USE OF MULTI-WALLED CARBON NANOTUBES IN MATRIX SOLID PHASE DISPERSION EXTRACTION COMBINED WITH GAS CHROMATOGRAPHY

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ABSTRACT

USE OF MULTI-WALLED CARBON NANOTUBES IN MATRIX SOLID PHASE DISPERSION EXTRACTION COMBINED WITH GAS CHROMATOGRAPHY

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The use of Multi-Walled Carbon Nanotubes (MWCNT) as solid sorbent in Matrix Solid-Phase Dispersion (MSPD) extraction and preconcentration method was presented to determine some commonly used organophosphorus insecticides/OPIs in honey samples using a Gas Chromatography Flame Ionization Detector (GC-FID). OPIs are poisonous compounds used to kill insects and rodents by affecting their nervous system. The limit of detections obtained after MSPD extraction were 7.0 ng/g for Malathion, Malaoxon and Fenitrothion and 33.3 ng/g for Isomalathion. The recovery of the insecticides from spiked honey, ranged from 83.6% to 103.3% with % RSD ranged from 9.8% to 12.3% (n=3). The correlation coefficient (R^2) of the calibration data varied from 0.9945 to 0.9987. Standard addition method was utilized to examine matrix-induced effects on analyte peaks, and to demonstrate the efficiency of the method. The MSPD extraction was successfully applied for the analysis of four honey samples but no insecticide residues were detected.

Key words: Multi-Walled Carbon Nanotubes, Matrix Solid-Phase Dispersion, Flame Ionization Detector, Honey, Organophosphorus Insecticides

ÖZ

ÇOK DUVARLI KARBON NANO TÜPLERİNİN GAZ KROMATOGRİFİ İLE BİRLEŞTİRİLMİŞ MATRİKS KATI-FAZ DAĞILIM EKTRAKSYONUNDA KULLANIMI

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Danışman: Prof. Dr. Rüveyde Sezer Aygün

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Bal örneklerinde, bazı sıklıkla kullanılan Organik Fosforlu İnsektisitlerin (OFI'ler), Gaz Kromatografi - Alev İyonlaşma Dedektörü (GK-AID) ile tayini için gerekli özütleme ve önderiştirme yöntemi olan Matriks Katı-Faz Dispersiyon (MKFD) metodu ile birlikte kullanılmak üzere, Çok Duvarlı Karbon Nano Tüpleri (ÇDKNT), katı sorbent olarak önerilmektedir. Organik Fosforlu İnsektisitler, zararlı böcekleri ve kemirgenleri, sinir sistemlerini etkileyerek öldürmek için kullanılan zehirli bileşiklerdir. MKFD özütleme işlemi sonucunda elde edilen tayin edilme sınırları; Malathion, Malaoxon ve Fenitrothion için 7,0 ng/g, isomalathion için ise 33,3 ng/g olarak bulunmuştur. Bu insektisitlerin katıldıkları bal ortamından geri kazanımları, %83,8 dan %103,3 aralığında değişen değerlerde elde edilmiştir. Bu geri kazanım değerlerindeki hata, % Bağıl Standart Sapma (%BSS) olarak % 9,8 ile % 12,3 (n=3) aralığında bulunmuştur. Kalibrasyon verilerinin korelasyon katsayıları (R^2) 0,9945 ile 0,9987 arasında değişmektedir. Matriks ortamının, analit pikleri üzerinde yarattığı etkiyi incelemek ve yöntemin etkinliğini göstermek amacı ile standart katma metodu çalışılmıştır.

Matriks Katı Faz Dispersiyon özütleme tekniği, Çok Duvarlı Karbon Nano Tüpleri ile birlikte, dört gerçek bal örneğinin analizinde başarılı bir şekilde uygulanmış ve örneklerde hiçbir insektisit kalıntısı bulunmamıştır.

Anahtar Kelimeler: Çok Duvarlı Karbon Nano Tüpleri, Matriks Katı-Faz Dispersiyon, Alev İyonlaşma Dedektörü, Bal, Organik Fosforlu İnsektisitler

To My Family and Islamic Development Bank (IDB)

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

1.1 Structural and Physicochemical Properties of Organophosphorus Insecticides (OPIs)

Organophosphorus insecticides are esters of pentavalent phosphorous acids (Figure 1.1). They have four oxygen atoms arranged around the phosphorus atom, and one of the oxygen that is doubly bonded is replaced by sulphur in some organophosphorus compounds. The two **R** groups are usually methyl or ethyl, and **X** is frequently a rather complex aliphatic, homocyclic or heterocyclic group. The **X** group is often referred to as the **leaving group**, because the physicochemical dissimilarities and toxicity of OPIs depends on this leaving group [1].

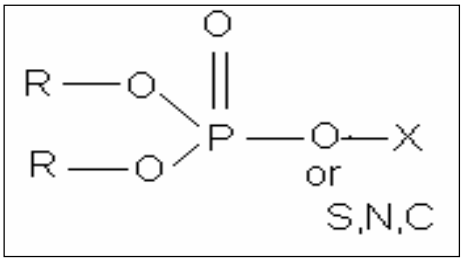
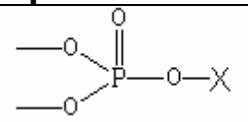
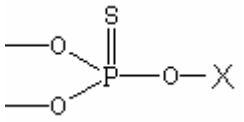
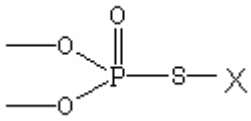
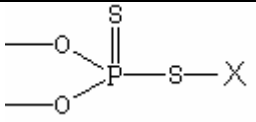
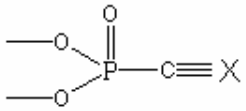
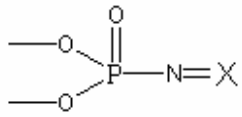


Figure 1.1 General structure of organophosphorus insecticides

Important groups of organophosphorus pesticides as shown in Table 1.1, give their systematic naming which partly depends on the atom (oxygen or sulphur) bonded to phosphorus via the double bond and on the atom (oxygen, sulphur, carbon or nitrogen) linking the leaving group to the phosphorus.

The structural variability of organophosphorus compounds accounts for both their physicochemical properties and their considerable biological properties upon which they differ in the mechanism they attack by enzymes [1]. They vary greatly in their physicochemical characters such as boiling point, vapor pressure, chemical stability in air, water (solubility), soil and their degree of toxicity. These variations are caused by the different leaving groups attached to the pentavalent phosphorus acid structure.

Table 1.1 Main chemical groups of organophosphorus insecticides (adapted from ref. 1)

Main group	**Structural representative**	**Examples**
Phosphates		Chlorfenvinphos, dichlorvos, mevinphos, phosphamidon
Thionphosphates		Bromophos, diazinon, fenitrothion, parathion, pirimiphos (methyl and ethyl)
Thiolphosphates		Oxydemeton-methyl, vamidothion
Dithiophosphates		Azinphos-methyl, dimethoate, disulfoton, malathion, menazon, phorate
Phosphonates		Trichlorphon, butonate
Pyrophosphoramides		Schradan

1.2 Agricultural Uses of Organophosphorus Insecticides

The wide spectrum of physicochemical and biological properties enables appropriate substances of the OPIs to possess a correspondingly wide range of uses in agriculture and in animal hygiene. Some are used as fumigants, others as contact poisons and yet others as systemic compound [1]. Some are used for crop protection early in the growing season, whereas some others are particularly valuable near harvest time [1]. Those used early in the season must be sufficiently persistent to avoid the necessity of multiple applications with its attendant increase in cost and effort. On the other hand, those used late in the season are specifically chosen because they do not persist very long; so that they can disappear sufficiently rapidly to ensure that negligible residues remain when the crop is harvested. Hence without the knowledge of these aforementioned time scale of insecticides and herbicides application by farmers and gardeners, lead to wrong timing application which can results in their distribution in the environment. Table 1.2 gives the uses for some organophosphorus compounds in crop production.

Table 1.2 Use of organophosphorus compounds against common pests of some representative crops (adapted from ref. 1)

Crop	**Pest**	**Chemicals**
Apples, pears, Some other top fruit	Aphids, Codling moth	Demeton-S-methyl, dichlorvos, dimethoate, fenitrothion, formothion, vamidothion Azinphos-methyl, Phosphamidon
Avocados	Soft brown scale thrips	Malathion
Bananas	Aphids, thrips, red spider	Malathion
Beans (several types)	Aphids Looper caterpillar	Malathion, demeton-S-methyl, dimethoate, disulfoton, menazon, mevinphos, formothion Dichlorvos
Cereals	Leatherjackets	Fenitrothion
Citrus	Mealy bug Mediterranean fruit fly Red scale	Malathion Parathion
Coffee	Antestis bug Leaf miner, stem borer	Parathion trichlorphon Parathion
Maize	Aphids Fruit fly, Stalk borer	Demeton-S-methyl PhorateTrichlorphon
Pea	Aphids, pea moth, thrips, weevils	Azinphos-methyl, fenitrothion

Table 1.2 (continued)

Potatoes	Aphids	oxydemeton-methyl
Stored grain	Weevils	Dichlorvos, malathion, pirimiphos-methyl
Sugar beet	Aphids (and virus)	As for potatoes; also mevinphos
Sweet potatoes	Leaf miners	Trichlorphon
Tobacco	Aphids Leaf miners	Dimethoate, malathion Phosphamidon
Tomatoes	American bollworm Aphids Red spider mite	Mevinphos Demeton-S-methyl, disulfoton, malathion, oxydemeton-methyl Formothion, oxydemeton-methyl
Vegetables (various, e.g. beetroot, carrot, onion, parsnip)	Mangold fly Aphids Carrot fly Celery fly Onion thrips	Dimethoate, formothion Demeton-S-methyl, oxydemeton-methyl, disulfoton Chlorfenvinphos, diazinon, phorate, disulfoton Malathion

1.3 Classification of Organophosphorus Insecticides

Organophosphorus insecticides are divided into five subgroups on the bases of their physicochemical properties and practical uses [1].

1.3.1 Low-Persistence Contact Poisons (*subgroup 1)*

These are compounds of low chemical stability. They are soluble in water and not readily hydrolysed in it. Their low hydrolytic stability precludes the possibility of long persistence in biological environment. They are used as contact insecticides. Some members of this group include mevinphos, tetraethyl pyrophosphate, tetrachlorvinphos etc.

1.3.2 Loco-Systemic/Persistence Contact Compounds *(subgroup 2)*

Substances of this group are less soluble in water but soluble in lipids. They have variable chemical stability and some of them can characteristically persist for few days or even few weeks after application. Their lipophilic nature enables them to enter into to the waxy cuticle of leaves and diffuse short distances from the point of original contact, sometimes traveling as far as the lower surface of the leaf. Once absorption has occurred, they are not readily removed by rain or sprinkle irrigation. Another characteristic of most member of this family is that they undergo oxidation before they reach their site of action in the nervous system of the insect.

Because their oxidation products are more polar than the parent molecule, they possess some systemic action even where the original molecule does not. Some members in this group are: malathion, methyl parathion, diazinon, fenitrothion and trichlorphon.

1.3.3 Systemic Insecticides *(subgroup 3)*

Compounds of this group have moderate to high chemical stability and their oil/water partition coefficients are such as to enable them both to enter in plants and to be translocated within them. Examples of some members of this group are: dimethoate, demeton-methyl, phorate, formothion and disulfoton.

1.3.4 Organophosphorus Compounds with a Fumigant Action *(subgroup 4)*

Compounds of this group have sufficiently high vapor pressure and low chemical stability that enable them to be used as Fumigants. e.g dichlorvos.

1.3.5 Organophosphorus Compounds Used Against Soil Organisms *(subgroup 5)*

Substances in this group are usually used to control soil organisms such as the carrot root fly larva which live in the soil or hatch from eggs laid on the soil surface and migrate to nearby crop plants. Examples of some members of this group are: chlorfenvinphos, phorate, diazinon and bromophos.

1.4 Adverse Effects of Organophosphorus Compounds

Organophosphorus pesticides are an integral part of agricultural production. They are used to improve farming and prevent disease transmission via pest. However their extensive use by farmers in crop fields, animal husbandry, home application and industrial application, can lead to unprecedented aggregation of them in environment. Moreover, the less persistent one can degrade into more toxic chemicals which may consequently, posed adverse health effects to higher animals including human beings [2]. They have varieties of symptoms such as depression, constant headache and feeling nausea. Which are prevailing sicknesses in our present societies, indicating the adverse effect of organophosphorus insecticides.

1.4.1 Mechanism of Toxicity of Organophosphorus Insecticides

All organophosphorus compounds share the same mechanism of toxicity, by inhibiting the action of acetylcholinesterase enzyme that is responsible of destroying neurotransmitter in nervous junctions.

During the action of nervous system, transmission of message from a nervous tissue to receptor organ is accomplished by neurotransmitters at nerve junctions. One of these neurotransmitters that conduct signals is called acetylcholine. During its action, the acetylcholine crosses the gap between the nerve tissue and receptor organ and then sends the signal to the receptor organ.

From being overwhelmed with acetylcholine or building up at the receptor organ, the acetylcholine broken down into its original components (acetyl and choline) as shown in Figure 1.2 by an enzyme called acetylcholinesterase (AchE) [3].

In the presence of organophosphorus insecticides in the nervous junction, the phosphate groups of the compound phosphorylate with the acetylcholinesterase enzyme and the product formed is relatively stable to hydrolysis. Thus, it deactivates the enzyme and therefore preventing the breakdown of acetylcholine [4].

The stability of the phosphorylate bond depends on the type of organophosphorus compound for either containing dimethyl phosphate or diethyl phosphate and the leaving group attached to phosphorus atom (P-X), which also depends on the electronegativity of the X group. In our study, two classes of organophosphorus: Phosphorothionates (e.g. fenitrothion) and Phosphorodithionates (e.g. malathion), which are relatively harmless chemicals [1, 3] were investigated. However, the body converts them to their corresponding oxygen analogs (oxons; fenitrothion-oxon and malaoxon respectively) which are the potent inhibitor of acetylcholinesterase enzyme [3].

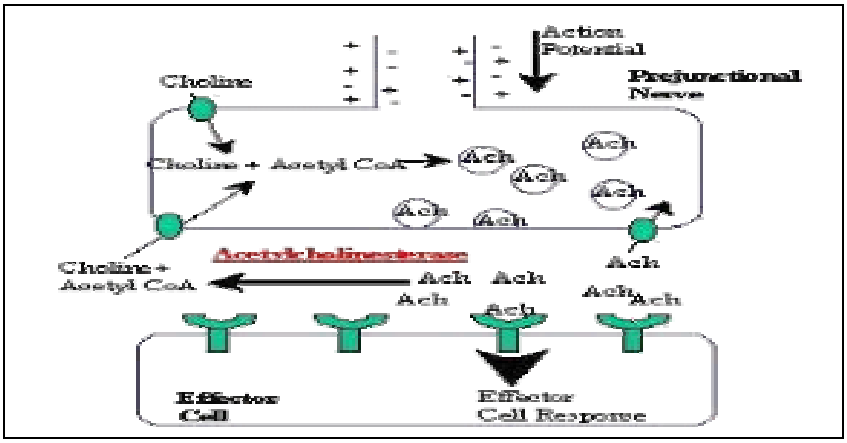


Figure 1.2 Transmission of signal by acetylcholine and its destruction by acetylcholinesterase between nervous junctions (adapted from ref. 3)

1.4.1.1 Malathion

Malathion is a nonsystemic broad-spectrum dithiophosphate with a **leaving group** of succinic acid ester S-$(CH.COOC_2H_5)_2$ (Figure 1.3). It has very low persistence in moist environment, and easily degrades to malaoxon. It is used in agriculture and regional pest eradication programs [5]. Malathion is suited for the control of sucking and chewing insects on fruits and vegetables, and is also used to control mosquitoes, flies, household insects, animal parasites (ectoparasites), and head and body lice.

