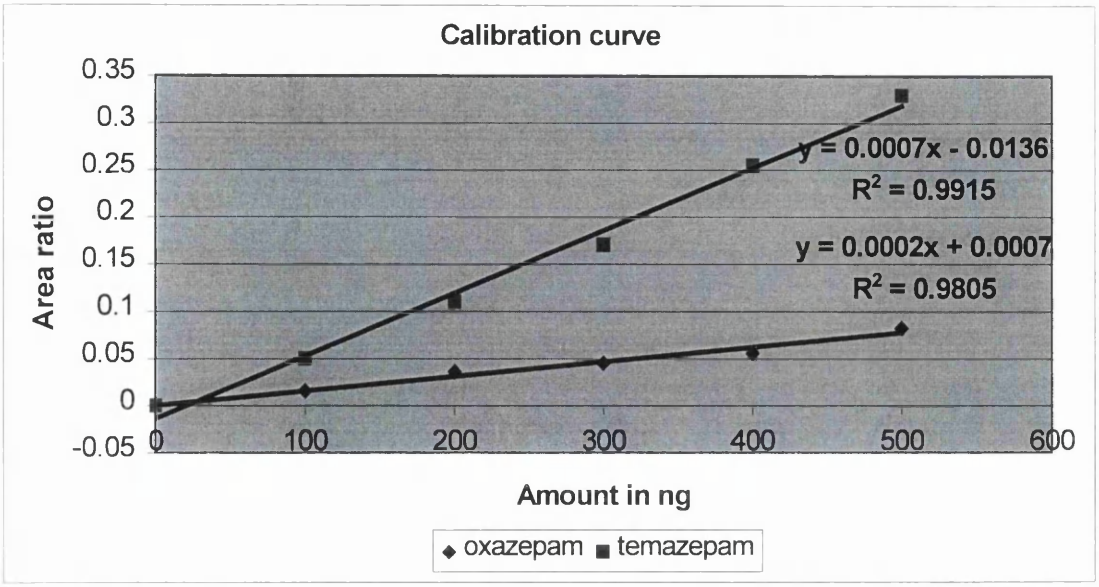


Figure 13.8 Calibration curve for temazepam and oxazepam with prazepam(IS)

13.3.4. Application to case samples

The developed method was evaluated using samples from autopsy and road traffic cases. Typical gas chromatograms for these analyses are shown in Figures 13.9 to Figure 13.12 below and the results are summarised in Table 13.3.

13.4. Conclusion

Although SPME is a simple technique for sample preparation, many factors have to be taken into consideration, especially for optimising the extraction. Extraction time and temperature are among the most important factors to be considered besides pH adjustment and addition of salt. In the present study it was found that SPME could be used to extract benzodiazepines from urine specimens. The technique is robust and solvent free, but the life span of the fibre can shorten due to substances present in the urine if appropriate care is not taken. Also, the fibre is quite brittle and easily bends even within the encasing sheath. A high pH will also destroy the fibre.

Table 13.3. Results from application of the proposed method to case samples.

Case No.	Concentration in ng/ml			
	Diazepam	Desmethyldiazepam	Temazepam	Oxazepam
Case1	Nd*	nd	nd	nd
Case 2	Nd	71.5	nd	nd
Case 3	34.8	nd	nd	nd
Case 4	89.8	86.5	231	54.2
Case 5	Nd	438.5	nd	nd
Case 6	59.3	nd	nd	nd
Case 7	12.5	nd	nd	nd
Case 8	56.2	46.2	nd	nd
Case 9	Nd	nd	132	nd
Case 10	45.3	42.3	nd	nd

*nd = not detected

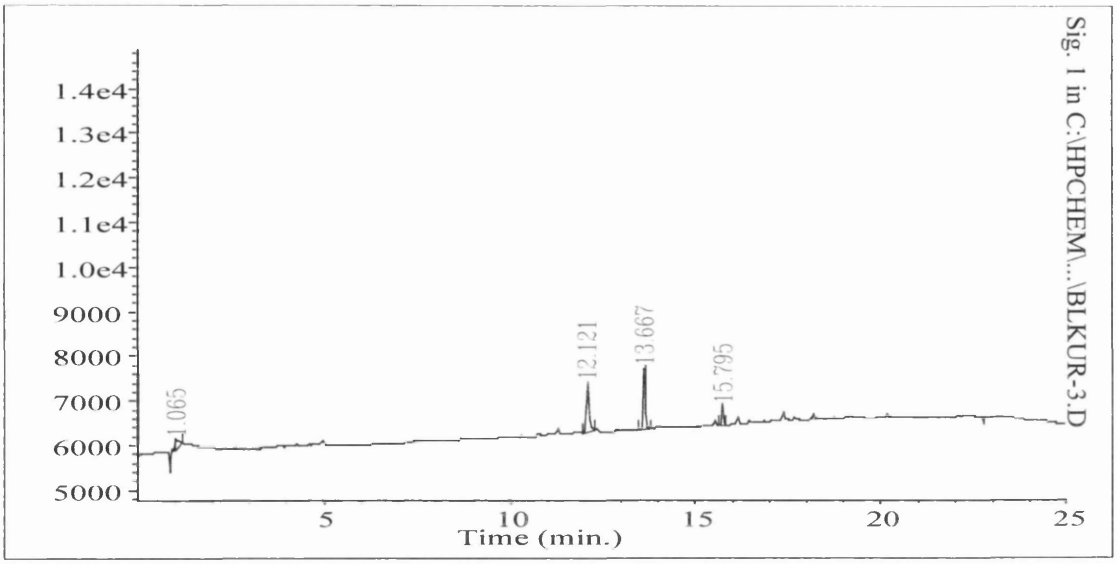


Figure 13.9 Chromatogram of blank urine.

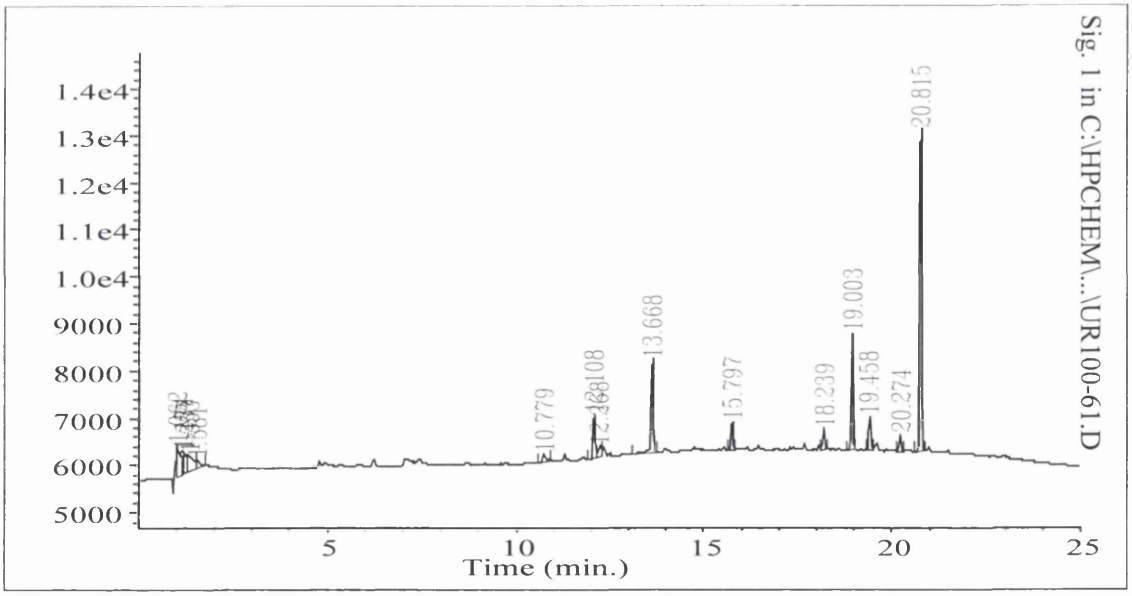


Figure 13.10 Chromatogram of spiked urine standard containing oxazepam (retention time 18.2 min), diazepam (19.0), DMD (19.4), temazepam (20.2) and prazepam IS (20.8). Concentration of each standard was 50 ng/ml.