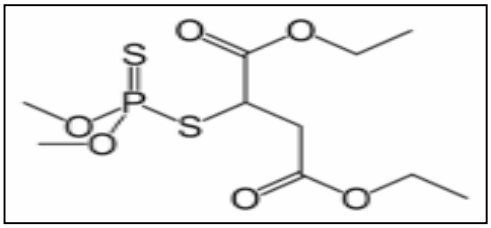


Figure 1.3 Structural representation of Malathion

1.4.1.2 Malaoxon

Malaoxon is the primary metabolic product of malathion and, under conditions of moisture or photolytic degradation, it is formed as an environmental breakdown product of malathion which makes it available for direct human exposure. It is more toxic than malathion, because the P=O group forms highly stable phosphorylate complex with the acetylcholinesterase enzyme than the P=S group (Figure 1.4) [5].

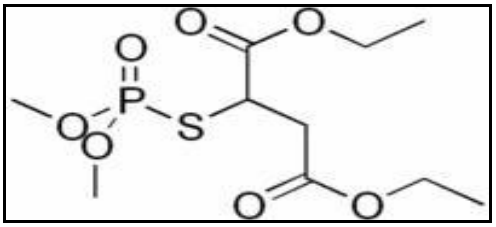


Figure 1.4 Structural representation of Malaoxon

1.4.1.3 Isomalathion

Isomalathion is one of the toxic by-product of malathion and found in both technical grade and end-use product of malathion (Figure 1.5). It is also more toxic than malathion and can improve its activity when present in malathion formulae [6]. The role of isomalathion to effect the toxicity of malathion, has been elucidated by Ryan and Fukuto (1985). These workers showed that when isomalathion was fed to rats in small amounts as a pre-treatment prior to administering malathion itself, it was found that less of the malathion was hydrolysed by carboxylesterases (an enzyme in vertebrates that breaks the carboxyl group of the compound) than occurred when pure Malathion was fed in otherwise identical circumstances [1].

These investigators concluded that isomalathion (which possesses a malaoxon-like **P═O** group), is a much more powerful inhibitor of carboxylesterase enzymes than is Malathion itself . This synergy was also observed from the degradation study of the insecticides in Section 3.6.

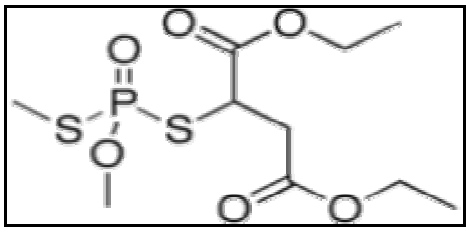


Figure 1.5 Structural representation of Isomalathion

1.4.1.4 Fenitrothion

Fenitrothion is a contact or *loco-systemic* organophosphorus insecticide. It is commonly used in the control of fly, mosquito, and cockroach residual contact spray for farms and public health programs. It is also used against flour beetles, grain beetles, and weevils. As shown in Figure 1.6 fenitrothion is a thionphosphate, in which the nitro toluene group attached to the oxygen atom.

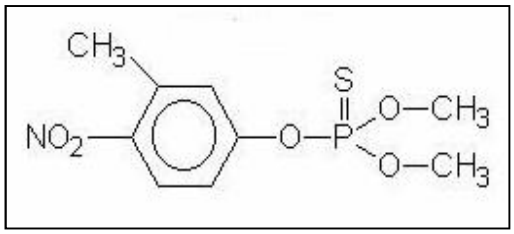


Figure 1.6 Structural representation of Fenitrothion

Some of the physical and biological characters of the organophosphorus insecticides used in this study are given in Table 1.3.

Table 1.3 The description and physical properties of malathion, malaoxon and isomalathion and fenitrothion (from ref. 6)

Compound	**Description**	**Boiling point** (^{0}C)	**LD_{50}** (mg/kg)
Malathion	Colorless or brownish-yellow liquid with garlic smell	156-157	800 to 1000
Malaoxon	Brownish yellow	145	26.2 to 39.2
Isomalathion	Yellow	150-156	89
Fenitrothion	Yellowish brown liquid	140-145	250 to 800

1.4.2 Acetylcholinesterase (AchtE) Activity

As mentioned before, the function of AchtE is to destroy acetylcholine back to its inactive precursor forms. During the action of the enzyme, Acetylcholine is splitted into choline and acetylated co-enzyme, which reacts rapidly with water to produce acetic acid that initiate the regeneration of acetylcholine (Figure 1.2) [3]. It has been calculated that AchtE enzyme can hydrolyze (break apart) 300,000 molecules of acetylcholine per minute at 37^{o}C [1]. It is the speed with which the enzyme goes back to its original form that allows it to interact with so many molecules of acetylcholine.

1.4.3 Acetylcholinesterase Inhibition

In the presence of organophosphorus pesticide, the phosphate group has higher affinity to the acetylcholine-esterase enzyme than the neurotransmitter. Thus, the pesticide phosphorylates the enzyme which is relatively stable to hydrolysis [1]. The enzyme is then inhibited from breaking the acetylcholine. Acetylcholine can then accumulate, causing overstimulation in the nervous system.

1.5 Organophosphorus Pesticide Poisoning

Organophosphorus pesticide poisoning can occur in two main ways namely, acute and chronic poisoning.

1.5.1 Acute Poisoning

Acute poisoning normally occurs shortly after the contact with a *single dose* of poisonous pesticide. Acute toxicity very often results from the disruption of an identifiable biochemical or physiological system of the victim and, in consequence, its responses are usually readily quantifiable [7]. A system used to measure acute toxicity is the 50 percent lethal dose, or $LD_{50;}$ (it is the amount of poison that kills half of the organism in a randomly chosen batch of a named species when applied in a particular way under stated experimental conditions). LD_{50} is usually expressed in terms of milligrams poison per kilogram body weight of the experimental animals (mg/kg) [1, 6].

When acute poisoning transpired, a variety of symptoms are possible because excess acetylcholine has deleterious effects at different types of receptors in both the autonomic and the central nervous systems. Symptoms of acute exposure to organophosphate or cholinesterase-inhibiting compounds include the following: numbness, tingling sensations, incoordination, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty in breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, convulsions or fatality [1, 3, 4, 6].

1.5.2 Chronic Poisoning

Chronic effects are defined as those symptoms which occur as a result of continued exposure to small and non-lethal doses of a poison but where no clinically observable acute symptoms had preceded them. People that are occupationally exposed to organophosphorus insecticides and the consumers from the accumulation of organophosphorus insecticides agricultural products are likely to encounter such chemicals quite often, and that mild symptoms of poisoning are difficult to quantify. Possible symptom of chronic poisoning is either mental acumen or reasoning capability [1].

1.6 Regulation and Tolerance Values of Organophosphorus Pesticides

In response to international and national concern about food quality, the United Nations established a commission called Codex Alimentarius Commission, the International Standard Organization (ISO) etc, and several national regulatory institutes in individual countries such as the United State Environment Protection Agency (EPA), the European Commission on Pesticides regulation, Turkish Standards Institution (TSE) and others, are also established to determine guidelines to safeguard the consumer and to issue certain recommendation [8].

The tolerance values usually expressed in terms of Maximum Residual Limit (MRL) is the tolerable amount of any toxic chemical in food. The MRL value for the UN Codex Alimentarius Commission for organophosphorus pesticides and veterinary metabolic products in animal products including honey, ranged from 0.05 to 0.5 mg/kg, indeed these values covers the highly toxic, moderately and less toxic chemicals where malathion and fenitrothion can be categorically based on their oxidation products. However in national levels the regulation of MRL values varies from region to region and the MRL value according to the Turkish food codex, Honey Bulletin (Bulletin No: 2005/49). The allowed limit of pesticides residue in honey is 0.01 mg/kg [9].

1.7 Contamination of Honey by Organophosphorus Insecticides

In the long term use of organophosphorus insecticides in agricultural production, the parent insecticides and their metabolic products accumulates in the fatty part of leaves and flowers. When bees come in contact with nectar and pollen from blossoms of these lands, the compounds can stick on their hair. Thus different pesticides can be introduced in the food chain by the bees through honey [10, 11], as Bees travel some distances and come in contact with nectar and pollen from blossoms in their beehives or in lands where pesticides were previously applied; therefore, the two principal routes of honey contamination with pesticides are shown in Fig. 1.7.

First: the direct contamination in which insecticides are directly used in beehives to control several pests and diseases.

Second: the use of these insecticides in agriculture can indirectly contaminate honey through pollen or nectar collected by bees in nearby treated fields [11].

Therefore, honey can indeed serve as a valuable and easily accessible environmental pollution indicator and biological indicator.

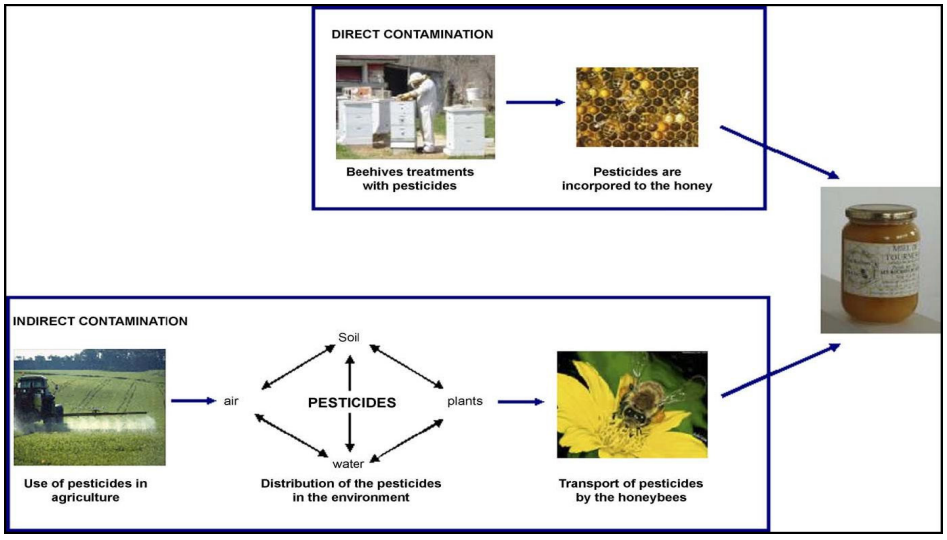


Figure 1.7 The direct and indirect contamination of pesticides in honey (adapted from ref. 11)

1.8 Composition and Analytical Methods for the Quality Control of Honey

Honey is a complex biological material that contains various components and this renders it possessing medical uses. Honey is primarily composed of sugars and water (79.6% sugar and 17.2% water). Honey also contains acids (0.57%), some protein (0.26%), a small amount of minerals (0.17%) such as calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. A number of other minor components including flavors pigments, hydroxymethylfurfural (HMF), and aroma substances, sugar alcohols and vitamins. The pH of honey may vary from approximately 3.2 to 4.5 (average pH= 3.9) which makes it inhospitable for attack by most bacteria [12].

Several techniques have been developed for the quality control and quality assurance of the composition of honey. These methods includes refractometry for moisture determination, HPLC and GC for HMF determination, sugars and protein determination, atomic spectroscopy for metallic elements determination and some other techniques [13]. However the contamination of honey by organic polluting substances can also change its quality and thus it becomes unsafe for consumption. The methods applied to determine these organic pollutants are usually chromatographic techniques [11].

1.8.1 Chromatographic Methods

Chromatography-based techniques are mainly used to identify and quantify pesticides residues in honey. As can be seen in Table 1.5, Gas Chromatography (GC) has been widely used for pesticides determination combined with the following detectors: (1) Electron capture detector (ECD) [14-18]; (2) nitrogen–phosphorus detector (NPD) [16-20]; (3) flame ionization detector (FID) [21]; (5) mass spectrometry detection (MSD) [20, 22-27]; (6) flame photometric detector (FPD) [28]; (7) thermoionic-specific detector (TSD) and pulsed flame photometric detection (PFPD) [29]. Most of them are specific detectors, for example ECD is used for compounds with electronegative atoms whilst NPD is used for compounds with nitrogen or phosphorus in their structures. However, some of them are non-specific detectors, like FID and MS Detectors. Moreover, MSD is a universal and non-specific detector that allows not only to detect and quantify the analytes, but also to identify these compounds on basis of their structure [11].

Some pesticide determinations by GC are not possible due to their low thermal stability and/or their insufficient volatility without further chemical derivatization. For such cases the alternative technique is the liquid chromatography. The most widely used HPLC detectors for pesticide analysis are diode array (DAD) [30-32,], UV [33-35], and MS detection employing Atmospheric Pressure Ionization (API), in positive and negative modes [30, 36, 37].

Table 1.4 Chromatographic methods used in pesticides determination

Techniques	**Pesticides**	**Limits of Detection**	**References**
GC-ECD	Trichlorofon, OCPs, Fluvalinate	3-0.003 mg/kg	14-18
HPLC-DAD	Acaricides	0.74-0.005 mg/kg	28, 30-32
HPLC-UV	Rotenone	0.015mg/kg	33-35
GC-MS	Chlordimeform, OCPs, OPPs bromopropylate	0.01-<0.003 mg/kg	18, 22-27
LC-APcI-MS	OPPs, OCPs, carbamates	0.5-<0.01 mg/kg	28, 34, 35
GC-FPD	OPPs	< 0.001 mg/kg	26
GC-NPD	OCPs, OPPs, coumaphos	<0.012 mg/kg	16-20
TLC	Atrazine, simazine	0.4-3.5 μg/spot	10

OCPs= Organochlorine Pesticides

OPPs= Organophosphorus Pesticides

1.8.2 Sample Preparation Methods

Sample pre-treatment is essential in the trace analysis of honey because of its complex matrix. During the last decades, different methodologies of sample handling have been proposed, these extraction on honey matrix include the following procedures: (i) solvent extraction (SE), (ii) supercritical fluid extraction (SFE), (iii) solid-phase extraction (SPE), (iv) matrix solid-phase dispersion (MSPD), (v) solid-phase microextraction (SPME) and (vi) stir bar sorptive extraction (SBSE). The percent recoveries of the extractions methods applied to honey are given in Table 1.6.

Table 1.5 Sample preparation methods applied in honey analysis

Method	**% Recovery**	**Reference**
Solvent Extraction (SE)	79-102	17, 19, 21, 31
Solid Phase Extraction (SPE)	63-98	16, 18, 21, 29, 35
Super Critical Fluid Extraction (SFE)	53-94	22, 34
Matrix Solid Phase Dispersion (MSPD)	80-113	20
Stir Bar Sorption Extraction (SBSE)	40-64	31
Solid Phase Micro Extraction (SPME)	81-115	36
Ultra Sonic extraction	92.3-94	10, 33

1.9 Gas Chromatography-Flame Ionization Detector (GC-FID)

Gas chromatography, specifically gas-liquid chromatography involves a sample being injected onto the injector port (head) of the chromatographic column and vaporized. The sample is then transported through the column by the flow of inert, gaseous mobile phase which is mostly argon or nitrogen or helium from the carrier gas tank, to the detector where its respond factor is displayed on data recording system as depicted in Figure 1.8 [38].

In this study, a newly arrived GC-FID as shown in Figure 1.9 was utilized to investigate the performance and efficiency of matrix solid-phase dispersion to extract some organophosphorus insecticides in honey sample, and the study of real honey samples. The detail part of the instrument are shown in Section 2.4

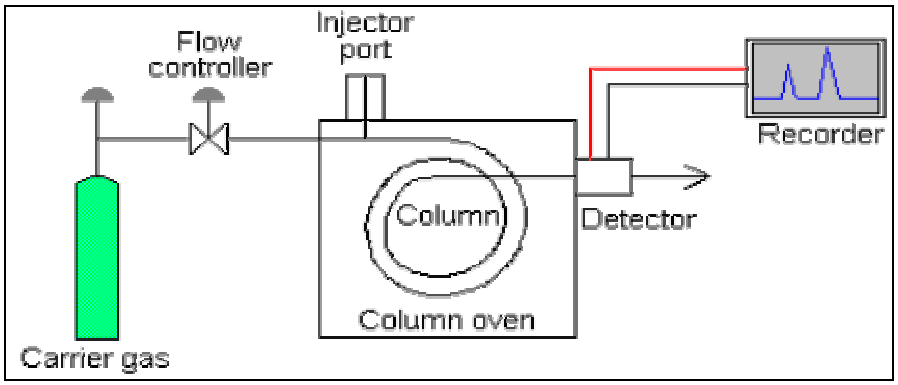


Figure 1.8 Schematic diagram of a gas chromatograph

Figure 1.9 Shimadzu gas chromatograph (GC-2010 Series A) with flame ionization detector used during the study

1.9.1 Columns

Gas-liquid chromatography is based upon the partition of the analyte between a gaseous mobile phase and a liquid phase immobilized on the surface of an inert solid. The columns system used in gas chromatography are open tubular or capillary and packed columns; capillary columns are of two basic types, namely, Wall-Coated Open Tubular (WCOT) and Support-Coated Open Tubular (SCOT).

Wall-coated columns are simply capillary tubes coated with a thin layer of the stationary phase. In support-coated open tubular columns, the inner surface of the capillary is lined with a thin film of a support material, such as diatomaceous earth. Generally, the efficiency of a SCOT column is less than that of a WCOT column but significantly greater than that of a packed column. Hence WCOT columns became more favorable column in the scientific community. At present WCOT columns are Fused-Silica Open Tubular columns (FSOT) (Figure 1.10).

Since the invention of FSOT columns in 1979, they had replaced the former WCOT glass columns types; this is because of their flexibility they can be bent into coils of few inches in diameter [39, 40].

In this research, the FSOT column, with the special features: length 30 m, 0.53 mm internal diameter and having a stationary phase of *95% dimethylpolysiloxane* and *5% diphenypolysiloxanel* with 5.0 µm film thickness was used for the separation of the organophosphorus insecticide analyte mixtures.

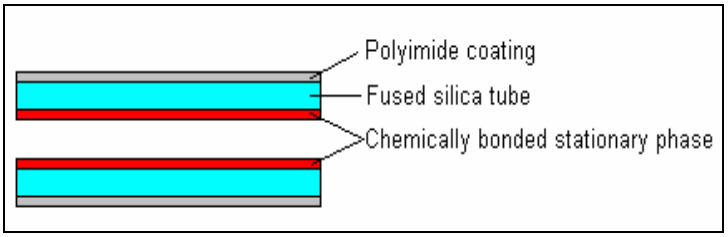


Figure 1.10 cross section of a fused silica open tubular column

1.9.2 Stationary Phase Composition: Polysiloxanes

Polysiloxanes are the most widely used stationary phases for packed and capillary-columns. They offer high solute diffusivities coupled with excellent chemical and thermal stabilities. The general representation of polysiloxanes is shown in Figure 1.11.

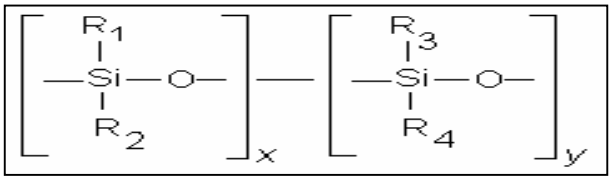


Figure 1.11 The general representation of polysiloxanes

Where the R-groups can be methyl, phenyl, $CH_2CH_2CF_3$, or $CH_2CH_2CH_2CN$, and X and Y indicate the percentage of an aggregate in the overall polymeric stationary phase composition. In our case, R_1 and R_2 are CH_3 groups and consist 95% of the polymeric composition, and R_3 and R_4 are phenyls consisting 5% in the polymeric material. Then it follows that, the stationary phase is non-polar [38, 40].

1.9.3 Flame Ionization Detector (FID)

The Flame Ionization Detector is a popular detector for the analysis of virtually all organic compounds; it has high sensitivity, a large linear response range, about 10^7, and low noise for hydrocarbons. It is also robust and easy to use, but unfortunately, it destroys the sample [40].

Principles of Operation

The FID consists of a small hydrogen-air diffusion flame burning at the end of a jet. The effluent from the column is mixed with hydrogen and air, and ignited, as can be seen in Figure 1.12. Organic compounds burning in the flame produce ions and electrons which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and the collector electrode is located above the flame and the current resulting from the pyrolysis of these organic compounds are measured. FID's are mass sensitive rather than concentration sensitive (responding to the number of carbon atoms entering the detector per unit time); this gives the advantage that changes in mobile phase flow rate do not affect the detector's response [39].

FID has very little response to functional groups such as N_2, H_2S, SO_3, NOx, NH_3, [40]. However, since the most organic compounds contain carbon atom, it means then FID can detect them from sample matrices if effective preconcentration method is applied. Therefore, in this study matrix solid-phase dispersion was used to preconcentrate the organophosphorus compounds.

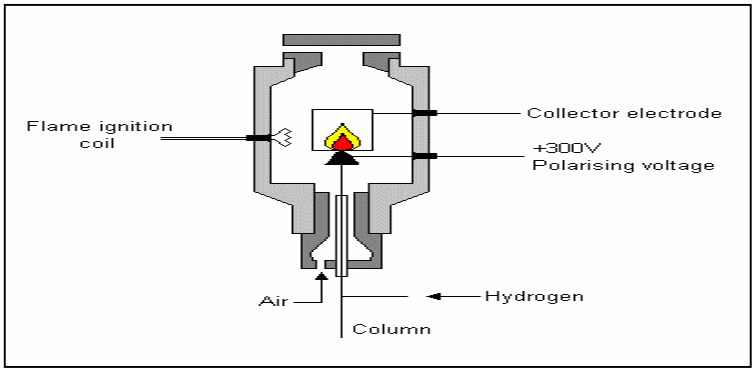


Figure 1.12 Schematic diagram of flame ionization detector (adapted from ref. 39)

1.10 Theory of Gas Chromatography Separation

Whether it is packed or open tubular, the column, which in the normal GC system is connected to the injector port of the gas chromatograph at one end and to the detector at the other Fig 1.8, is adjusted to some suitable temperature and continuously swept with the mobile phase (carrier gas). When a mixture of volatile components is introduced to the inlet end of the column, each solute in that sample engages in a highly dynamic equilibrated partitioning between the stationary phase and the mobile phase in accordance with their distribution constant, (*Kc)* [40].

$$Kc = C_S/C_M \quad (1.1)$$

Where:

C_s is the concentration of solute molecules in stationary phase,

C_M is the concentration of solute molecules in mobile phase.

If we consider a single band of solute at one point in time: as the solute molecules in the gas phase are swept forward by the carrier gas, those in the stationary phase are carried down the column at finite distance. At that instant, the equilibrium distribution is violated at the back of the band (where C_S is finite and C_M is zero) and at the front of the band (where C_S is zero and C_M is finite). To reestablish the distribution constant throughout the band, the dominant partitioning is from stationary phase to mobile phase at the rear of the band, and from mobile phase to stationary phase at the front of the band [39].

In other words, the flow of carrier gas (F) disrupts the equilibrium distribution at the front and rear of each chromatographing solute band, causing continuous evaporation at the rear and reestablishment at the front of each solute band as it chromatographs through the column (Fig. 1.13). Because all solutes are injected simultaneously, separation is obviously contingent on differences between the *Kc* values of the individual solutes. The proportion of a solute in the mobile phase at any given time is a function of the vapor pressure of that solute; molecules of those components exhibiting higher vapor pressures partition more toward the mobile phase. They are swept toward the detector more rapidly and are the first solutes eluted from the column [39].

Other solutes may exhibit lower vapor pressures, either because they are higher-boiling; example malathion or because they engage in interactions with the stationary phase that effectively reduce their vapor pressures under the chromatographic conditions employed.

Individual molecules of these solutes venture into the mobile phase (carrier gas) less frequently, their concentrations in the mobile phase are lower, and they require longer periods of time to reach the detector; hence separation is achieved [39, 40].

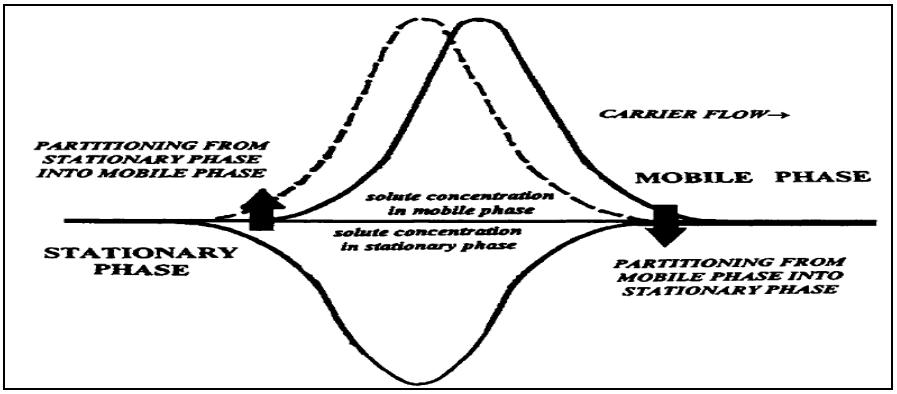


Figure 1.13 Partitioning of solute between mobile and stationary phases (adapted from ref. 39)

1.11 The Extraction Method

Some of the several methodologies of extractions used in sample preparation of solid and semi-solid matrices are shown in Section 1.8.2. As being a simple and effective matrix destruction method for viscous sample, the matrix solid-phase dispersion extraction encouraged us in the analysis of the organophosphorus insecticides in honey.

1.11.1 Matrix Solid-Phase Dispersion (MSPD)

MSPD is an extraction technique that is based on the principles of chemistry and physics which involve: (i) the mixing and interactions of the sample matrix and target analytes with a solid support bonded-phase or the surface chemistry of solid support materials. (ii) The use of force applied to the mixture of sample and sorbent material by mechanically blending them to produce a complete sample disruption and total distribution of target analytes on the surface of the sorbent material respectively.

The mixture, which then served as solid-phase material is filled in a column/cartridge to ready for elution with proper solvent(s). The sorbent has several functions: (1) works as abrasive compound breaking the physical structure of the sample, (2) adsorbs the compounds of the matrix, (3) it works as a solid support for filling the column and (4) allows the fractionation of the sample. Matrix solid phase dispersion (MSPD) has found particular application as an analytical process for the preparation, extraction and fractionation of solid, semi-solid and/or highly viscous biological samples [41].

Its simplicity, flexibility and minimum solvent consumed, have been cited as a contributing factor to being chosen over more classical methods such as Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE). The main difference between MSPD and SPE is that the sample is dispersed throughout the column and retained in not only the first few millimeters [42].

Since its introduction in 1989, matrix solid-phase dispersion (MSPD) has been cited as the extraction method employed in over 250 publications on complex biological, pharmaceutical and environmental matrices which are solid or semi-solid [43]. It has provided recoveries between 80% and 113% (see Table 1.6) and detection limits of 30 to 50 µg/kg [44]. These features attract and encourage us to apply it in the extraction and preconcentration of some organophosphorus insecticides from honey sample.

1.11.2 Factors to Consider in Performing MSPD extraction

Several factors that have been examined for their effect in conducting MSPD extractions include: (1) the effect of average particle size. As expected, very small particle sizes (3–10 µm) lead to extended solvent elution times and the need for excessive pressures or vacuum to obtain adequate flow. The appropriate particle sizes change from 40 to 100 µm. (2) Non-end-capped vs. end-capped materials or materials having a range of carbon loading (8–18%), (3) the character of the bonded-phase. Depending on the polarity of the phase chosen, rather dramatic effects on the results may be observed. Applications requiring a lipophilic bonded-phase may use octadecyl silyl (C-18) and octa silyl (C-8) materials interchangeably. (4) The use of underivatized silica or other solid supports, which will exercise thorough abrasion and sample disruption during the blending process [41]. However, the further disruptions of the sample and component dispersion will only occur to the degree that the components interact with the particulate surface with each other.

Silica-based support materials (derivatized silica, silica gel, sand, Florisil) have been almost exclusively reported for use in MSPD. Most methods reported to date use reversed-phase materials, such as octa silyl (C_8) and octadecyl silyl (C_{18})-bonded silica as the solid support. The lipophilic character of the reversed-phase materials is believed to facilitate disruption, dispersion and retention of lipophilic entities [42]. (5) The ratio of sample to solid support material; the most often applied ratio is 1 to 4, (6) Chemical modification of the matrix or matrix solid support blend; addition of chelating agents, or acids, or bases, etc at the time of blending affect the distribution and elution of target analytes from the sample. (7) The optimum choice of elution solvents and the sequence of their application to a column [43].

1.11.3 Types of Solid Sorbents Used in MSPD

The selectivity of an MSPD procedure depends on the sorbent/solvent combination used, several types of solid support has been applied; for example, silica treated with sulphuric acid has been used for fat removal and simultaneous selective MSPD of PCBs from pork, with limitation of the use of silica or Na_2SO_4 as drying agent being a main aim [44]. The extraction of atrazine and avoparcin, an antibiotic, using a polymer as the solid support 4 g of a 1:1 mixture of cross-linked methacrylate polymer (XAD-7) and diatomaceous earth were used for the extraction of atrazine from 0.5 g of beef kidney [45]. Sea sand was also used for the selective MSPD of xanthines and flavanones in root bark [46]. Neutral alumina has been found useful for extracting sulfonamides from chicken muscle [47].

In this study, we want to apply multi-walled carbon nanotubes in powder form as solid sorbent material for extraction and preconcentration of organophosphorus insecticides in honey sample.

1.11.4 Carbon Nanotubes

Carbon nanotubes (CNTs) are novel carbon materials which were first found in 1991 by S. Iijima [48]. Carbon nanotubes are made of perfect graphite sheets which are rolled up into a cylinder and closed by two caps; they are bristle in texture (Figure 1.14). CNTs have attracted much interest that was directed toward exploiting their unique thermal, mechanical, electronic and chemical properties. CNTs have the potential to extend their use in scanning probe microscopy, catalysis, field-effect transistors, hydrogen storage media, electrochemical sensor [49]. which is due to their unique electronic, metallic, and structural characteristics. They are divided into single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) according to the carbon atom layers in the wall of the nanotubes.

MWNTs were selected in this study because of its extremely large surface area and the multiple walls/layers with hexagonal arrays of carbon atoms in the graphite sheets surface provide strong interaction with many kinds of organic compounds. It is also the most commonly used carbon nanotubes in solid phase extractions [49].

Multi-walled carbon nanotubes have great analytical potential as an effective solid phase extraction adsorbent for chelates or ion pairs of metal ions, organic compounds and organometallic compounds [50]. It has been successfully used as the sorbent for the preconcentration and separation of dioxin, bisphenol and phthalate esters, to determine dichlorodiphenyltrichloroethane and its metabolites, and in the determination of ten different sulfonamides [49, 51].

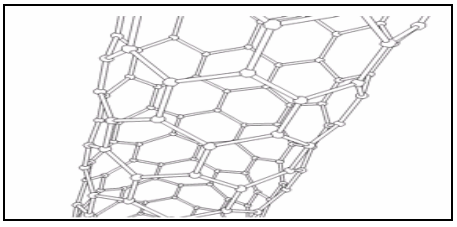


Figure 1.14 The hexagonal arrays of CNTs

From the facts mentioned above, MWNTs have a great potential as matrix solid-phase dispersant sorbent and can be used in the extraction and preconcentration of some organophosphorus insecticides in the analysis of complex honey samples.

For personal safety, in order to have protection from the dust of the powdered CNT, laboratory eye goggles, nose mask and gloves were used in the study during weighing and extraction procedures.

1.12 The Aim of the Study

The aim of the study was to apply a novel material namely: multi-walled carbon nanotubes as a sorbent material in matrix solid-phase dispersion, for the extraction and preconcentration of Malathion and Fenitrothion organophosphorus insecticides from honey sample using gas chromatography with flame ionization detector. Matrix solid-phase dispersion is an extraction and analyte preconcentration technique that had been applied for the trace analysis of solid and semi-solid biological and environmental samples. However, since its introduction, several types of sorbent materials had been utilized (see Section 1.11.3), but to our knowledge, multi-walled carbon nanotubes have never been applied.