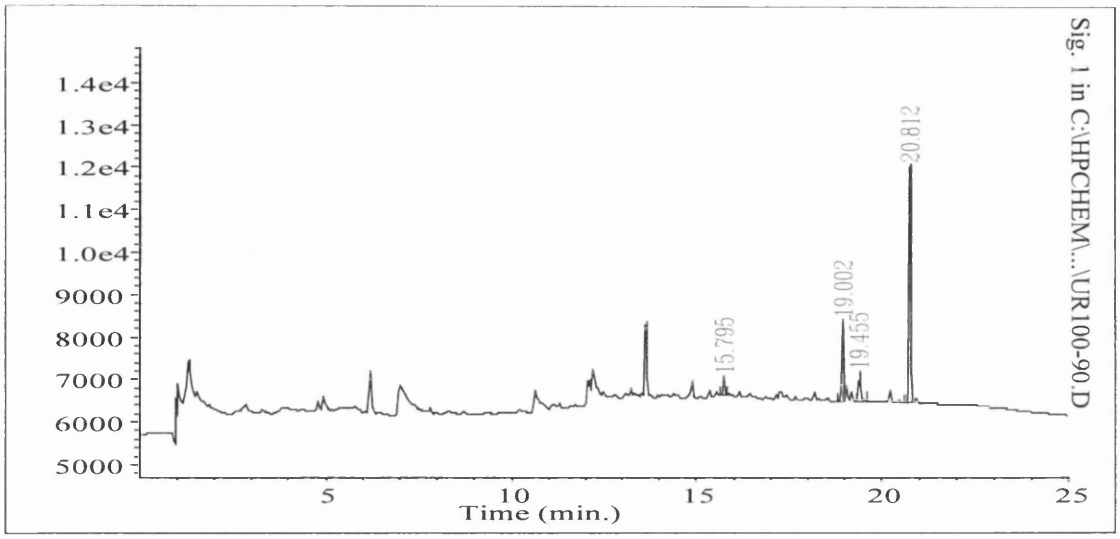


Figure 13.11 Case 8. Positive diazepam at 19.0 min and desmethyldiazepam 19.4 min.

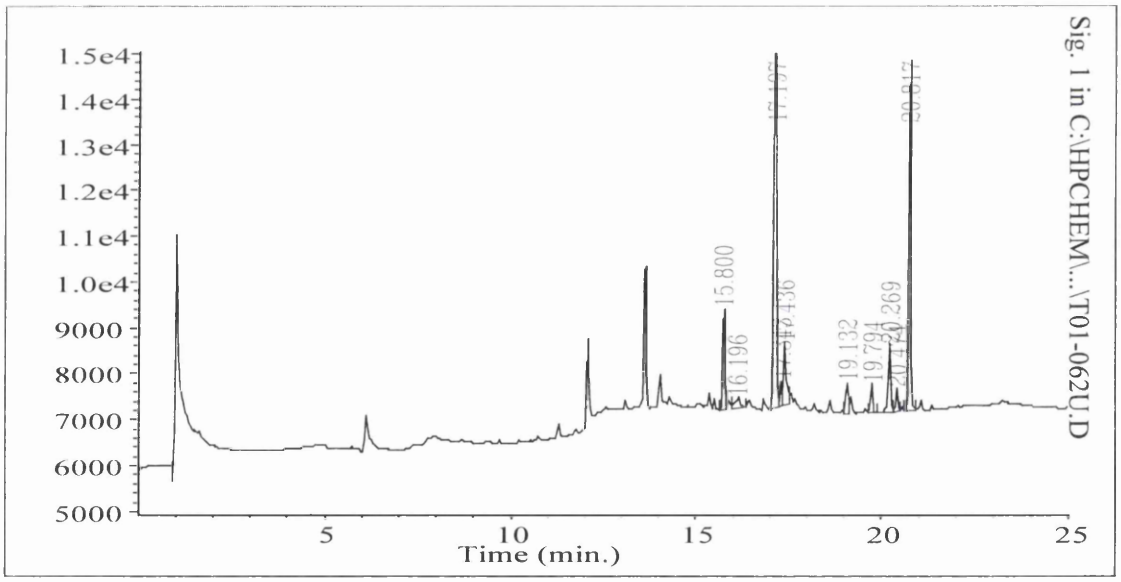


Figure 13.12 Case 9 Positive for temazepam at 20.2 min.

14. OVERALL CONCLUSIONS

The Department of Chemistry (DOC) comprises a network of laboratories throughout Malaysia that provides analytical, investigatory and consultancy services to government agencies for the enforcement of laws and regulations and the implementation of programmes related to public health in the areas of food and water safety, environmental quality, public order and security, consumer protection, and occupational safety and health. It also provides technical inputs for the evaluation of supplies for government contracts, the classification of customs tariff and administration of justice. It is divided into 8 main divisions namely Forensic, Environmental Health, Trade Tariff Classification, Industry, Research and Quality Assurance, Project Implementation, Administration, and Technical Support.

The Forensic Division is subdivided into the Narcotic Section, Serology/DNA Section, Criminalistics Section, Toxicology Section, and Document Examination Section. In the Toxicology Section activities and functions consist of:

- Alcohol analysis in blood, urine and vitreous humor.
- Human poisoning. (intentional and unintentional, including drugs and pesticides)
- Animal poisoning
- Heavy metal poisoning
- Dangerous drugs in body fluids
- Scheduled poisons

Even though it has an enormous function very little input has been placed on developing new techniques, especially in sample preparation. Emphasis needs to be given to revamping the whole set of methods that have been used for many years, using liquid-liquid extraction as the means of sample preparation. Areas such as minimising the usage of organic solvents should be looked at in depth. With the onset of the global effect (Greenhouse Effect), effort needs to be made to minimise the use of solvents that contribute to depleting the ozone layer, especially chlorinated substances such as chloroform which are usually employ as extraction media. Also, minimising the use of so-called carcinogenic solvents such as hexane, acetonitrile that

can contribute toward health hazardous is important. Problems arise from waste disposal that could create an environmental catastrophe if not handled properly. Stacks container of solvent lying around could be considered as environmental time bombs ready to go off with disastrous effects, if care is not taken

The work described in this thesis has achieved most of the goals set at the beginning of the research in minimising the use of solvents in analytical procedures, especially with regard to sample pre-treatment/preparation. Methods employing the use of newly developed modes of extraction were tried and successfully implemented in analysis of organophosphorus pesticides in biological specimens, including blood and urine. The technique was also used in the determination of benzodiazepine drugs. A comparison with SPE was also made.

In the first part of the thesis, work was done on method development in the use of solid phase microextraction in headspace, mainly in blood. The technique of SPME employs a coated fiber, usually polydimethylsiloxane (other coatings such as polyacrylate are also available), to extract and concentrate non-polar analytes which are then desorbed in the injection port of a gas chromatograph for analysis. The extraction of a sample by SPME can be conducted directly, with the coated fibre immersed in a liquid sample, or in the headspace (HS), where the extracting fiber is suspended above the sample, usually in a closed system. The HS approach is preferred when the sample matrix contains undissolved particles or non-volatile dissolved material which may be transferred to the GC injector or non-polar non-volatile material which may contaminate the coated fiber. The theory of HS-SPME has been described in detail by Zhang and Pawliszyn [148]. The SPME device is commercially available or it can be easily assembled from readily available components.

The HS-SPME technique is usually applied in an equilibrium situation with the analyses distributed between the fiber coating, the HS gas, and the aqueous and/or any non-aqueous non-polar phase present in the sealed HS container. The amount of an analyte present in the coating compared to that added or present in the HS vial is a measure of the extraction efficiency, or percent extracted for that analyte under the conditions of that analysis. This equilibrated amount is usually related to that from an appropriately spiked matrix for the HS-SPME quantitation. The addition of salt to the

aqueous matrix is often used to increase the extraction efficiency. Non-polar analytes with low vapour pressure can be analysed by HS-SPME but the time required to attain an equilibrium for these analytes is greatly increased. Furthermore, the particular sample matrix, the stirring efficiency in the aqueous phase and that imparted to the gas phase, the amount of sample, the size of the HS container, the ratio of the HS to aqueous phase and the position of the coated fiber in the HS can all affect the time required for the analyte to equilibrate between the HS vial contents and the SPME fiber coating. Increasing the analyte vapour pressure by increasing the temperature will decrease the equilibration time although the equilibrated extraction efficiency will be reduced. Analytes can also be determined in a non-equilibrated state providing adequate repeatabilities for the analyses are demonstrated. This approach is practical with slow equilibrations where the incremental increase in extracted analyte at the time of analysis is small. Alternatively, suitable internal standards, preequilibrated with the target analyses before the SPME step, can be used. If the sample contains lipid material, as in some food samples, the equilibrated analyte extraction efficiencies can be severely reduced as the non-polar food components compete with the SPME coating for analyses.

SPME was initially employed for the direct analysis of non-polar volatiles in water, and has been applied successfully to the direct or HS extraction of non-polar/polar, semi-volatile pesticides and drugs in blood and urine. These procedures employ various detection techniques of differing selectivities and sensitivities and report method detection limits (MDL's) of low ng/ml, while at the same time maintaining acceptable extraction times.

There are a few points of interest to note from the experiences with SPME reported here. First of all, the fibre assembly is fragile, thus making it possible to bend the outer syringe needle or damage the fiber. Although the fiber assembly is easy to use, great care must be demonstrated to avoid damaging the fiber. Second, 100 μm bonded polydimethylsiloxane fiber is used because of its robustness. Bonded phase fibres can be submersed directly into a liquid matrix during sampling and can be rinsed with organic solvents. Another point of concern involves injection seals. The diameter of the outer syringe needle cores standard septa easily, requiring them to be replaced

frequently. Thus, the used of a pre-drilled septa instead of a normal septum was selected. Pre-drilled septa work better and last longer, but the septum retainer nut has to be tightened beyond normal torque to keep them from leaking. Due to the nature of the septum retainer nut, the fiber assembly will not rest firmly unless it is held in place. Using SPME guide ring stand with a wrench clamp works well for holding the fiber assembly in place during desorption.