CHAPTER 2

EXPERIMENTAL

2.1 Reagents and Chemicals

Acetone, n-hexane, acetonitrile, ethylacetate and dichloro-methane used in the experiments were HPLC grade (J.T Baker, Holland). The insecticide standards: fenitrothion and isomalathion were purchased from LGC Promochem (Germany) and malathion and malaoxon standards were purchased from Referans Kimya (Ankara, Turkey), and all were 99.9% pure.

Anhydrous sodium sulphate (J.T. Baker, Holland) and silicagel 60 (0.063-0.200 mm) (MERCK, Germany), and Multi-Walled Carbon Nanotubes (MWCNT) (Nanocyl 7000) purchased from Nanocyl, S.A. Belgium. Glass columns for extraction have been prepared by the glass blowing workshop in the Department of Chemistry and were 18.0 cm long and 1.0 cm in internal diameter and the polyethylene frits (6Ml) (SUPELCO, USA) were placed at the bottom. Millipore membrane filters of pore sizes 0.20 and 0.45 µm (Corporation Bedford UK) were tested for filtering the impurities.

2.2 Preparation of Standards

The standard solutions of each insecticide were prepared by dissolving 20.0 mg of each compound in 50.0 mL of acetone so that a stock solution of 400.0 µg/mL is obtained. They were stored at 4 ^{0}C in a refrigerator.

Individual standard working solutions were also prepared in acetone from the stock solutions daily and were used to identify retention times of analytes. A mixture of the insecticide standards was prepared in 100-mL volumetric flask at concentration of 100.0 µg/mL from which working mixture of the insecticide standard solutions ranged from 1.0 to 7.0 µg/mL were also prepared in acetone. They were used to determine the resolution, precision and sensitivity of the instrument. All the preparations for the standards were done under a well ventilated fume hood.

2.3 Washing of Glasswares

The glassware were always washed a day before use. They were first washed with detergent in hot water and then rinsed with hot water again. After that, they were rinsed with tap water for three times and finally rinsed with distilled water for two or three times. Following washing, all the apparatus were dried in closed box so that no dust or flying particles will come in contact.

In every working day, before dilution and extraction, the glassware and MSPD columns were rinsed with *n*-hexane which is then followed by acetone.

2.4 Instrumental Conditions

Chromatographic analyses were carried out using a SHIMADZU GC-2010 series A equipped with flame ionization detector and a capillary column (30 m, 0.53 mm i.d., 5 μm film thickness) with a stationary phase of 95% dimethylpolysiloxane and 5% diphenylpolysiloxane. Helium (99.999% pure) was used as the carrier gas. Dry air (99.9% pure) and hydrogen (99.999% pure) were used as detector gases at flow rates of 400 and 40 mL/min respectively.

2.5 Sample

Honey samples with different trade marks, collected from different regions of Turkey, were purchased from markets. They were stored at 4 ^{0}C in a refrigerator. Before preparation for the extraction, honey sample was placed in an oven for 5 min at 45 ^{0}C, so that its moisture content and viscosity are reduced.

2.6 Sample Preparation

2.6.1 Apparatus Used in Extraction Method

Matrix Solid Phase Dispersion extraction was performed with a laboratory made glass column of 18.0 cm length and 1.0 cm internal diameter in which a polyethylene frit was inserted at the end in order to support solid materials (sorbent and co-sorbent).

The agate mortar and pestle were used to blend the solid sorbent and the honey sample. During the process, eye goggles nose mask and gloves were used for personal safety, especially when working with MWCNT powder.

The solid phase material was multi-walled carbon nanotubes in the powder form, with specification shown in Table 2.1. Silica gel and anhydrous sodium sulphate were used as clean-up co-sorbent and drying agent respectively. The organic solvents; n-hexane, acetone, acetonitrile, dichloromethane and ethyl acetate, were used as proper eluting solvents. A linear peristaltic pump was used to extract the column content.

Table 2.1 Characteristics of the MWCNT (Nanocyl-7000)

Property	**Value**
Average Diameter	9.5 nm
Average Length	1.5 μm
Carbon Purity	90%
Metal Oxide (impurity)	10%
Surface Area	250-300 m^2/g

2.6.2 Procedure of MSPD Extraction

MSPD extraction was performed as shown in Figure 2.2 in the following procedures. An amount of 50.0 mg of the honey sample was spiked with 10.0 μL of the insecticide standards mixture in agate mortar, then 200.0 mg carbon nanotube powder were added and the mixture was thoroughly blended with an agate pestle for 1.0 min.

During blending, the sample matrices are fragmented and the analytes are released and then trapped within the hexagonal layers of the carbon nanotubes.

The mixture then was loaded in to a glass column containing a frit at the bottom, 3.0 g of silica gel and 50.0 mg of anhydrous sodium sulphate. The frit at the bottom provides support to the sorbent (WWCNT) the clean-up sorbent (silica gel) and the drying sorbent (anhydrous sodium sulphate). A second frit was placed over the dispersed sample with slight compression as shown in Figure 2.1. The analytes were eluted from the cartridges with 20.0 mL n-hexane–acetone (8:2) mixture. The eluate was then evaporated to 1.0 mL with a stream of nitrogen under a fume hood. It was then filtered through a 0.20 µm membrane filter and injected into the GC-FID system which was already optimized as stated in Section 2.4. The optimization process and the optimized conditions for GC-FID system are given in Section 3.1 and Table 3.1.

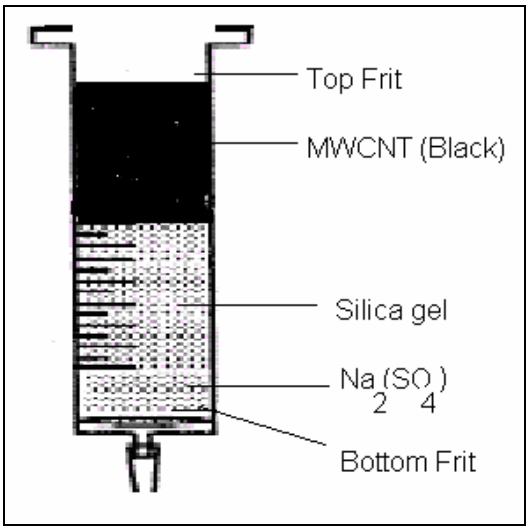


Figure 2.1 The loaded glass column ready for elution

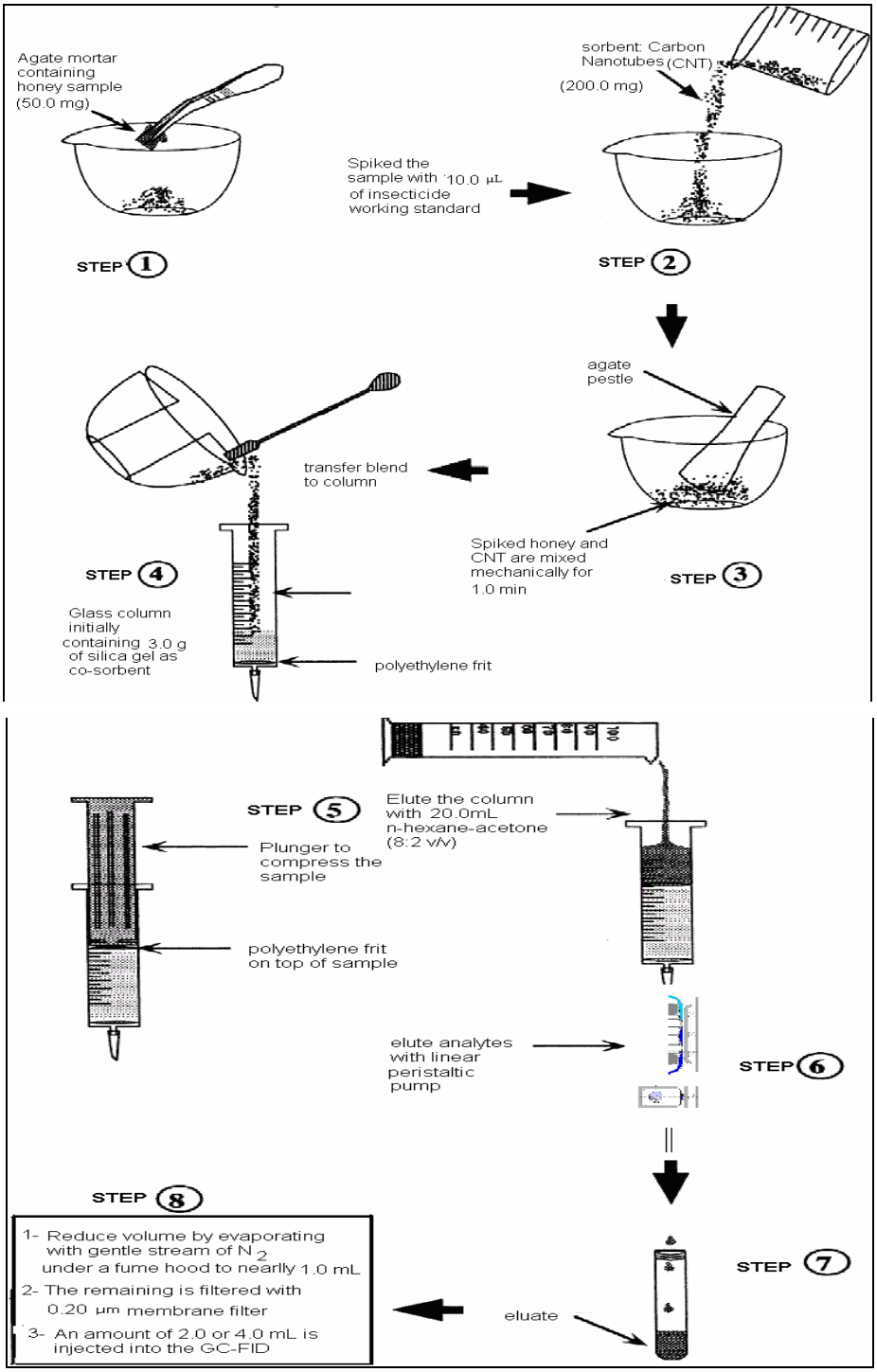


Figure 2.2 The extraction, and preconcentration step in MSPD sample preparation procedure (adapted from ref. 41).

2.7 Standard Addition Method

A standard addition (SA) method was used for the quantification of matrix effect on the analyte peaks and to evaluate the efficiency of the silica gel clean-up co-sorbent for removing interferences.

MSPD extraction, mentioned above, was carried out without spiking the honey sample with insecticides standards. The eluates were then filtered through the 0.20 µm membrane filter and then divided into four aliquots of equal volume. Working mixture of the insecticide standards were added to three of the aliquots in increasing order of concentration, and the fourth one was without spiking (it is the controlling blank aliquot). They were all diluted to final volume of 10.0 mL with acetone. The blank aliquot with zero analyte was first subjected to analysis and no peak was observed in its chromatogram. However, when the other three aliquots were injected, the peaks for analytes were observed in the chromatograms. The comparison of the five standard calibration curve to that of calibration curve obtained by standard addition method, for any insecticide standard, indicated that there is significant matrix induced effect as shown in Figures 2.3- 2.6, as expected.

The curves were plotted for the ranged of the smallest detectable concentration at 5.0 µg/mL to 15.0 µg/mL and they were linear within this range and the curves of standard addition have similar linearity, however the negative intercepts in Figures 2.4 and 2.6 could not be explained.

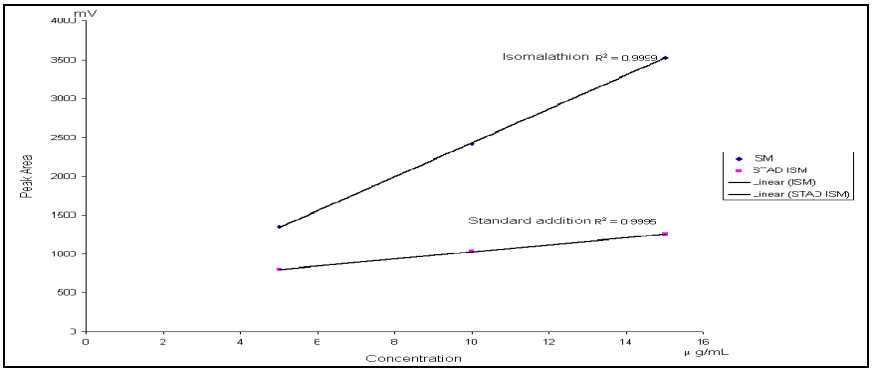


Figure 2.3 Comparison of insecticide standard calibration curve and standard addition method calibration curve of Isomalathion (n=3)

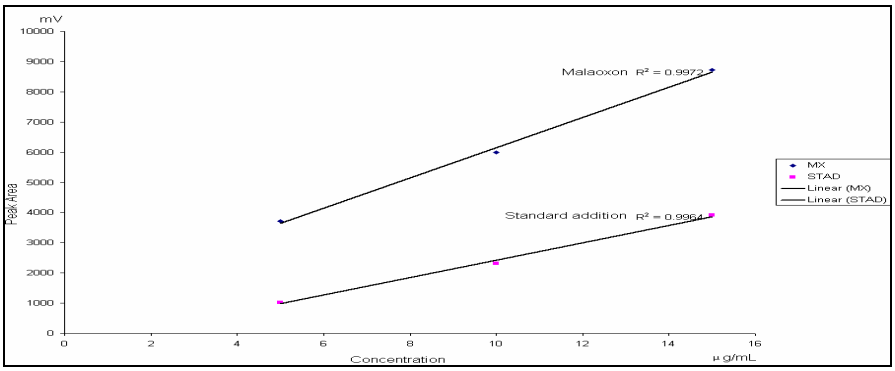


Figure 2.4 Comparison of insecticide standard calibration curve and standard addition method calibration curve for Malaoxon (n=3)

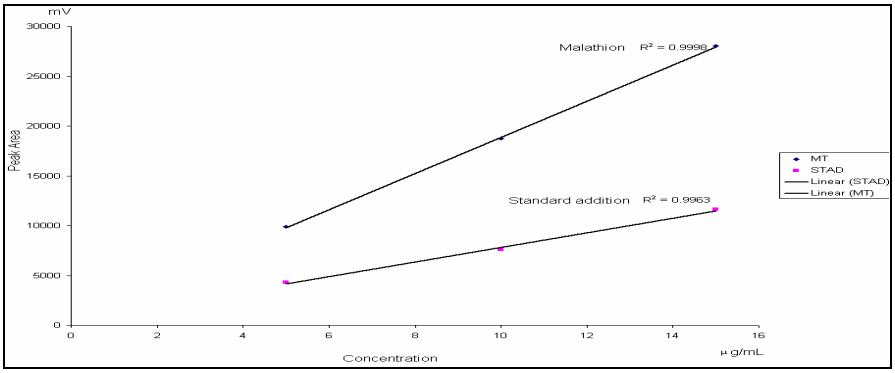


Figure 2.5 Comparison of pure insecticide standard calibration curve and standard addition method calibration curve for Malathion (n=3)

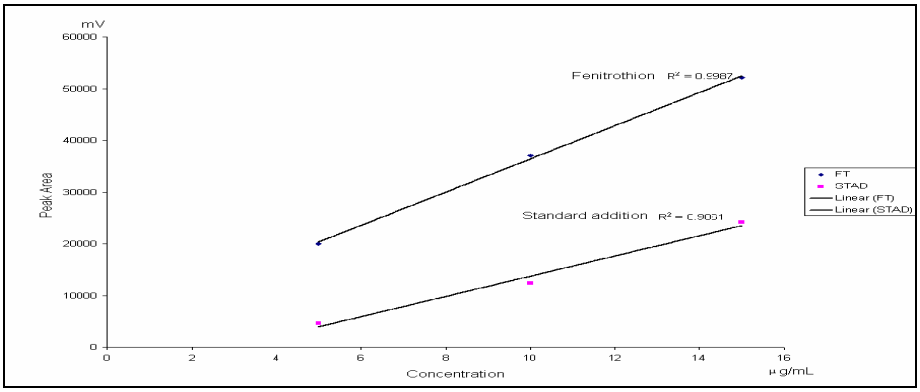


Figure 2.6 Comparison of pure insecticide standard calibration curve and standard addition method calibration curve for Fenitrothion (n=3)

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization of GC-FID Parameters

Optimization of gas a chromatographic separation requires careful attention to number of important variables such as column pressure, total flow of carrier gas, solute-stationary-phase interaction, temperature of column, retention times and resolution of peaks and their interaction to one another [40]. In the GC-FID system, such variables as column pressure and column temperature affect the retention time and resolution of peaks. For instance, increasing the column pressure increases the total flow rate of the carrier-gas and decreases the retention times of the peaks but reduces the resolution of peaks (as the case of isomalathion and malathion). The solute-stationary phase interactions mechanisms, such as dispersion, dipole, acid/base, and hydrogen bond donors/acceptors affects the retention time. For example, a column containing a polar stationary phase displays greater retention for a solute having polar functional group, while on non polar stationary phase (which is our case) nonpolar solutes are retained more [40]. That is why, fenitrothion which is more non polar with solubility in water of 21.0 mg/L (at 20 ^{0}C) than other insecticides, displayed greater retention time (21 min) in the nonpolar stationary phase.

In this study, six variable (except the stationary phase) operational parameters of the instrument which are: column temperature, column vapor pressure, total flow rate of the carrier gas, injector and detector temperatures, and volume of injection were examined. Their effects in the separation of organophosphorus insecticide peaks are discussed below.

3.1.1 Preliminary Study

In the preliminary study to optimize the operational parameters of the GC-FID (Section 3.1), the temperature-program used in a previous study was utilized [52]. First the retention time for the solvent peak were examined by conducting solvent analysis and the retention times of the individual insecticide peaks were determined by injecting each analyte standard individually. In this study the following instrumental conditions which were adapted from the reference. The temperature program was: initial temperature 150 ^{0}C hold time 3 min then rose to 250 ^{0}C with a rate of 10 ^{0}C/min and hold up time of 10 min. injection port at 250 ^{0}C and detector temperature at 260 ^{0}C. The initial chromatograms obtained under these conditions are shown in Figures 3.1 to 3.4.

It should be noted that, in every daily experiment, the column is first cleaned before analysis by raising the column temperature to 260 ^{0}C and kept at this temperature for at least 24 min (analysis time) without injecting any samples.

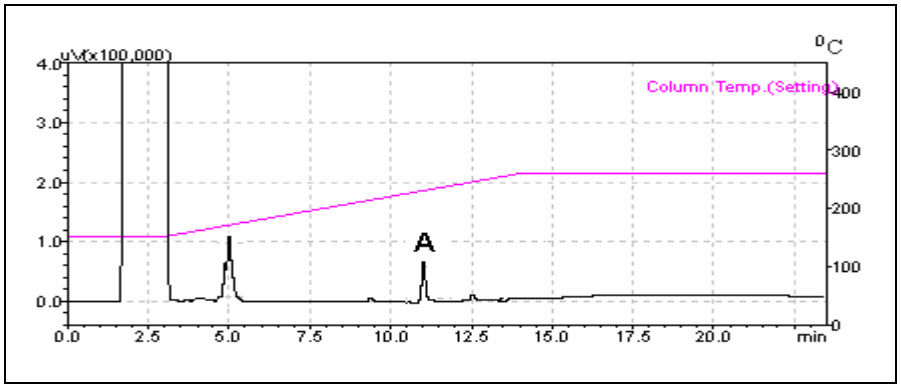


Figure 3.1 Malaoxon peak (A) at retention time of 11.24 min ± 0.02 (n=5)

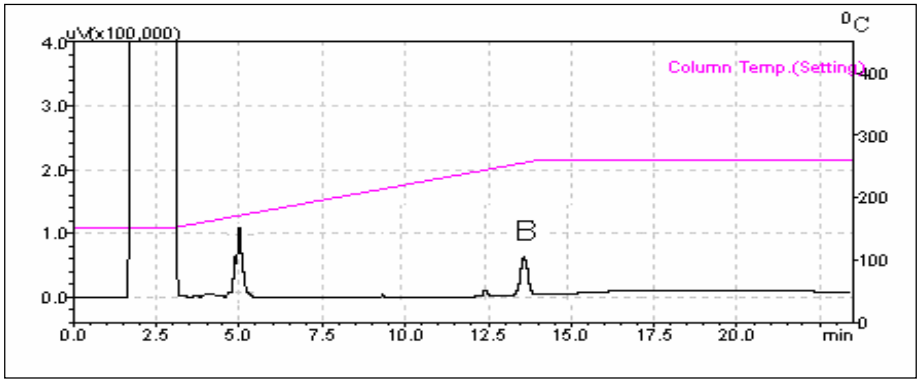


Figure 3.2 Isomalathion peak (B) at retention time of 13.31 min ± 0.03 (n=5)

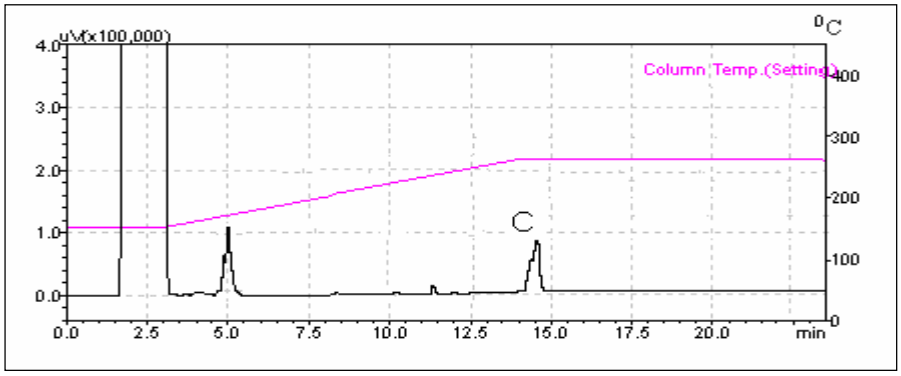


Figure 3.3 Malathion peak (C) at retention time of 14.52 min ± 0.02 (n=5)

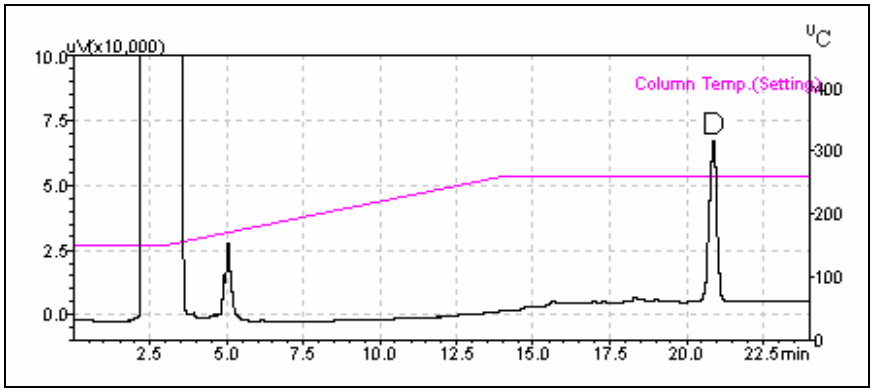


Figure 3.4 Fenitrothion peak (D) at retention time of 21.28 min ± 0.24 (n=5)

3.1.2 The Influence of Column Temperature

The column temperature affects the thermodynamic partitioning process of the analytes between the mobile gas phase and the coated substances of the stationary phase; and the partition coefficients of the analytes (Section 1.10) which are also influenced by solute-column specific interactions such as polarizability and hydrogen bonding [40]. On the other hand, thermodynamic partitioning has also being controlled kinetically by the rate of exchange of solute between stationary phase and mobile phase. These two chromatographic processes which are subjected to change with temperature give rise to differential change on analyte relative retention times.

Initially, the initial temperature of 100 to 200 ^{0}C was scanned and 150 ^{0}C was chosen in order to separate the solvent peak coming very close to malaoxon peak. The final temperature was set to 260 ^{0}C so that large range of temperature could be scanned as shown in Table 3.1.

In order to decide about ultimate temperature program, several initial and final hold up times were tried. The optimized temperature program was: initial temperature of 150 ^{0}C held isothermally for 3.0 min and then raised to final temperature of 260 ^{0}C at a rate of 10 ^{0}C/min and held isothermally for 9.0 min completing the analysis within 23.0 min.

3.1.3 Effect of Column Pressure

In the gas chromatography with flame ionization detector, the influence of column pressure overrides the influence of total flow rate of carrier gas. The pressure affects the retention time of the analyte peaks in inverse proportion. The choice of optimum pressure for the quantitative determination of the organophosphorus insecticides in honey sample was based on the reproducibility of the peak retention times and good resolution.

During the pressure optimization, the pressure was varied from 15 to 30 kPa, as shown in Figure 3.5; increasing the pressure of the column, decreased the retention time of the analytes. Although there was little change of retention time with increase in pressure from 15 to 22.5 kPa, but after this value the reproducibility of the analyte peaks at the specific retention times were very poor with percent RSD varying from 15 to 19%. Hence, based on the reproducibility of the retention times, the pressure 20 kPa was selected as the optimum value.

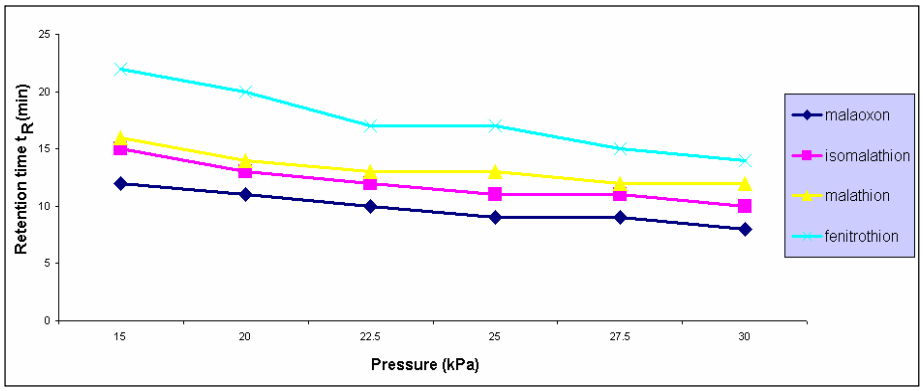


Figure 3.5 The influence of column pressure on peak retention time of organophosphorus insecticides (n=3), using the optimize column temperature program

3.1.4 Total Flow Rate of Carrier Gas

The flow rate (F) of the carrier gas has little effect on the retention time and peak area of the analytes and indeed small impact on the sensitivity of the detector. The effect of the carrier gas flow rate was examined by changing it from 20 to 200 mL/min. When the flow rate was increased from 20 to 100 mL/min, the peak areas and retention times of all the analytes remained constant. Even increasing it from 100 to 200 mL/min with the increments of 50 mL/min, no significant changed in the peaks width and retention times of the analytes were observed as shown in Figure 3.6.

Since flow rate of the carrier gas offered no significant effect on the resolution and retention times, column pressure was more important. Because as observed in this study, changing the pressure will directly change the flow rate, however changing flow rate, does not affect pressure significantly.

So the optimum column pressure was set to 20 kPa, while the total flow rate was automatically adjusted to 15.1 mL/min, controlled by the instrument.

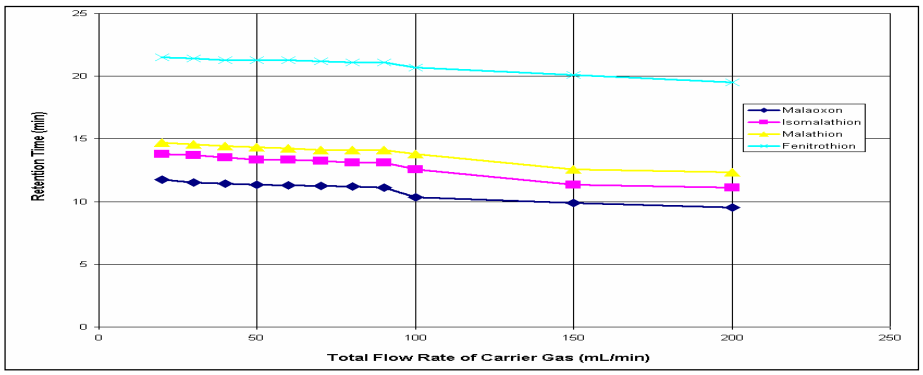


Figure 3.6 The effect of total flow rate of carrier gas on retention of analytes, using the optimized column temperature program

3.1.5 Effect of Injector Port Temperature

Gas chromatography is related with volatility phenomenon that involves the vaporization of the sample followed by the downstream carry-over of it by the carrier gas through the column. The initial encounter of high temperature at the injector port is very important to vaporize the insecticides and thus diffuse through the column.

During the optimization of the injector temperature, the temperature was varied from 100 ^{0}C to 250 ^{0}C. At 100 ^{0}C no analyte peaks could be observed in the chromatogram, but as the temperature was increased to 150 ^{0}C, they started to appear except a peak for fenitrothion; although their peak areas were relatively small.

When the temperature was increased to 200 ^{0}C, peak areas were also increased and fenitrothion peak was observed but its retention time was not reproducible with so that % RSD ranged from 12.3 to 19.0. However by adjusting the temperature to 250 ^{0}C, better reproducibilities that ranged from 2.4 to 6.3% were obtained for the retention times for all the analytes.

3.1.6 Effect of Detector Temperature

The detector temperature affects very little to the reproducibility of the peak areas of analytes except fenitrothion. As can be seen from the graph in Figure 3.7, from 260 ^{0}C to 270 ^{0}C, there is no change in the peak areas of analytes, but as temperature increases from 270 to 300 ^{0}C, the response of the analytes varies greatly in fenitrothion. At the temperatures 290 ^{0}C and 300 ^{0}C, the reproducibility in the analyte peak retention times was very poor for fenitrothion with % RSD value of 10 to 14%. Therefore 270 ^{0}C was selected as the optimum value for the detector temperature.

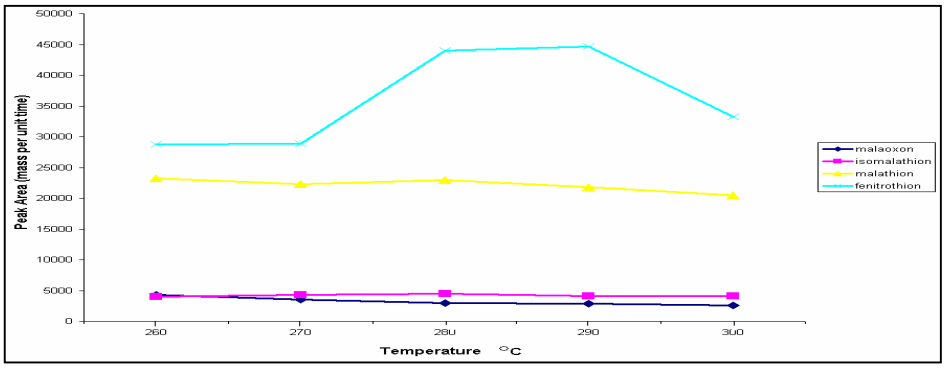


Figure 3.7 The effect of detector temperature on peak area (n=3), using the optimized column temperature program

3.1.7 The Effect of Injection Volume

The peak areas of the analyte are directly proportional to the volume (µL) of sample introduced into the injection port of GC-FID system. Volumes of injection from 1.0 to 5.0 µL were examined. Clean chromatograms were obtained when 1.0 and 2.0 µL were injected, but the peak areas of the analytes were small.

However when an amount of 3.0 to 5.0 µL, were used there were some other unresolved peaks coming close to the malathion peak and several unidentified peaks have also appeared, probably due to some trace impurities coming from standard chemicals. Results are shown in Figure 3.8.

It was observed that when low concentration of the analyte such as 0.50 µg/mL was needed to be injected 4.0 µL of volume should be used to observe the analyte peaks. Therefore, although in some cases 2.0 µL volumes are used, the optimum volume of injection was selected as 4.0 µL.