This experiment presented an opportunity for the use of a relatively new, simple, yet rapid, solventless technique to extract polar and non-polar semi-volatile organophosphates and benzodiazepines directly or from the headspace region of samples. A typical experiment involving SPME requires minimal sample preparation and analysis times are under 70 min. Multiple analyses can be performed within the time span allotted for a typical laboratory period. SPME offers a relatively inexpensive way to analyse fairly complex samples such as blood and urine that might otherwise be difficult to analyse by conventional headspace analysis.

The solid phase extraction technique was also tried out. It gave a good extraction precision and recovery for most of the analytes of interest. A study on parameters affecting the extraction was also made. Dilution of the sample must be optimised such that the analytes of interest are not lost due to the increase in solvation. Time for percolating the sample through the cartridge also plays a significant role in the recovery. Solvents used for elution were examined and a few were suitable but ethyl acetate was chosen due to the high recoveries obtained and because it is the least toxic of the solvents evaluated. The protocol can be utilised for future work to extract alkyl phosphate metabolites of the organophosphorus pesticides.

However SPE has some important limitations - plugging of the cartridge or blocking of the pores by matrix components, especially with regard to putrefied whole blood, and high elution volumes. Moreover, it is a multistep step process and is therefore subject to analyte loss. Finally, SPE often involves a concentration step through solvent evaporation and in this way it is not applicable to extraction of volatile or thermolabile compounds.

Another area that has been examined is minimising the effect of degradation of the organophosphate malathion in blood. It is unstable in aqueous solution and especially in blood because of the presence of esterases. Complete degradation of organophosphates during storage was frequently observed. Organophosphates are degraded more rapidly by esterase activities than by other chemical mechanisms. In contrast the organophosphorothioates are hydrolysed chemically in aqueous solution but are stable in biological specimens and are not metabolised by esterase. Also the effect of adding preservatives was studied. The results from the study indicate that, to prevent enzymatic hydrolysis of and thus to avoid any loss of OP compounds during storage, the addition of sodium fluoride was not recommended but instead EDTA/K or Li/Heparin. If prompt submitting to laboratory cannot be performed, storage at 0-4°C is the most suitable procedure or better at -20 °C [149].

Although SPME offers promising features that have certain advantages over more conventional techniques, it should not be seen as a panacea, a substitute or an opponent of existing standard methods such as SPE. It should be instead consider as a complimentary technique, which offers an attractive alternative to more conventional systems.

In Progress and Future Work

Initial investigation of the application of SPME for isolation of organophosphates in vitreous humour (headspace/direct) has been done during this project but no actual cases have been reported. Also work can be done in the use of HS-SPME for the determination of paraquat diene and monoene in blood and urine.

At present, investigation is in progress of analysis of hair samples, coupling freezer mill (liquid nitrogen) and HSSPME for the analysis of organophosphates in agricultural person/s exposed to pesticides. This approach can also be applied to drugs of abuse as well.

It would also be good to look at the technique for Systematic Toxicological Analysis since it offers solventless extraction with the sample not destroyed. Sensitivity can be

enhanced by using different types of fibre because the primary focus will be on qualitative analysis.

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16. ANNEXES

CONFERENCE PRESENTATIONS IN SUPPORT OF THIS DISSERTATION

16.1. Optimisation of Solid Phase Microextraction (SPME) Conditions for Headspace Analysis of Organo-Phosphate Pesticides in Whole Blood.

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OPTIMISATION OF SOLID PHASE MICROEXTRACTION (SPME) CONDITIONS FOR HEADSPACE ANALYSIS OF ORGANO-PHOSPHATE PESTICIDES IN WHOLE BLOOD.

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Abstract

Organo-phosphate (OP) pesticides are a significant cause of fatal poisoning in many countries. Problems exist in detecting the parent substances in autopsy blood when putrefaction has occurred. This study evaluated the use of SPME as an extraction technique for the analysis of OP pesticides in whole blood.

Malathion and diazinon were used as model compounds, added to whole blank blood. Parameters affecting the adsorption of analyte on the SPME fibre were sampling time, temperature, agitation, and modification of the ionic strength and pH. The best conditions for extraction were at 90°C, with a conditioning time of 10 minutes, exposure time of 30 minutes and desorption time of 5 minutes. All samples were agitated during the extraction process. The primary detection and quantification technique was GC-NPD but GC-MS was also used as a confirmatory identification technique. The minimum detectable level by GC-NPD was 100 ng per 0.5-ml whole blood and the linear quantifiable range was from 200 ng-1000 ng per 0.5-ml whole blood (correlation coefficient 0.993). Recoveries were approximately 0.39% with a coefficient of variation of 6.8%.

The rates of *in vitro* change of Malathion concentrations in blood during storage at ambient temperatures in the range 20-25°C and at 4°C were also measured. The time limit of detection at 20-25°C was 4 hr but at 4°C was more than 11 days. No interferences from the putrefying blood were observed. From the results it was concluded that headspace-SPME could be used as an alternative technique for sample preparation which is simpler and more rapid than liquid-liquid extraction or conventional solid phase extraction.

Keywords: Solid Phase Microextraction, Organo-phosphates, Whole blood

1. Introduction

In 1990, SPME was introduced by Arthur and Pawliszyn [1]. This method is a new, fast, and simple analytical technique which employs a stationary phase of polydimethylsiloxane coated on a fused-silica fibre to extract analytes from aqueous or gaseous samples in sealed vials using direct immersion or headspace techniques respectively. The subsequent analyses are currently performed by GC and HPLC and the analytes are desorbed in the injector of the gas chromatograph. This method represents a further advance as a solvent-free alternative to the extraction of organic compounds from biological samples [2,3]. Headspace SPME had been considered suitable only for extraction of volatiles but the results for tricyclic antidepressants [4] and local anaesthetics [5] seem to open the applicability of headspace SPME to a number of other solid drugs and poisons of medium-sized molecular weights.

More recently headspace SPME of biological fluids was applied to analyses such as cyanide in human whole blood [6], and organophosphates and carbamates in blood and urine [7,8].

SPME is a process dependent on equilibrium rather than total extraction: the amount of analyte extracted at a given time is dependent on the mass transfer of an analyte through the aqueous phase. The principle behind SPME is the equilibrium partition process of the analyte between the fibre coating and the aqueous solution (including the gaseous phase for headspace analysis). The time to equilibrium is a function of the analyte and conditions used (e.g. fibre chemistry and thickness)

We will look at these factors by focussing on the analyses of Malathion and Diazinon (as internal standard) in blood.

2. Experimental

2.1. Materials

Malathion and Diazinon were purchased from Promochem Limited UK. Stock solutions containing 100 μg per ml were prepared in methanol. Sodium chloride and hydrochloric acid (HCL) were AR Grade and were used to prepare saturated sodium chloride and 0.1M HCL solutions.

Headspace vials (4.0-ml amber, screw top) with septum caps (PTFE/Silicone septa)

were heated and agitated with a Corning hot plate stirrer. SPME sampling stands (holding 8 vials) and magnetic stirrer (10x3 mm covered with PTFE) were used. A manual assembly for SPME, with replaceable 100 μm extraction fibre coated with polydimethylsiloxane, was obtained from Supelco Sigma-Aldrich Company Ltd, UK.

2.2. Instrumentation

Gas chromatography used a Hewlett-Packard Model 5890 Series II Gas Chromatograph equipped with a nitrogen-phosphorus detector, a 0.75 mm splitless insert and an HP1 capillary column (30 m x 0.53 mm i.d., 0.88 μm phase thickness). The column oven was programmed from an initial temperature of 100°C (held for 2 minutes) to 300°C at a ramp rate of 10°C per minute. Injector and detector temperatures were 250°C and 280°C respectively.

2.3. Headspace SPME Procedure

A polydimethylsiloxane (PDMS) coated fibre (diameter 100 μm) was exposed to a temperature of 250°C in the GC injection port overnight prior to the analyses. This removed contaminants as well as conditioning the fibre.

3. Determination of Extraction Procedure

3.1. Optimisation of Desorption Conditions

The SPME fibre, exposed to 1.0-ml blood containing malathion and diazinon at a concentration of 10 ppm, was inserted into the GC inlet and left for various time intervals (1- 5 minutes). A check was made for carry over or traces of peaks of no interest

3.2. Effect of Temperature

A set of 4.0-ml vials was prepared with magnetic stirrer and 1.0 ml of blood containing a concentration of 10 ppm malathion and diazinon. The vials were place in a vial receptacle on a hot plate/stirrer at 80°C and maximum speed. After heating for 10 minutes, the septum-piercing needle of the SPME was passed through the

septum (Fig.1). The pre-treated fibre was pushed out from the needle and exposed in the headspace for 5, 10, 15, 20, 30, 40, 50 and 70 minutes to allow the adsorption of the compound. The fibre was withdrawn into the needle and pulled out from the vial. It was then inserted into the injection port of the gas chromatograph; the fibre was exposed for 5 minutes to ensure complete desorption of the compound. The same procedure was applied to a second set of samples at an incubation temperature of 90°C.

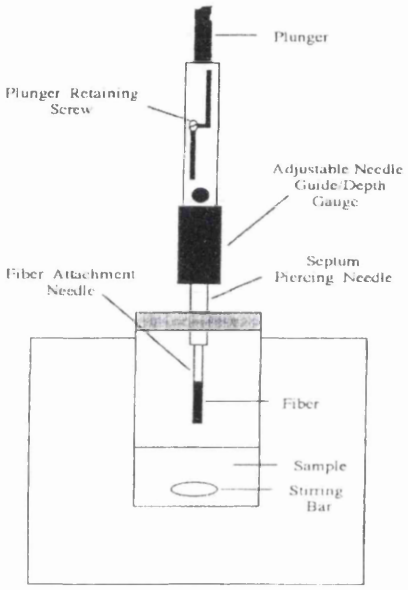

Fig. 1. Schematic illustration of the headspace SPME method.

3.3. Effect of Agitation

Two sets of blood samples were prepared as in Paragraph 3.2 and placed in the heating block at 80°C. One set was agitated and the other not. Time extraction profiles were established from 0-70 minutes.

3.4. Additives and pH Adjustment

Three sets of 6 vials were prepared containing 0.5-ml blood spiked with 1000 ng of malathion and diazinon. The first set was diluted with 0.5-ml distilled water. The second set was diluted with 0.5-ml 0.1M HCL. The third set was diluted with 0.5-ml water, 100 µL 0.1M HCL and 100 µL saturated sodium chloride. All three sets were treated as above (see 3.2.) but at a sample temperature of 90°C.

The results of the work described in Paragraphs 3.1-3.4 formed the basis for optimal conditions for subsequent studies.

3.5. Limit of Detection (LOD)

Aliquots of blood (0.5-ml) containing malathion at concentrations of 500 ng, 300 ng, 100 ng and 80 ng malathion per 0.5-ml blood were analysed in triplicate with the developed procedure. Blank injections were performed between each run to ensure carry over, if present, was negligible.

3.6. Reproducibility

The analysis was repeated ten times at malathion concentrations of 200 ng and 800 ng per 0.5-ml whole blood using the optimised conditions.

3.7.Linearity / Calibration Curves.

Calibration standards with malathion concentrations in the range 200 ng to 1000 ng per 0.5-ml whole blood were prepared. Internal standard diazinon was added at an amount of 60 ng per vial. The precision of the method at 200 ng and 800 ng / 0.5 ml was measured by analysing 10 samples at each concentration.

3.8. Stability of malathion during storage in a fridge (0-4 ℃) and at ambient temperature (20-25 ℃).

Fresh blood (20-ml) was spiked with malathion to a concentration of 10 ppm. The sample was split into 2 halves. One half was kept in the fridge (0-4°C) and the other was exposed to a room temperature of 20-25°C. The specimens were analysed every 30 minutes for the first 8 hours and then each day for 11 days.

4. Results

4.1. Effect of Agitation and Temperature

The equilibrium state was achieved faster for malathion at the higher temperature but the amount extracted was lower compared to that at the lower temperature. This due

to the effect of the temperature on the fibre. At higher temperatures the diffusion coefficient in blood is higher and the extraction time is shorter, but the partition coefficient in the fibre is also lower. For diazinon, the higher the temperature the better the response and equilibrium was also attained faster at the higher temperature.