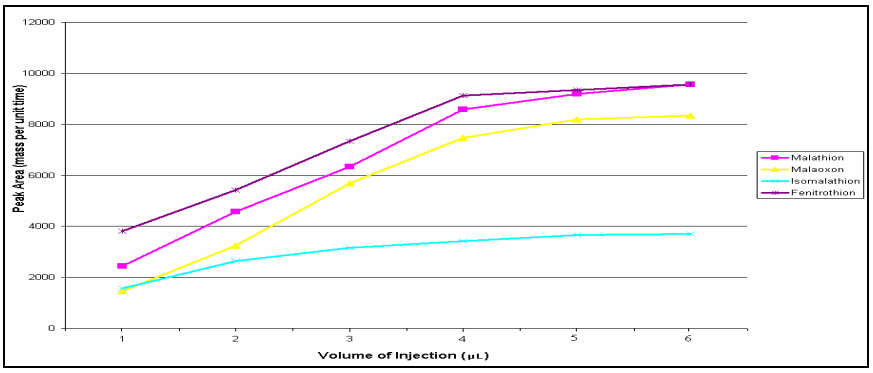


Figure 3.8 The effect of volume of injection to peak area (n=3), using the optimized temperature program

Therefore by using the adjusted temperature programming as summarized in Table 3.1, and under the other optimum conditions, malaoxon eluted first, followed by isomalathion, malathion and then fenitrothion as their retention times given at Table 3.2.

Table 3.1 Selected parameters of the GC-FID

Parameter	**Observed Conditions**	**Selected Condition**
Column Temperature Programming	Initial 100 ^{0}C to 200^{0}C Hold 1 min and 4 min Final: 260 ^{0}C Hold 9 min and 10 min	Initial: 150 ^{0}C, 3 min rate of 10 ^{0}C/min to Final:260 ^{0}C ,9min
Column Pressure	15 - 30 kPa	20 kPa
Detector Temperature	260 - 300 ^{0}C	270 ^{0}C
Injector Temperature	100 - 250 ^{0}C	250 ^{0}C100
Injection Volume	2.0 and 4.0 µL	1.0 – 4.0 µL
Analysis	Split and Splitless	Splitless
Carrier Gas	-	Helium, 15 mL/min
Makeup Gas	From manual	Helium, 30 mL/min
Fuel	From manual	Hydrogen gas, 40 mL/min
Oxidant	From manual	Dry Air, 400 mL/min

The typical chromatogram obtained under these selected instrumental conditions for the analyte mixture each in … mg/mL is shown in Figure 3.9.

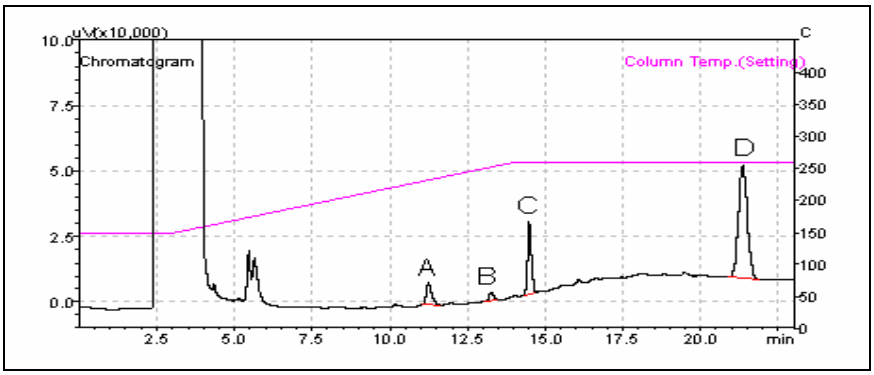


Figure 3.9 The chromatogram obtained using the optimum parameters, A-malaoxon, B-isomalathion, C-malathion, D-fenitrothion

Table 3.2 The precise retention times of the organophosphorus insecticides (n=5)

Organophosphorus Insecticides	**Retention time (min)**
Malaoxon	11.40 ±0.02
Isomalathion	13.31 ±0.03
Malathion	14.94 ±0.02
Fenitrothion	21.29 ±0.24

3.2 Analytical Figures of Merit for Gas Chromatography Method

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. Since gas chromatographic methods are used for different purposes, the method validations may also be different [40]. For example, several publications outline guides to validate methods for pharmaceuticals, pollutants etc. methods such as the United States Pharmacopoeia (USP) [53], International Conference on Harmonization (ICP) [54], and the Food and Drug Administration (FDA) [55-57]. In general, methods must include studies on selectivity, linearity, accuracy, precision, dynamic range of response, limit of detection (LOD), limit of quantification (LOQ), and repeatability.

In the initial stage of the study the analytical figures of merit for the instrument with optimized parameters were examined with working standard mixture of the insecticides and these are Limits of Detection (LOD), Limits of Quantification (LOQ), Precision and Linearity of response of instrument (Table 3.3).

Concentrations of the insecticides standard from 1.0 µg/mL to 30.0 µg/mL were prepared in acetone. Five times injection were carried out for each concentration and the volume of injection was 4.0 µL starting from 1.0 µg/mL. As shown in Table 3.3 and Figure 3.10 there was linearity of response from the instrument as the concentration increases from 1.0 to 30.0 µg/mL.

The detection limit is defined as the lowest analytical concentration that yields a signal-to-noise of at least 3:1 (S/N of 3) and it was experimentally determine as given Section 3. 2.1. The limit of quantification (LOQ) is estimated as the concentration of analyte which yields a signal-to-noise of at least 10 and the values are given in Table 3.3.

Table 3.3 The analytical figures of merit of the GC-FID system for the insecticides standard mixture in acetone

Compounds	LOD (µg/mL)	LOQ (µg/mL)	R^2	% RSD (n=5)
Malaoxon	0.17	1.8	0.9993	±4.3
Malathion	0.17	1.8	0.9963	±5.6
Fenitrothion	0.17	1.8	0.9967	±7.1
Isomalathion	0.33	3.3	0.9910	±3.4

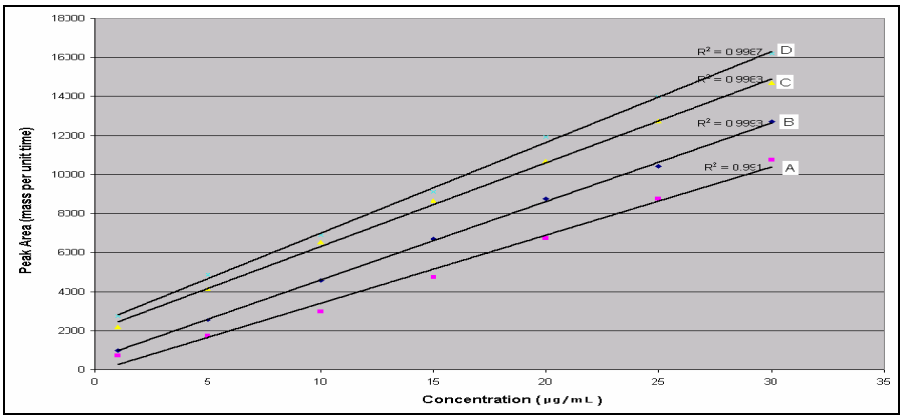


Figure 3.10 The linear calibration of insecticide standards in pure solvent, A- Isomalathion, B- Malaoxon, C- Malathion and D- Fenitrothion

3.2.1 Determination of Instrument Detection Limit for Optimized Parameters

In chromatography systems the instrument detection limit is defined as the lowest analytical concentration that yields a signal-to-noise of at least 3:1 (S/N of 3) [38, 40].

Although the insecticides shared common atoms such as phosphorus and oxygen, their limit of detection by the GC-FID system depends on the whole individual compound. This is because of their dissimilarities of physicochemical properties.
The instrument detection limit was examined by first injecting high concentration of the analyte mixture of 10.0 µg/mL into the instrument. The large peak areas showed that lower concentration could be used. Another three working solutions containing mixture of the insecticide with concentration of 0.50, 1.0 and 5.0 µg/mL were then prepared. They were injected starting with the highest concentration again. The peaks for malathion, malaoxon and fenitrothion were seen, except for isomalathion at the lowest concentration. The concentrations of the three insecticides were further decreased to 0.10 µg/mL, but this time the peaks disappeared.

Concentration of 0.30 and 0.20 µg/mL was prepared for isomalathion, still no peak was observed. The limit of detection was then calculated as stated in the definition and the results obtained for all the analytes are given table 3.3

3.3 Optimization of Matrix Solid-Phase Dispersion (MSPD)

3.3.1 Effect of Solid Sorbents

As mentioned in section 1.11.2, several solid sorbents have been used as solid sorbent in MSPD. In this study, Multi-Walled Carbon Nanotubes (MWCNT) were used as solid support and dispersant in the extraction of some organophosphorus pesticides from honey samples.

Preliminary experiments were carried out to assess the effect of two sorbents, MWCNT and silica gel on the yield and selectivity of the MSPD process. The experiments were performed for investigating the one which would give clean chromatograms and high recoveries of the analytes. The ratio of honey sample to solid sorbent was 1:4 (g/g) for both solid materials, and silica gel was used in both cases as clean-up co-sorbent. N-hexane-acetone (10 mL, 1:1 v/v) was selected as the eluting solvent as used in previous studies [43, 58].

Extractions with MWCNT and silica gel without spiking honey sample were performed. The blank extract of silica gel extracts displayed very crowded chromatogram with matrix interference peaks. Almost all the analyte peaks were affected and a hump was persistently observed at the retention time of malathion. Some other interference peaks appeared at the same retention times of the analytes as can be seen in Figure 3.11, however in the chromatograms of the MWCNT blank extracts, the number of interference peaks were decreased and even the hump that blocked the malathion peak, disappeared (Figure 3.12).

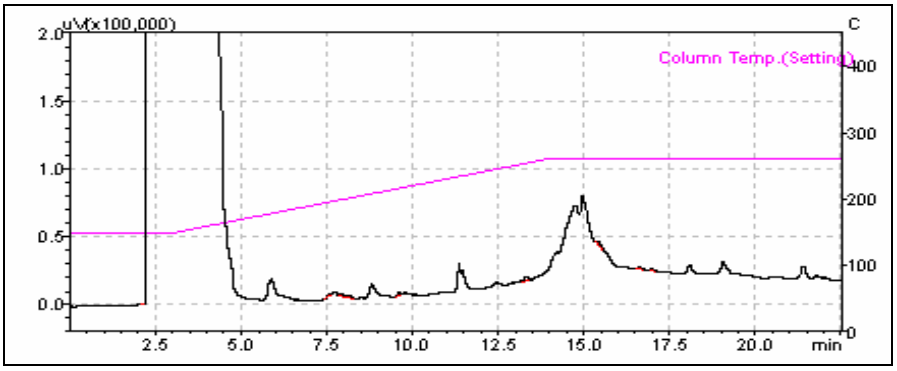


Figure 3.11 Chromatogram obtained without spiking honey sample with silica gel as the sorbent

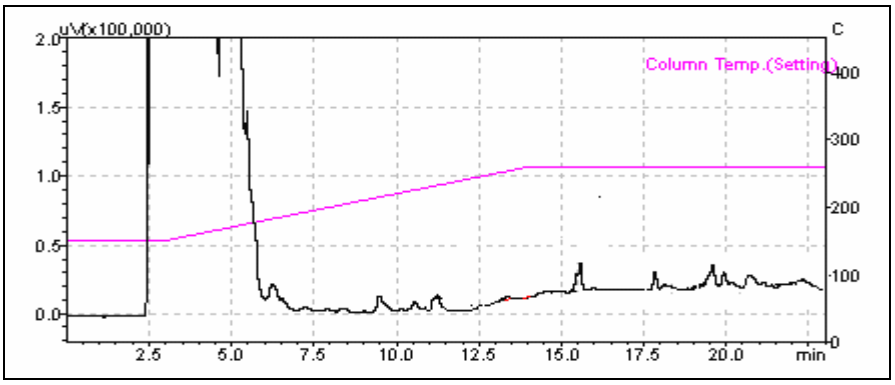


Figure 3.12 Chromatogram obtained without spiking honey sample with MWCNT as the sorbent

3.3.2 Effect of the Mass Ratio of Carbon Nanotubes to Honey sample

The second step in the method set-up was the comparison of solid support to sample mass ratio (g/g) that will allow complete disruption of the honey matrix, the adsorption of insecticides on the surface of the dispersant solid sorbent and finally facilitate the transfer of the mixture into the column.

The sorbent to sample ratios of 1:1 to 4:1, were examined. As shown in Figure 3.13, increasing the amount of solid sorbent showed an increase in the recovery (percent) of the analytes. This is due to large amount of the MWCNT available to fractionate the honey sample matrix and released the analytes which are then trapped within the hexagonal arrays of MWCNT. Therefore, the optimum ratio of 4:1 (i.e. 0.2 g of solid sorbent to 0.05 g of sample), was chosen.

It has been suggested that the duration of grinding has little or no effect [59]. Therefore, two blending times, 1.0 and 5.0 min, were tested. The results showed that increasing the dispersal time did not enhance the extraction efficiency. On the contrary, lower extraction efficiencies were observed when the dispersal time was 5.0 min. A grinding time of 1.0 min was therefore chosen for all the following experiments. Table 3.4 shows the recovery percent and percent relative standard deviation for three replicate extractions, for the different ratios of the MWCNT to honey sample carried out under the optimum condition given at Table 3.1

Table 3.4 The % Recovery and % RSD of the insecticides of mass ratio of sorbent to honey sample (n=3)

Mass ratio of MWCNT to Honey sample (g/g)	% Recovery and % RSD			
	Malaoxon	Isomalathion	Malathion	Fenitrothion
1:1	22 ±6.3	25 ±5.0	37 ±4.8	24 ±3.5
2:1	50 ±3.8	36 ±6.0	52 ±5.6	45 ±4.5
3:1	61 ±4.0	56 ±4.0	69 ±3.0	67 ±3.9
4:1	91 ±6.7	86 ±7.4	94 ±4.3	89 ±5.8

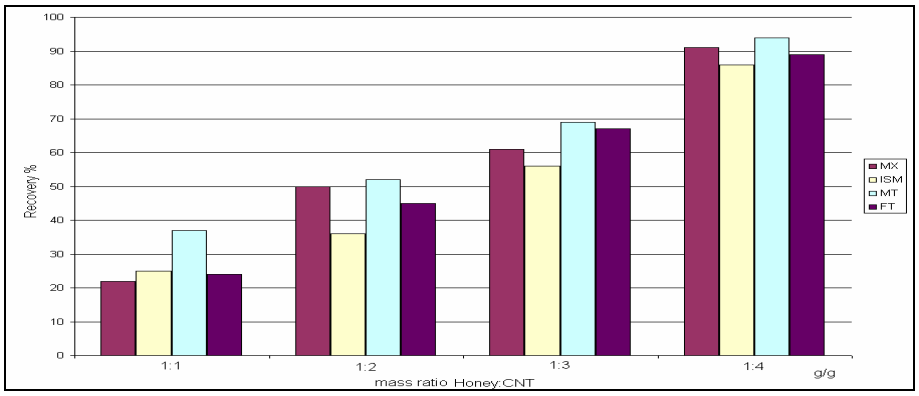


Figure 3.13 The effect of the ratio of sample to sorbent mass

3.3.3 Mass of Clean-up Co-Sorbent

In some extraction techniques or sample preparations, interference removal through the washing or cleaning of extracts is a critical step. It is the stage where high probability of loosing the analytes is inevitable and sometime it requires a lot of time in the sample preparation step. However, MSPD offers the possibility of performing extraction and clean-up simultaneously.

Silica gel was used as the cleanup sorbent and its mass is important to remove the interference of the sample. The clean-up sorbent mass of 1.0 to 4.0 g was examined; as shown in Figure 3.14, the recoveries of the analyte were better as the mass of sorbent increases and at masses of 3.0 g and 4.0 g constant recovery were obtained. But for economic reasons, 3.0 g of sorbent was used for the rest of the studies. The efficiency of silica gel in removing the interferences was further investigated by standard addition method studies followed by the comparison of calibration graphs as shown in Section 2.7. Their precisions are shown in Table 3.5.