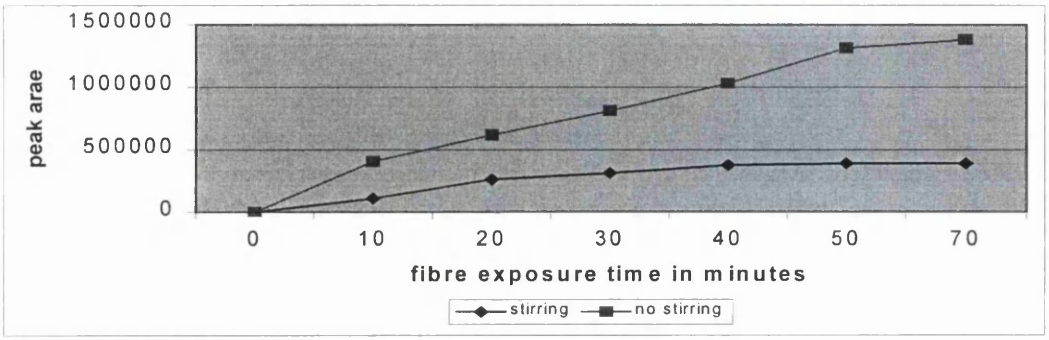

Fig.2 Effect of stirring on analysis of malathion

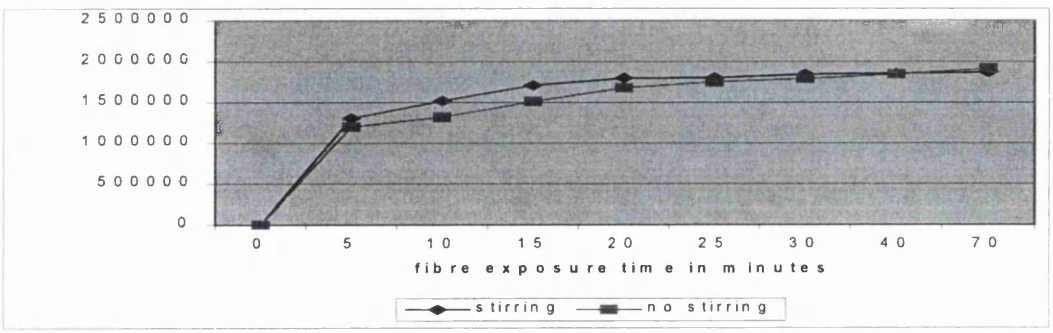

Fig.3 Effect of stirring on analysis of diazinon

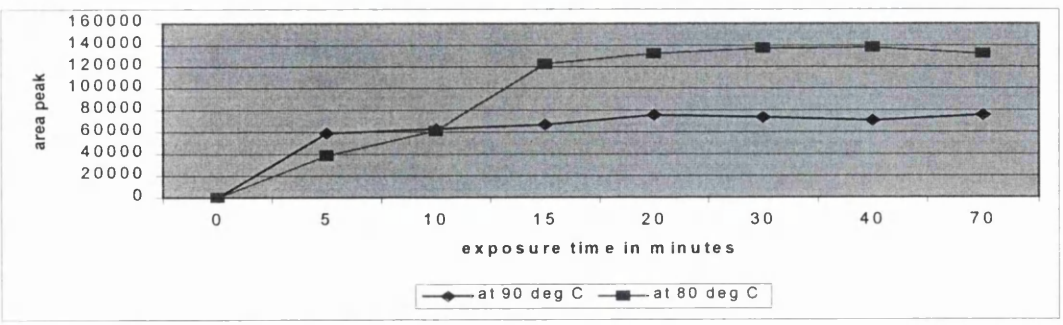

Fig.4 Extraction time profile for malathion in blood

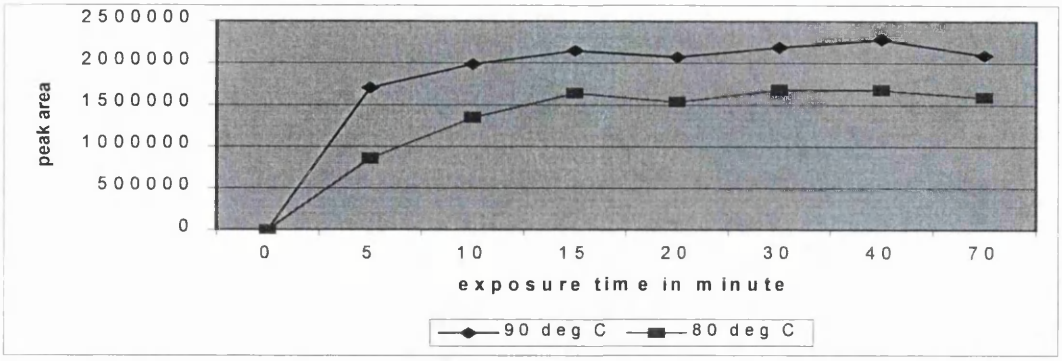

Fig.5 Extraction time profile for diazinon

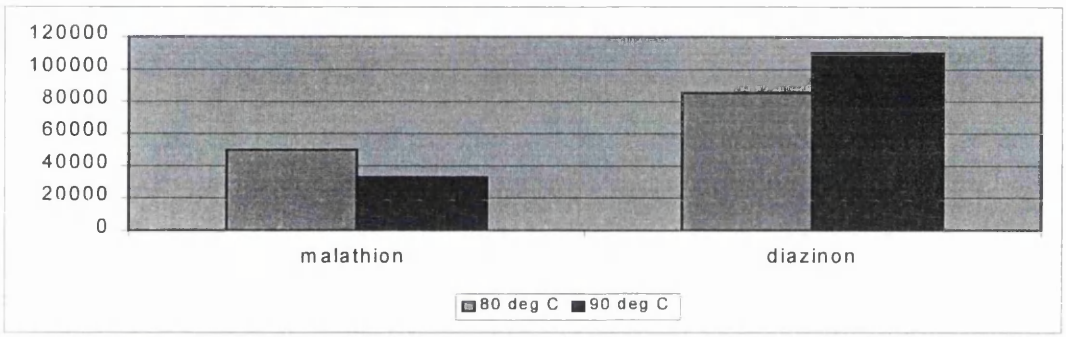

Fig.6 Extraction at 80 and 90 °C

4.2. Salting Effect and pH Adjustment

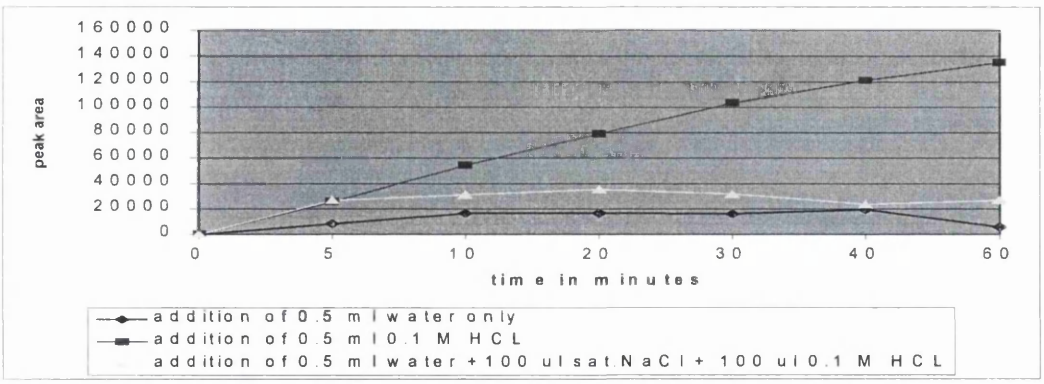

Fig.7 Extraction time profile of malathion after acid and salt addition

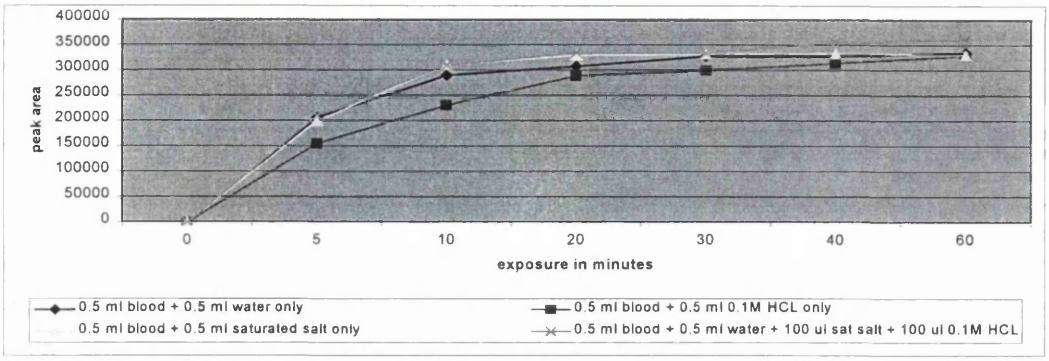

Fig.8 Extraction time profile of diazinon after acid and salt addition

Two other common techniques to enhance extraction are addition of salt and pH adjustment. From Fig.7-10, the results indicated that both of these influenced the amount extracted. By addition of salt and pH adjustment the equilibrium state can be attained much more rapidly.

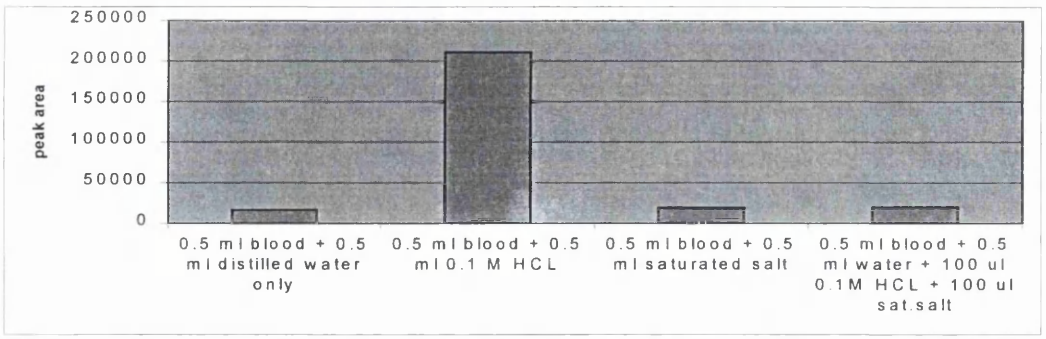

Fig.9 Malathion extracted after acid and salt addition

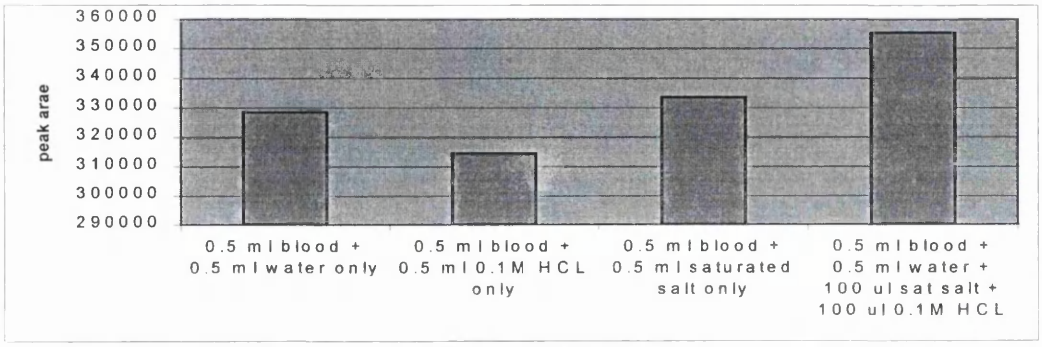

Fig.10 Diazinon extracted after acid and salt addition

If only acid is used, the amount extracted is higher in the case of malathion but

equilibrium is not achieved even after 60 minutes fibre exposure, whereas after diluting with water the equilibrium was achieved faster but the amount extracted was lower compared to that of salting and acidification. Acidification reduces the response for diazinon. Diluting the blood also had a significant impact on the amount extracted.

Based on the results given in 4.1 and 4.2 above, the developed extraction method is as follows:

A blood sample (volume 0.5 ml) is diluted with 0.5 ml distilled water followed by addition of 100 µL of 0.1M HCL and 100 µL saturated sodium chloride solution. Extraction consists of 10 minutes sample equilibration and 30 minutes fibre exposure to the sample headspace. Desorption time in the GC inlet is 5 minutes.

4.3. Detection Limit, Reproducibility Study, Quantifiable Level and Linearity.

The limit of detection was found to be 100 ng per 0.5-ml blood. Retention times for malathion and diazinon (internal standard) were 15.9 minutes and 14.1 minutes respectively. Recovery at 200 ng per 0.5 ml blood was 0.39% and coefficient of variation 6.8% whereas at 800 ng per 0.5 ml blood was 0.38 % and coefficient of variation6.5% (n=5). The method was linear in the range 200 ng to 1000 ng per 0.5 ml blood (n=3) and the correlation coefficient (r^2) was 0.993. At concentrations of 200 ng and 800 ng / 0.5 ml blood, the method was found to have a coefficient of variation of 4.40% and 8.14% respectively.

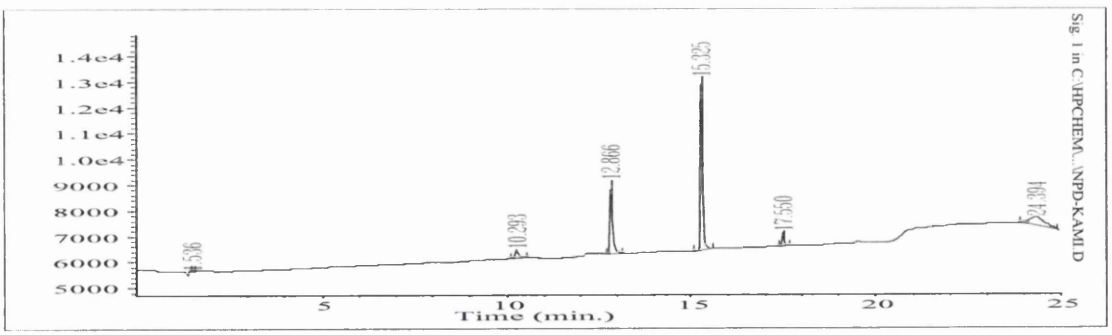

Fig.11 Blank blood

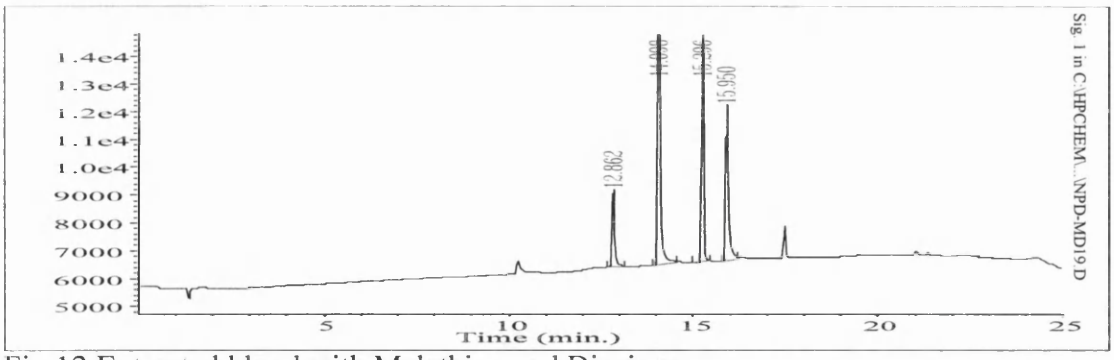

Fig.12 Extracted blood with Malathion and Diazinon

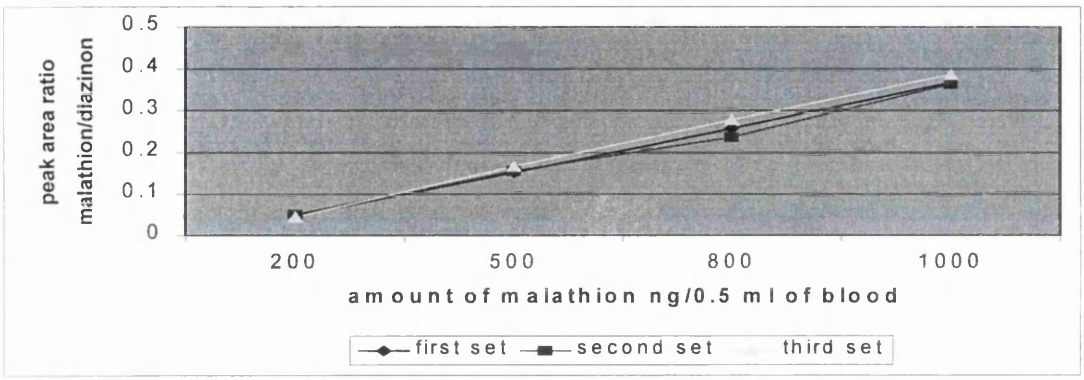

Fig.13 Calibration curve for malathion at 90 deg C

4.4. Stability of malathion in whole blood.

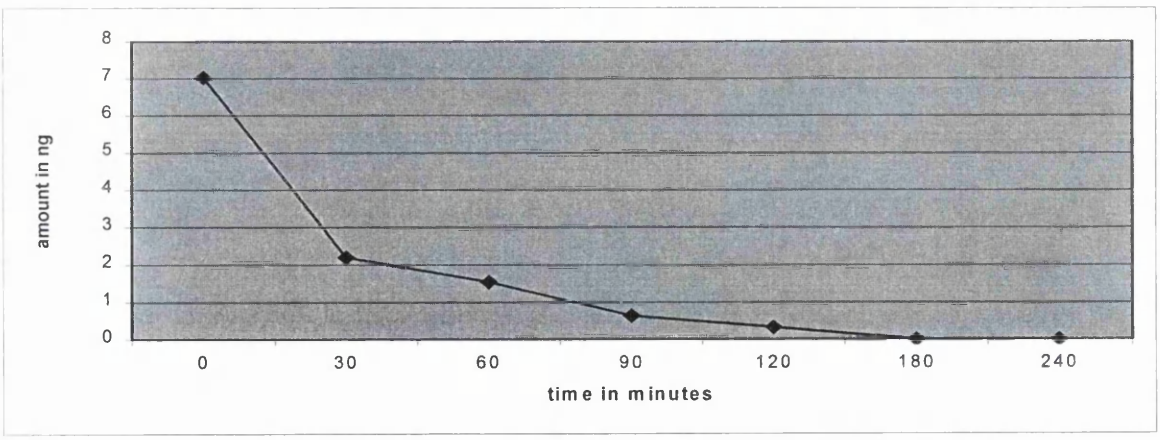

Fig.14 Rate of malathion disappearance at 20-25 °C

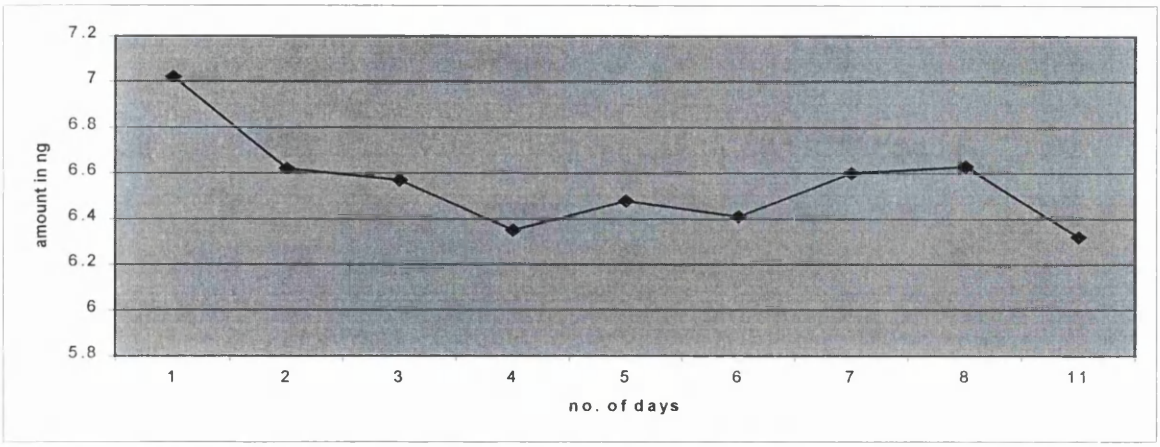

Fig.15 Stability of malathion in blood at 0-4deg C

Malathion disappeared (not detected) from fresh blood after about 4 hours at a room temperature of 20-25°C (Fig.9). The half-life ($t_{1/2}$) was found to be 1.1 hours at pH 6-7. Malathion was found to be stable if the sample was kept under cold conditions without much deviation (concentration of 10 ppm) but the measured concentration still dropped initially.

5. Discussion

Since malathion and diazinon each have a low Henry's constant (semi-volatile pesticide), the sample is heated in order to increase their concentrations in the gas phase in HS-SPME. Although heating is often useful to enhance sensitivity it has a double impact: at higher temperature, diffusion coefficients in blood are higher and the extraction time is shorter but the net partition coefficients are lower. The difference arises from the fact that, with SPME, there are three phases and heating alters the partitioning of analyte between the headspace and the fibre to favour the headspace. Two temperature levels were evaluated, i.e. at 80° and 90°C. At the higher temperature the amount of malathion extracted was reduced (Fig.4) due to a reduction in the distribution constant in the fibre but examination of the extraction time profile showed that equilibrium was attained faster at the higher temperature.

Agitation improved the equilibrium attained and the time required for malathion (Fig.2 and Fig.3) though these were not so marked for diazinon. Also, modification of the ionic strength and the pH had an impact on the sorption (affinity)

of the analyte for the fibre coating and equilibrium was achieved much faster and with a higher recovery. Reducing the solubility of the analyte in the aqueous phase can increase the amount of the analyte extracted by the fibre. This can be achieved by the addition of salt and/or by pH adjustment. It is important to remember that in SPME neither complete extraction of analytes nor full equilibrium is necessary [9], but consistent sampling time, temperature, fibre immersion depth and headspace volume are crucial to reproducibility. However, extraction at nonequilibrium results in a lower degree of reproducibility of the analysis.

6. Conclusion

The technique was applied successfully to study the rate of disappearance of malathion from whole blood. The technique is simple but careful consideration has to be taken into account. Advantages of SPME are that not much preparative work needs to be done and the use of solvent is eliminated, thus reducing pollution and health hazards. Also, only a small sample volume is needed for extraction. The major drawbacks are the fragility of the fibre and time consuming nature of the technique if done manually.

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16.2. Screening of Post-Mortem Blood in Pesticide Poisoning Cases By Headspace Solid Phase Microextraction.

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SCREENING OF POST-MORTEM BLOOD IN PESTICIDE POISONING CASES BY HEADSPACE SOLID PHASE MICROEXTRACTION.

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Abstract

Since the introduction of Solid-Phase Microextraction (SPME) a decade ago, this technique has become a method of choice for sample preparation. This paper presents results obtained from the application of a published headspace-SPME (HS-SPME) method to the analysis of autopsy case samples.
25 specimens of post-mortem blood, collected at autopsy in Malaysia, were examined using HS-SPME as a routine screening procedure for pesticides. Of these samples, 3 positive cases of malathion poisoning were identified and quantified using GC-NPD with HS-SPME as the method of extraction. The limit of detection was 100 ng/0.5 ml blood and the correlation coefficient of the standard curve was 0.995 in the range 0-500 ng/0.5 ml of blood. Recoveries were found to be 0.34% with average coefficient of variation 5.4% (at a concentration of 100 ng/0.5 ml blood). The three cases confirmed as malathion poisoning had malathion concentrations of 51.6 ng, 134.5 ng and 6025 ng per 0.5 ml blood respectively. Dual column GC-NPD was used for the confirmation of malathion in postmortem blood from cases of suspected suicidal intoxication. A stability investigation was carried out by exposing samples to a room temperature of 17-20°C. After 2 hours malathion was no longer detectable.