Table 3.5 The % Recovery and % RSD of analytes at different mass of cleanup co-sorbent (n=3)

Mass of Co-Sorbent (g)	% Recovery and % RSD			
	Malaoxon	Isomalathion	Malathion	Fenitrothion
1.0	20.8±10.3	17.9±12.4	25.3±7.3	26.1±9.6
2.0	40.8±5.6	35.6±8.6	43.7±8.2	45.6±10.1
3.0	60.4±7.9	58.7±9.3	68.7±5.1	69.3±7.7
4.0	70.0±8.1	63.4±10.3	70.9±6.2	71.2±6.3

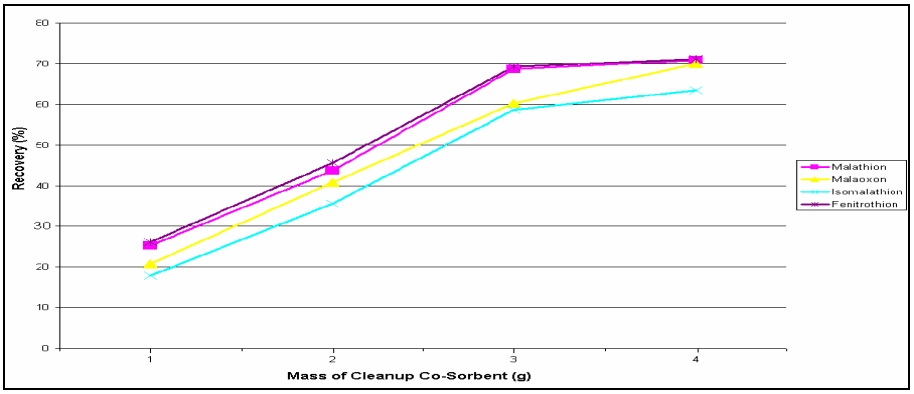


Figure 3.14 The effect of mass of cleanup co-sorbent to analyte recoveries

3.3.4 Choice of Eluting Solvents

3.3.4.1 Effect of Individual Solvents on Extraction Efficiency

A considerable number of single solvents have been used for the elution step in matrix solid-phase dispersion. These solvents were n-hexane, acetone, dichloromethane, acetonitrile, and ethyl acetate. The selection of suitable eluting solvent depends on the recovery percent and removal of contaminants from the extracts. In general, the elution solvent polarity, miscibility, or partitioning characteristics govern its ability to display excellent recovery percent. Figure 3.15 shows the recovery percent of the individual analytes. Normal hexane resulted the cleanest extracts and the percent recoveries of the analytes ranged from 45.0 to 67.5%. The extraction with acetone gave percent recovery of the analytes changing from 58.0 to 69.5%; besides the chromatograms obtained were very much crowded.

The extracts from dichloromethane also showed more interference peaks on the chromatogram than all the other solvents, the recoveries of some of the analytes were from 47.8 to 68.7%. Although the chromatograms of acetonitrile and ethyl acetate indicated cleane extracts, the recoveries of most of the analytes were relatively low, varying from 39.9 to 60.1% and 34.0 to 58.7% respectively. Moreover, the evaporation of acetonitrile and ethyl acetate with nitrogen gas flow was obstinately difficult and took quite a very long time. Their low percent recoveries might be due to loss of some of the analytes during the long evaporation process. The precisions for percent recoveries all of the solvents were varied from 5.2 to 13.1% as shown in Table 3.6.

Table 3.6 The % Recovery and % RSD of the individual solvents (n=3)

Eluting solvents	% Recovery and % RSD			
	Malaoxon	Isomalathion	Malathion	Fenitrothion
Hexane	56.2 ±13.1	57.9 ±7.5	45.0 ±8.2	67.5 ±7.3
Acetone	69.5 ±8.1	67.6 ±10.0	58.0 ±10.8	65.4 ±5.2
Acetonitrile	39.9 ±12.2	42.2 ±6.5	60.1 ±8.2	51.2 ±7.7
Dichloro-methane	56.1 ±6.7	68.7 ±6.0	47.8 ±9.4	48.1 ±7.4
Ethyl acetate	34.2 ±9.1	34.0 ±7.8	58.7 ±10.5	53.9 ±5.7

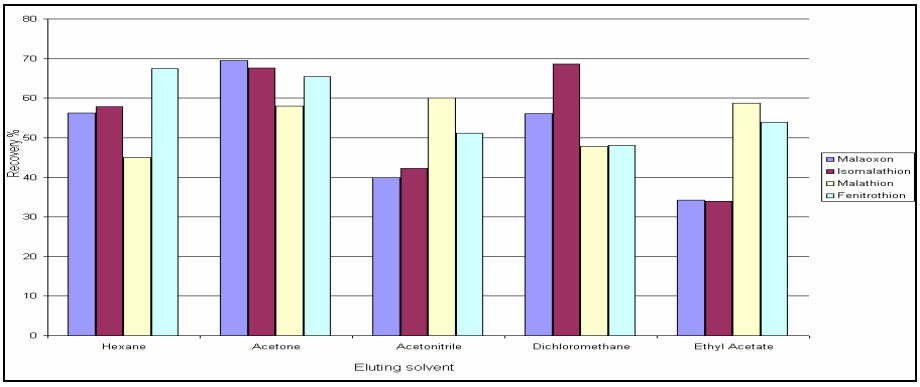


Figure 3.15 Percent recovery obtained after eluting with individual solvents

3.3.4.2 Elution with Binary Solvent (n-hexane-acetone)

Following the study of single solvent elution, several mixtures of solvents were examined. A combination of acetone-dichloromethane was first tested, the chromatograms were very crowded and some of the interference peaks overlapped with the analyte peaks.

Since honey is a complex matrix as stated in Section 1.8, its effects in using relatively polar organic solvent was not a surprise. The combination of n-hexane and acetone in different ratio was then applied. As shown in Figure 3.16, the volume ratios of the two solvents were varied from 1:9 to 9:1 (v/v). From this study, a clear chromatogram was observed and considerable recovery percentages were obtained. The relative standard deviations for the mixtures ranged from 3.7 to 14.1% (Table 3.7) which is quite appreciable level in sample of such a complex matrix. The optimum composition of the n-hexane-acetone mixture used for eluting the insecticides from the MSPD column was 8:2, and applied in further experiments

Table 3.7 The % Recovery and % RSD for the different n-hexane-acetone ratios (n=3)

Ratio of Hexane/ acetone	% Recovery and % RSD			
	Malaoxon	Isomalathion	Malathion	Fenitrothion
1:9	34 ±8.3	32 ±6.0	56 ±7.5	54 ±6.1
2:8	58 ±10.2	47 ±5.2	60 ±9.5	67±7.4
3:7	67 ±7.8	59 ±5.6	77 ±5.9	68 ±6.5
5:5	59 ±9.1	60 ±6.0	68 ±3.7	71 ±7.3
8:2	71 ±7.9	69 ±6.8	75 ±5.9	72 ±6.9
9:1	56 ±14.2	57 ±5.6	51 ±7.1	50 ±6.8

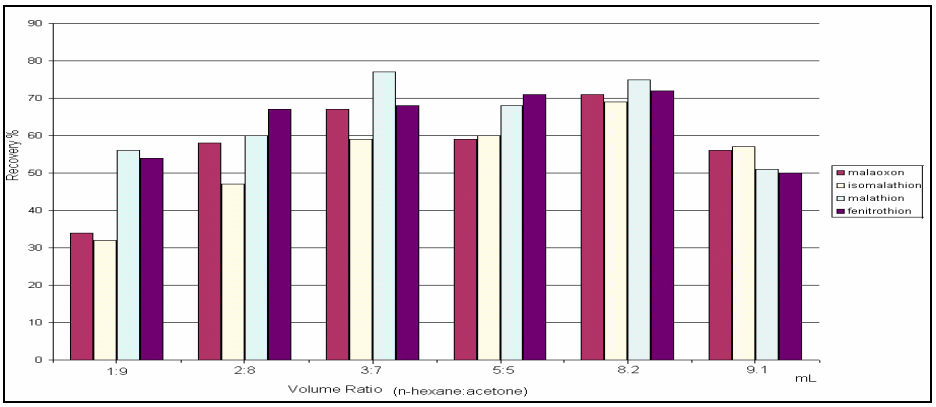


Figure 3.16 The ratio of hexane to acetone mixture and their percent recovery

3.3.4.3 Effect of Volume of Elution on Extraction Efficiency

In the elution step, the selection of the volume (mL) of eluting solvent(s) is important to ensure that the adsorbed analytes are completely removed from the solid dispersant and are carried to the liquid phase. Before eluting, the peristaltic pump is applied for 2.0 min; this is to ensure that all the water content in the column was removed.

The volumes of solvent (mixture at ratio 8:2 of n-hexane-acetone) from 5.0 to 20.0 mL were tried. The same ratio was maintained in all cases. As shown in Figure 3.16, the recovery percent increases linearly as the volume was raised from 5.0 to 15.0 mL. At 15.0 to 20.0 mL, the percent recovery remained almost constant. This shows that the optimum volume is between 15.0 to 20.0 mL and to be on the safe side of ensuring total analyte recovery, 20.0 mL volume of elution was chosen as the optimum value. The precision of the eluting volume for three replicates as shown in Table 3.8, ranges from 5.0 to 10.1%, indicated the uncertainty in extraction/sample preparation methods from the true value if there were no systematic error.

Table 3.8 The % Recovery and % RSD of the volume of elution (n=3)

Volume of elution (mL)	% Recovery and % RSD			
	Malaoxon	Isomalathion	Malathion	Fenitrothion
5	63.5 ±7.1	67.5 ±6.3	52.6 ±6.3	47.3 ±6.2
10	92.3 ±6.7	75.6 ±7.5	60.5 ±4.4	65.9 ±8.3
15	95.7 ±5.8	76.7 ±7.8	88.7 ±7.0	88.0 ±10.1
20	100.7±9.9	93.9 ±6.7	98.1 ±7.6	98.4 ±5.0

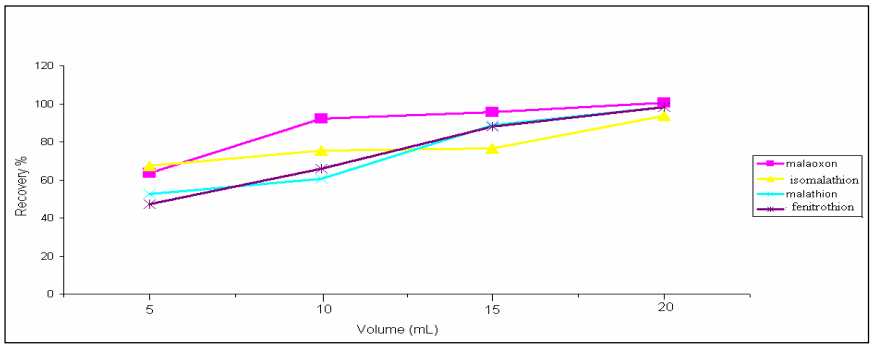


Figure 3.17 The effect of eluting volume on extraction efficiency

3.3.5 Method of Concentration of Analytes in the Extract Eluates

In the study of the best method of volatilization, mixture of the analyte concentration of 10.0 µg/mL was used for spiking the honey sample. The honey was then subjected to the optimum MSPD conditions. The use of nitrogen gas and vacuum pump concentration methods were tried to concentrate the analytes in the eluate. The volume of eluates was approximately 15.0 mL after eluting with 20.0 mL of the elution solvent.

In the utilization of the vacuum pump, the time for concentration was at minimum but most of the analytes were lost. Decreasing the eluate volume to approximately 1.0 mL, the peak areas of the analytes were decreased. These results indicated the inefficiency of this concentration method, which is probable due to the loss of analytes during the pumping.

Then, a second volatilization method using nitrogen gas was tried. During the process, gentle stream nitrogen gas was sent into the vial containing the extract, when the volume of extract was reduced to one milliliter and the peak areas of the analytes obtained were still high. Reducing the volume to 0.50 mL resulted in a crowded chromatogram and the analytes peaks were also affected. At volume 2.0 mL although there was clear chromatogram, the detection of the analytes was. Hence the optimum concentration volume was chosen to be at 1.0 mL.

3.3.6 Selection of the Pore size of the Membrane Filter

Two pore sizes of 0.20 µm and 0.45 µm membrane filters of Durapore type were examined in order achieved good filtering of large interference substances. With the 0.45 µm pore size, the chromatogram was too crowded and the detection of the analytes was greatly affected. When the 0.20 µm pore size filter was used, many of the interference peak disappeared thus this membrane was selected for use in the rest of the study.

3.3.7 Effect of Extraction Rate with Peristaltic Pump on Extraction Efficiency

Like liquid chromatography and solid phase extraction, the pressure applied to move or elute the mobile phase with the target analytes from the column is very important for the efficiency of MSPD. Most MSPD elutions have been conducted by gravity flow [59, 60].

In some instances it is stated that flow was initiated by the application of pressure to the head of the column or by placing the columns on a vacuum box and briefly applying suction [61]. In this study, we initially applied gravitational flow; it worked for silica gel, but did not work for the multi-walled carbon nanotubes. Therefore the use of peristaltic pump became necessary. In this occasion, the pump was connected at the end of the luer tip of the MSPD column via a Teflon tube with inner diameter of 0.0812 inch.

Since the pump consists of flow rate regulator; the speed of rotation of the peristaltic was increased from 1 to 5 rpm while the extraction flow rate varied from approximately, 1 mL/min to 5 mL/min. As shown in Figure 3.18 the recovery of the analytes decreased as the rate of extraction increased. This can be explained by the decreasing of the minimum time that the solvent should spend to percolate and carry over for elution.

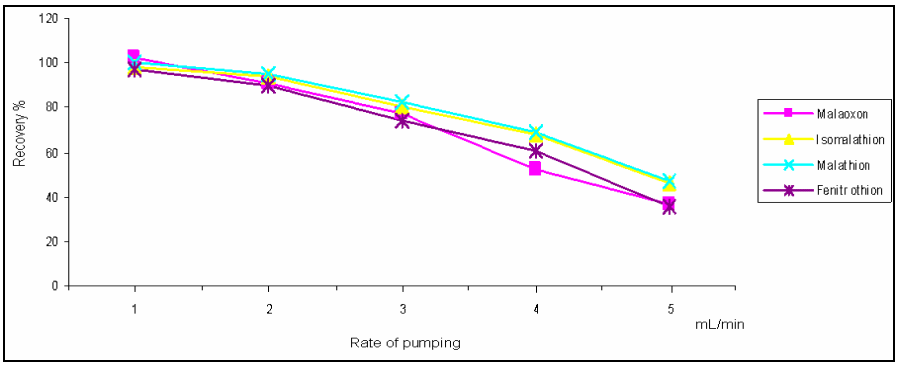


Figure 3.18 Effect of extraction rate (mL/min) on extraction efficiency

Although the time of extraction at rate 1.0 mL/min was long (approximately 20.0 min), it had better reproducibility and very good recoveries of analytes as shown in Table 3.9. Hence this extraction rate was selected as the optimum rate in MSPD process.