Keywords: Headspace Solid Phase Microextraction (HS-SPME), routine screening, dual column, stability.

1. .Introduction

In Malaysia, as in many other countries in the Asian continent, pesticides are one **of**

the most significant causes of fatal and non-fatal poisoning [1-3], exceeding those due to other substances such as prescribed or illicit drugs, which are more prominent in Europe and the United State [4-5].

These poisoning cases form an important part of the work of Forensic Toxicology in Malaysia [6] and it is essential that an appropriate and adequate range of analytical procedures should be available for the detection and measurement of these substances in biological specimens. There is a need for robust, facile and inexpensive methods of analysis that can be used even in laboratories that do not have GC-MS or LC-MS available. 25 specimens of post-mortem blood, collected at autopsy in Malaysia, were examined using HS-SPME as a routine screening procedure for pesticides

2. Materials and Methods

2.1. Reagents

Diazinon and Malathion were purchased from Promochem Limited Hertz, England. Stock solutions (1 mg/ml) was prepared by dissolving an appropriate amount in MeOH. Blank blood was screened negative for organophosphorus pesticides before used.

2.2. Apparatus

A manual assembly was used for SPME with replaceable extraction fibre, coated with 100 μm poly-dimethylsiloxane (from Supelco UK, Figure 1). Headspace vials (4.0 ml, amber glass) were fitted with screw top caps with with hole and PTFE/Silicone Septa. Vials were equilibrated in a Corning hot plate/stirrer with SPME sampling stand (holding 8 vials) and PTFE covered magnetic stirrers (10x3 mm). All of these items were purchased from Supelco UK.

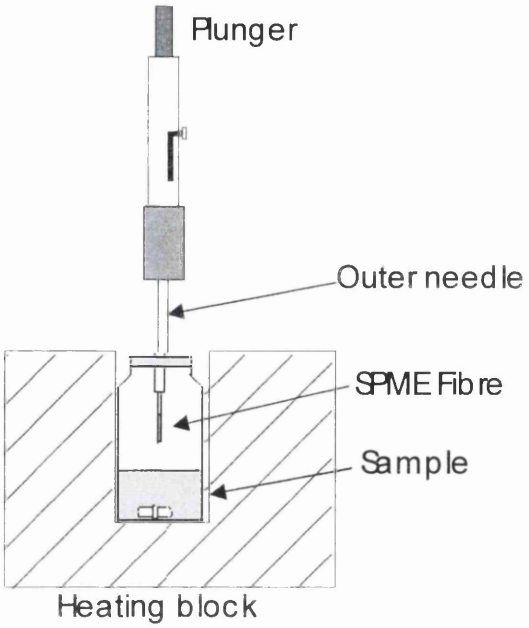

Figure 1: Headspace SPME system

2.3. Gas Chromatography NPD dual Column.

Gas Chromatography was carried out using a Hewlett-Packard Model HP 5892 Series II Plus chromatograph equipped with a nitrogen-phosphorus detector. The oven was fitted with two columns: (a) RTX01, 15 m x 0.32 mm with 0.25 μm phase thickness and (b) DB1301, 15 m x 0.25 mm with 0.25μm phase thickness. The GC was programmed from 120°C (held for 0.5 minute) to 300°C at 20°C/minute. The injection port temperature was set at 250°C and detector at 280°C. Splitless injection mode was used with a purge time of 0.5 minutes.

2.4. Extraction Technique

Spiked/case blood samples, volume 0.5 ml, were diluted with 0.5 ml distilled water followed by addition of 100 μL of 0.1M HCL and 100 μL saturated sodium chloride solution. The extraction sequence consisted of 10 minutes sample conditioning followed by 30 minutes SPME fibre exposure to the sample headspace at a temperature of 90°C. Samples were agitated continuously during the exposure period using miniature magnetic stirrers.

2.5. Linearity / Calibration Curves

Calibration standards in the range 100 ng to 500 ng malathion per 0.5-ml blank whole blood were prepared. The Internal Standard (Diazinon) was spiked at amount of 10 ng/sample.

3. Results

The limit of detection was found to be 100 ng malathion per 0.5-ml blood. No interferences were found in the analysis. Figures 2-3 show representative chromatograms obtained with blank blood containing the internal standard, diazinon, and with a blood specimen that is positive for malathion. Confirmation was obtained using a second column (RTX01) run in parallel with column DB1301, having another NP Detector (Fig.4).

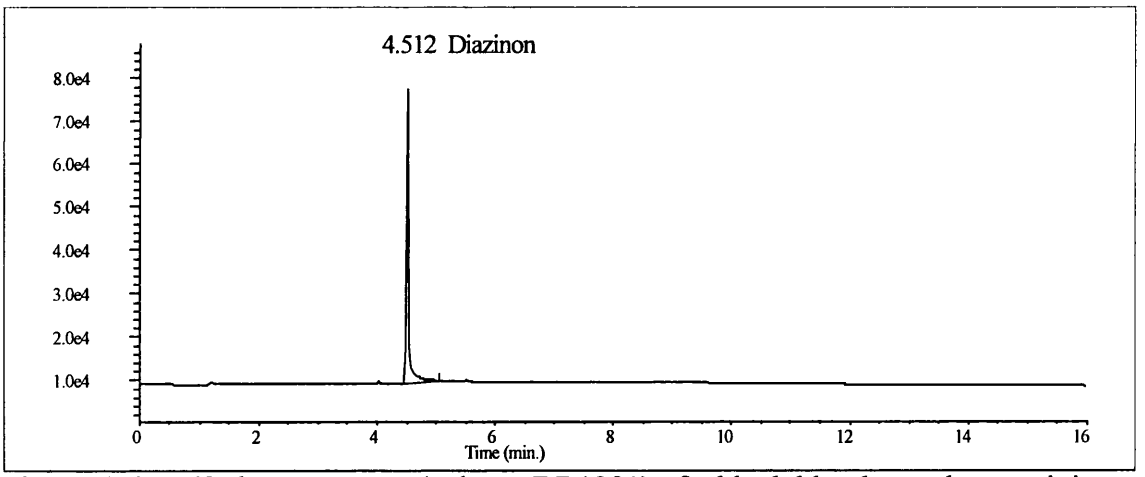

Fig.2 HS-SPME chromatogram (column DB1301) of a blank blood sample containing the internal standard, diazinon.

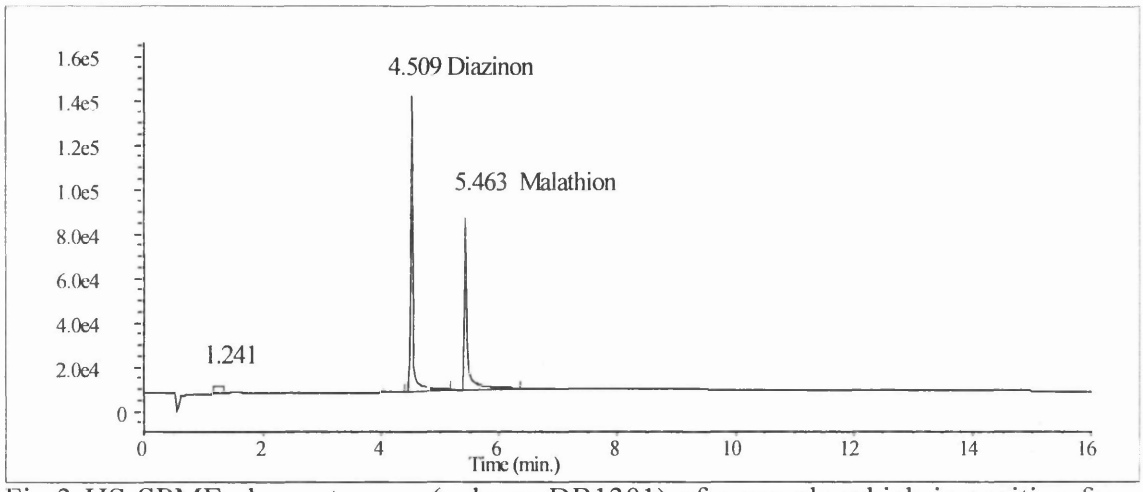

Fig.3 HS-SPME chromatogram (column DB1301) of a sample which is positive for malathion

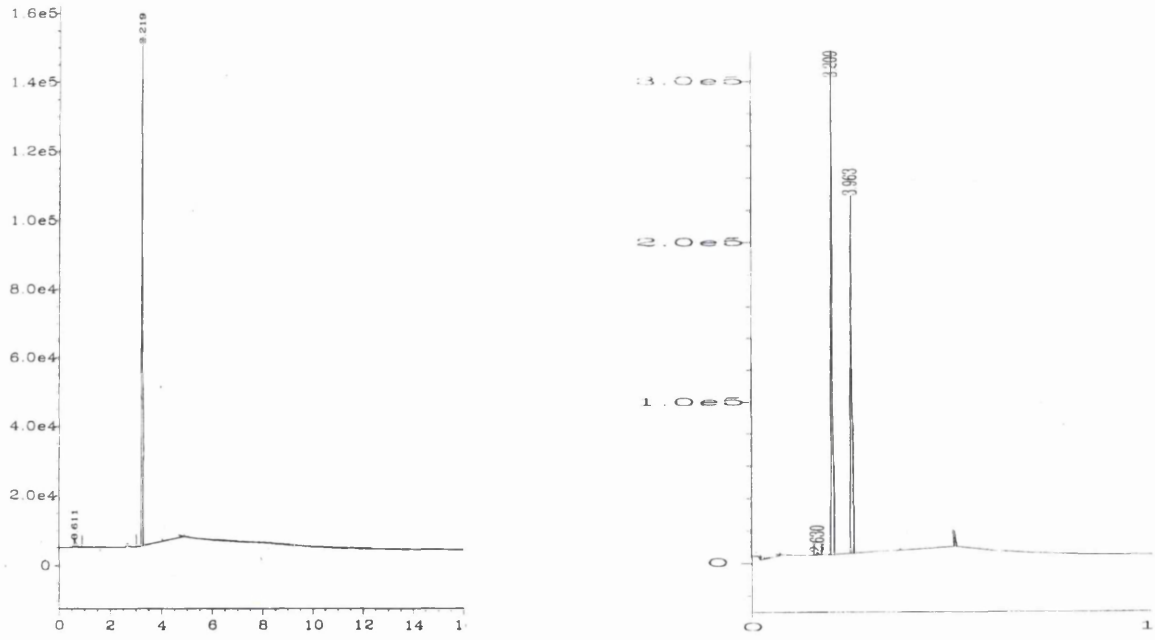

Fig.4 Confirmation by the second column (RTX01) running in parallel with DB1301. On the left, blank blood-containing diazinon as internal standard (retention time 3.2 minutes). On the right, positive sample with malathion at 3.9 minutes.

The method was linear in the range 100 ng to 500 ng per 0.5 ml blood (n=3) and the linear correlation coefficient (r^2) was 0.995 (Figure 5). Recovery of malathion at a concentration of 100 ng per 0.5 ml blood was 0.35% and coefficient of variation 5.2% (n=10). Precision measurements under repeatable conditions for blood samples (n =

10) of concentration 100 ng/0.5 ml gave a coefficient of variation of 5.4%.

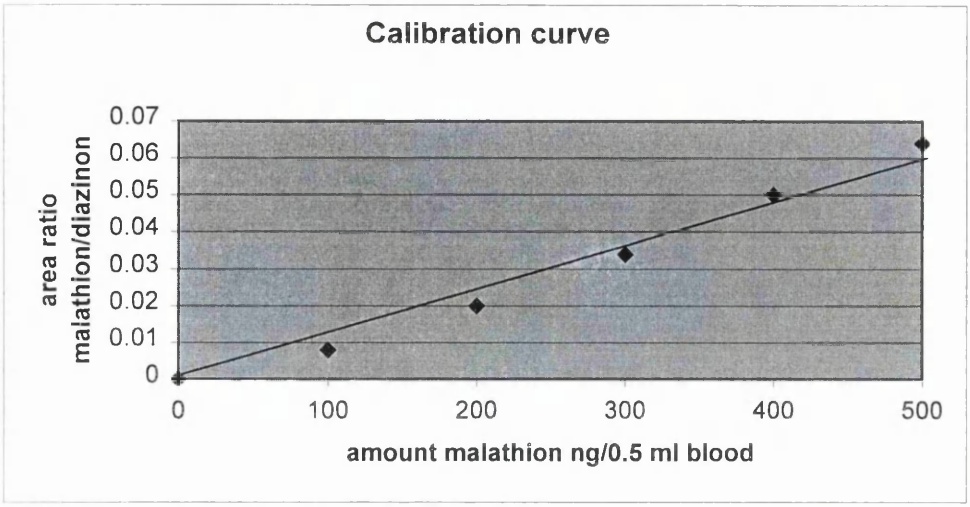

Fig. 5 Calibration curves for HS-SPME analysis of malathion in blood using column DB1301. The equation of the regression line and r^2 values are $y = 0.000131x-0.00352$ and $r^2 = 0.995$.

Analysis of 25 specimens of blood obtained at autopsy in Malaysia found three positive poisoning cases involving malathion, which are described below. In addition, the stomach contents were analyzed using a liquid-liquid extraction method with diethyl ether as extracting solvent. Malathion was found to be present in all three cases.

Case 1: A unemployed Indian man, age 44, alcoholic, was found to have stomach contents positive for malathion. The blood malathion concentration was 135 ng/0.5 ml (270 ng/ml)

Case 2: A Filipino maid, female, ages 27, was found dead. Stomach contents were positive for malathion. The blood malathion concentration was 104 ng/ml (in this case, 1 ml blood was used).

Case 3: Indian female, unemployed, age 18 took her own life by drinking malathion. The stomach contents were found to be positive for malathion and the blood malathion concentration was 6025 ng/0.5 ml (12050 ng/ml).

4. Discussion

Pesticide screening is a routine procedure in the Toxicological Unit in Kuala Lumpur. A simple, robust, sensitive, solvent free and easy to handle technique was needed, especially for sample extraction. SPME seems to offer an alternative procedure for sample preparation compared to the normal technique of liquid-liquid extraction using hexane or diethyl ether after deproteination. In the present study, the use of HSSPME was introduced. The technique was found to be quite easy to handle and not much sample preparation was required. The method can be used as a screening procedure, especially for volatile and semivolatile pesticides

5. Conclusions

The HSSPME method was simpler and more rapid than the conventional liquid-liquid extraction method. The method showed good linearity and excellent results for quantitative measurements, even though the SPME extraction method was based on an equilibrium process. Headspace SPME is suitable as an alternative extraction technique, especially for the analysis of pesticides

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16.3. Headspace Solid Phase Microextraction (HSSPME) In Cases Of Fenthion Poisoning

By Kamarruddin Asri, R.A.Anderson and A.K.Jackaria

In

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HEADSPACE SOLID PHASE MICROEXTRACTION (HSSPME) IN A CASE OF FENTHION POISONING.

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Fenthion (O,O-dimethyl-O-[3-methyl-4-(methylthio)-phenyl]-thiophosphate) is an organophosphate insecticide. A method is presented for the quantification of fenthion in post-mortem blood by solid-phase microextraction (SPME) using a 100μm polydimethylsiloxane (PDMS) fibre (Catalogue No. 57300-U) from Supelco followed by gas chromatography with nitrogen phosphorus detection (GC-NPD) on a Hewlett Packard Model 5890 Series II instrument. Gas chromatography–mass spectrometry (GC–MS) with a Finnigan-Thermoquest Trace instrument was used for confirmation. Diazinon(O,O-diethyl-O-[2-isopropyl-6-methylpyrimidin-4-yl]-phosphorothioate) was selected as the internal standard. For sample extraction, a simple headspace solid-phase microextraction procedure was chosen after comparison with traditional liquid–liquid extraction procedures. Optimisation of sample preparation such as selection of fibre, temperature of sample, extraction times, adjustment of pH and addition of salts was also evaluated. The detection limit was found to be 50 ng/0.5 ml blood and recovery was 2.8% ± 0.6% at 200ng/0.5ml blood. Precision was found to be 9.9% (same day) and 3.6% (interday) at a concentration of 200ng/0.5ml blood. The technique was applied to real cases involving acute intoxication by fenthion.

Keywords: fenthion, organophosphate, acute intoxication, SPME, blood analysis

16.4. Determination of Benzodiazepines (free form) in Urine by Solid Phase Microextraction and Gas Chromatography with Nitrogen Phosphorus Detection

By Kamarruddin Asri and R.A.Anderson

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DETERMINATION OF BENZODIAZEPINES IN URINE BY SOLID PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY WITH NITROGEN PHOSPHORUS DETECTION.

Kamarruddin Asri, Robert A. Anderson, Department of Forensic Medicine and Science, University of Glasgow, Glasgow G12 8QQ, UK.

An analytical technique based on solid phase microextraction (SPME) was developed for the determination of benzodiazepines, especially diazepam, desmethyldiazepam, temazepam and oxazepam in urine. Each urine sample was diluted with water and adjusted to pH 6.0 ± 0.5 with the addition of 0.1 M NaOH. Saturated aqueous sodium chloride solution was added to increase the ionic strength of the sample. After conditioning and agitating the sample for 10 minutes, a fibre coated with polyacrylate (PA) from Supelco (Catalogue No. 57304) with manual holder was inserted and immersed in the urine matrix for another 30 minutes, with continuous magnetic stirring. The fibre with the extracted benzodiazepines was inserted into the heated injection port of a Hewlett Packard model 5890 series II gas chromatograph in splitless mode, and left for 5 minutes to desorb. Analytes were separated on an HP-1 capillary column and detected using a nitrogen/phosphorus detector. The method was shown to be reproducible and robust. When 2 ml urine samples were analysed, detection limits were in the range of 5-50 ng/ml. Reproducibilty at 100 ng/2 ml for each benzodiazepine was in the range 5.0-13.9% and recoveries at the same concentration were in the range 0.2-7.3%. The regression curve had a linear correlation coefficient of 0.980-0994 (n=5) over the range 0-100ng/2 ml and 100-500ng/2 ml. The method was applied successfully to some authentic case specimens.

Keywords: Urine, SPME, Benzodiazepines, Authentic case specimens