Table 3.9 The % Recovery and % RSD of the rate of extraction

Rates of extraction (mL/min)	% Recovery and %RSD			
	Malaoxon	Isomalathion	Malathion	Fenitrothion
1	102 ±6.5	98 ±5.2	100 ±6.4	97 ±8.1
2	91 ±12.0	94 ±6.8	95 ±7.2	90 ±13.0
3	77 ±8.4	80 ±8.5	82 ±9.8	74 ±7.8
4	52 ±8.8	68.1 ±7.2	69.4 ±7.3	61 ±9.5
5	37 ±7.8	46 ±7.6	47 ±9.5	35 ±9.1

Table 3.10 Summary of the observed and optimum parameters of MSPD extraction

Parameters	**Conditions studied**	**Selected conditions**
Type of solid sorbent	CNT and Silica gel	CNT
Ratio of sorbent mass to sample mass	1:1, 2:1, 3:1, 4:1	4:1 (g/g)
Mass of clean-up co-sorbent (silica gel)	1.0 to 4.0 g	3.0 g
Elution solvent	Hexane, Acetone, Dichloromethane, Ethyl acetate and binary mixtures	Hexane and Acetone
Ratio of Binary eluting solvent	1:9, 2:8, 3:7, 9:1, 8:2, 7:3 (v/v)	Hexane: Acetone 8:2 v/v
Volume of elution	5.0 mL, 10.0 mL, 15.0 mL, 20.0 mL	20.0 mL
Rate of peristalsis	1 to 5 ramp	1 ramp
Dispersal time	1.0 min, 2.0 min and 5.0 min	1.0 min
Size of membrane filter	0.20 and 0.45 µm	0.20 µm
Method of Evaporation of eluate	Vacuum evaporation and Nitrogen gas	Nitrogen gas

3.4 Matrix Effects on GC-FID Response

The inherent difficulty in the extraction of honey sample is the co-extraction of matrix components that are also soluble in the extraction solvent. Official pesticide-residue laboratories have published guide lines for analyzing pesticide residues in complex matrix samples such as honey, fruits and vegetables stating that calibration solutions must be prepared in a blank extracts of the sample matrix, in order to improve accuracy of the calibration step [62]. However, on the basis of characterization of matrix effect, some researchers showed that data should be obtained from indirect calibrations carried out using two types of calibration standard: (i) chemical standards prepared from pure analyte dissolved in pure solvent and (ii) reference materials prepared incorporating the matrix co-extractives in the standard solution (SRM with specific matrix) [63, 64]. This approach will allow a reliable quantitation of pesticides in samples with important matrix effects, such as fruit and vegetable samples; however, the method has limitation for being applied in samples in which certified reference materials can be available.

As mentioned in Section 1.8 the pH and moisture conditions of honey made most pesticides to have very low stability in it. This in effect precludes availability of reference materials that contained pesticides in honey. Therefore, in the analysis of pesticides in honey sample, the matrix induces effects on analyte peaks could be evaluated by performing standard addition calibration method (Section 2.7) and preparing standard solution from blank extracts of honey.

3.4.1 Calibration of Standards Prepared by Blank Extracts

For the quantitative determination of the response factor of the instrument detector to the analytes in the presence of honey sample matrix, a mixture of the insecticide working standard solution was prepared from the eluates of honey sample extraction without spiking (blank extraction).

During the preparation of this standard, calculated volumes (mL) of the pure insecticide standard mixture with concentrations 9.0 µg/mL, 45.0 µg/mL, 90.0 µg/mL 135.0 µg/mL and 180 µg/mL were spiked in volumes of the eluates from the blank extracts of honey sample so that concentrations of 1.0, 5.0, 10.0, 15.0 and 20.0 µg/mL respectively are be obtained in a 10.0 mL volumetric flask. When the concentration of 1.0 µg/mL was first injected, the analyte peaks were not observed, but when concentrations from 5.0 to 20.0 µg/mL injected the peaks were observed. This indicates the effect of matrix t the flame ionization detector response factor since the low concentration of the insecticide standards could be observed in the presence of honey matrix.

The calibration curve of the insecticide showed lines which gently slope with 465 to 789, which is quite small when compared with the slopes of lines of the standards in pure acetone solvent. This indicates the low sensitivity of the detector in the presence of sample matrix.

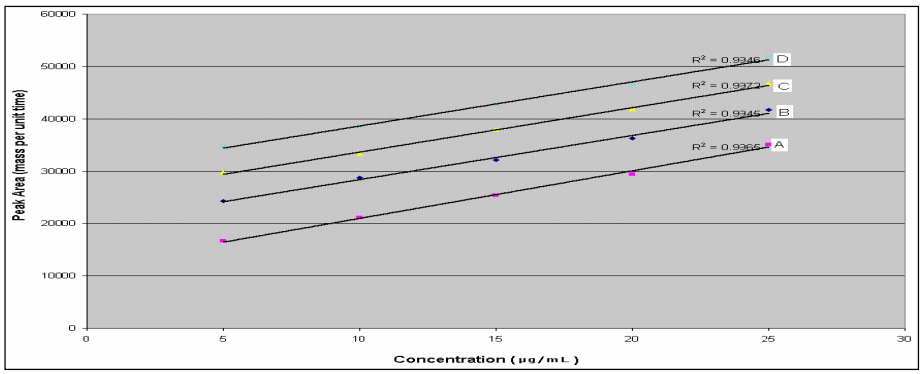


Figure 3.19 The calibration curve of the mixture insecticide standards prepared from blank extracts (A-isomalathion, B-malaoxon, C-malathion, D-fenitrothion)

3.5 Calculation of Percentage Recovery and Analyte Preconcentration Factor

The extraction efficiency of MSPD was evaluated through the calculation of percentage recovery of the analytes after elution. In general, the recovery percent is defined as the ratio of the amount of analytes extracted to the amount percolated [65, 66]. In consideration of the matrix effect on analytes peaks, blank extractions were made for the different optimization conditions. The GC-FID system was very sensitive to the honey matrix, thus some interference peaks appeared at the retention time of the target analytes. Therefore in the calculation of the recovery percent, the average peak area of the blank extracts at the retention time of the target analytes were subtracted from the total peak area obtained for the extract of the spiked honey sample, and the result is divided with the peak area of the analytes in the pure standards and the result was multiplied by hundred (100) as shown in equation 3.1.

$$\%\,\text{Recovery} = \frac{\text{Total peak area of spiked extract} - \text{Average peak area of blank extract}}{\text{Peak area of the pure standard used for spiking}} \times 100 \quad (3.1)$$

The analyte Preconcentration Factor (PF) is defined as the ratio between the concentration (5.0 µg/mL) of analyte standard prepared from blank extracts (C_b) to the lowest concentration (0.1 µg/mL) of the analyte standard spiked into the honey sample (C_S). Through this definition (Equ 3.2), the average EF for all analytes was 50.

$$PF = \frac{C_s}{C_b} \quad (3.2)$$

3.6 Degradation of Spiked Insecticides in Honey with Time

An amount of 2.0 g of honey sample was treated with 20.0 µL of 10.0 µg/mL of the insecticide mixture, so making the concentration in the honey sample as 0.10 µg/g. The treated sample was kept in a transparent glass vial at room temperature. Immediately after treatment, 0.05 g was taken, extraction was carried out. The process was repeated for six days in the same period of time. The recovery percents and relative standards deviations were calculated in day to day basis, result are given in Table 3.11.

As it is shown in Figure 3.20 malaoxon concentration increased in the next day while concentration of malathion, isomalathion, and fenitrothion were erratically decreased. Initial increase in Malaoxon is because the oxidation of malathion to malaoxon.

The synergic relationship of isomalathion and malathion in their degradation with time, also reflects how the toxicity of malathion can be raised in the presence of isomalathion. Fenitrothion has much less tendency to degrade than malathion.

Table 3.11 The % Recovery and % RSD of the decomposition of analytes in spiked honey sample with time

Time (days)	%Recovery and %RSD			
	Malaoxon	Isomalathion	Malathion	Fenitrothion
0	99.3 ±6.8	97.6 ±5.9	98.7 ±5.5	99.0 ±4.9
1	104.0 ±10.9	46.4 ±11.2	43.5 ±8.6	45.8 ±8.0
2	77.9 ±9.8	23.7 ±11.5	28.9 ±9.5	30.3 ±11.5
3	44.6 ±11.6	12.6 ±8.5	10.4 ±11.2	22.4 ±6.8
4	43.2 ±9.6	9.6 ±9.5	6.8 ±7.6	19.0 ±7.7
5	18.3 ±12.0	4.6 ±9.5	3.1 ±6.9	11.3 ±7.4

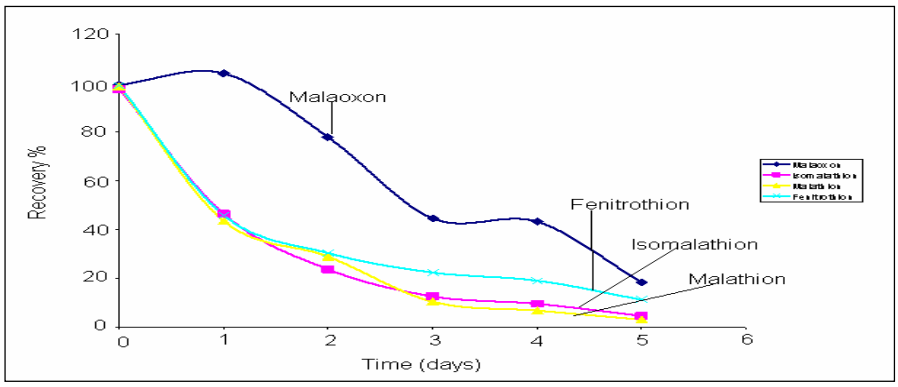


Figure 3.20 The degradation of insecticides spiked in honey sample with time

3.7 Analytical Figures of Merit for the Overall Method

The efficiency and performance MSPD extraction and preconcentration of the insecticides from the honey sample, was showed by combining it with the optimized conditions of the GC-FID system (Table 3.1). The extraction efficiency was achieved from percent recovery calculation (Equ 3.1) and the overall figures of merit of the extraction method combined with the GC-FID system were evaluated in terms of precision, limit of detection, limits of quantification and linearity of response of the instrument of analytes in honey sample.

Concentrations of insecticide standards mixture from 0.10 µg/mL to 20.0 µg/mL were prepared in acetone. During extraction, 10.0 µL of the prepared insecticide standards were spiked in 50.0 mg of honey sample so that, the final concentrations in honey are 0.20 µg/g to 4.0 g of insecticide to honey.

The optimum parameter of the extraction method was then applied and the results were obtained after injected into the instrument as shown in Figure 3.21. The extraction efficiency and the all figures of merit are shown in Table 3.12. From the calibration curve shown in Figure 3.22, there was linear response of the instrument for the analytes. However this time the slopes were from 145.9 to 378.2.

Table 3.12 The % Recovery and figures of merit of spiked honey with 0.1 µg/g of insecticide standards (*n*=3)

Compounds	% Recovery	LOD (ng/g)	LOQ (ng/g)	% RSD	R^2
Malathion	103.3	7.0	70.0	±12.1	0.9945
Malaoxon	96.5	7.0	70.0	±10.9	0.9987
Fenitrothion	101.2	7.0	70.0	±9.8	0.9962
Isomalathion	83.6	33.3	333.3	±11.1	0.9961

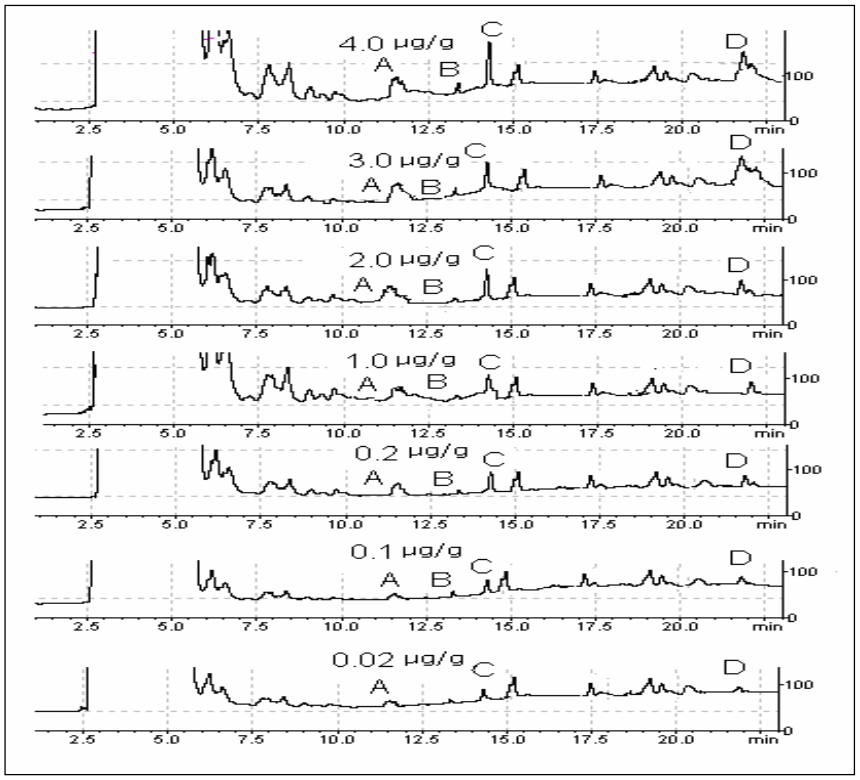


Figure 3.21 Overlay of chromatograms of the insecticides with increasing concentration from 0.02 to 4.0 µg/g, A-Malaoxon, B-Isomalathion, C-Malathion and D-Fenitrothion

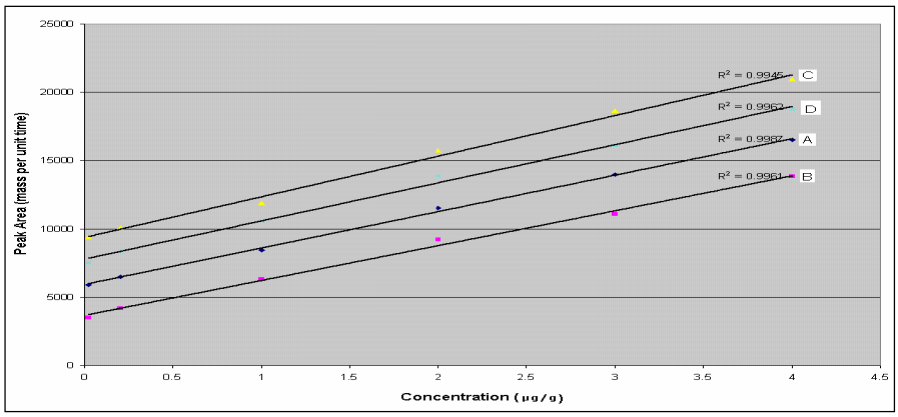


Figure 3.22 The calibration curve of analyte mixture after MSPD extraction, A-Malaoxon, B-Isomalathion C-Malathion and D-Fenitrothion

3.8 Real Sample Analysis

The developed method of MSPD was applied for the determination of the studied insecticides in four honey samples purchased from supermarkets. Table 3.12 shows the different honey product from different producers collected from different regions of Turkey. the result after performing MSPD combined with GC-FID system are shown in the chromatograms of Figures 3.22 to 3.25.

Table 3.13 Various types of honey used during real Analysis and the regions collected

Code	**Sample Name**	**Type of honey**	**Region**	**Result**
Sample 1	Çamlıbelde Balı	Flower honey (Çiçek balı)	Aegean Region (West Anatolia)	No detection
Sample 2	Ankara Balı	Süzme çiçek balı	Middle Anatolia (Ankara) (ATATÜRK ORMAN ÇİFTLİĞİ)	No detection
Sample 3	Binboğa Balı	Flower honey	East Mediterranean Region (Adana-Kozan)	No detection
Sample 4	Balparmak Balı	Süzme çiçek balı	Southeast Anatolia (Batman-Sirnak)	No detection

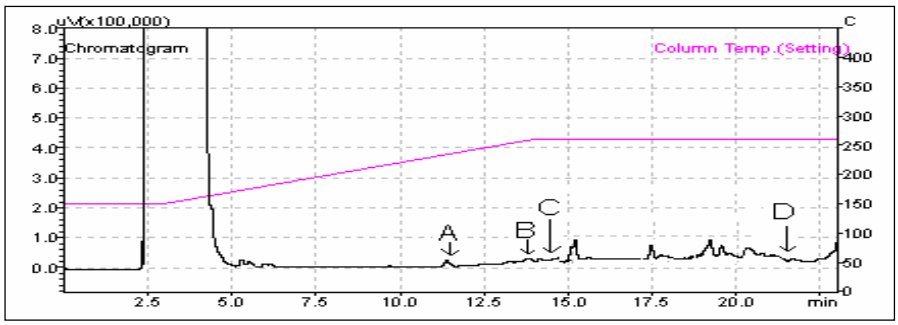


Figure 3.23 Sample 1 Çamlibelde Bali

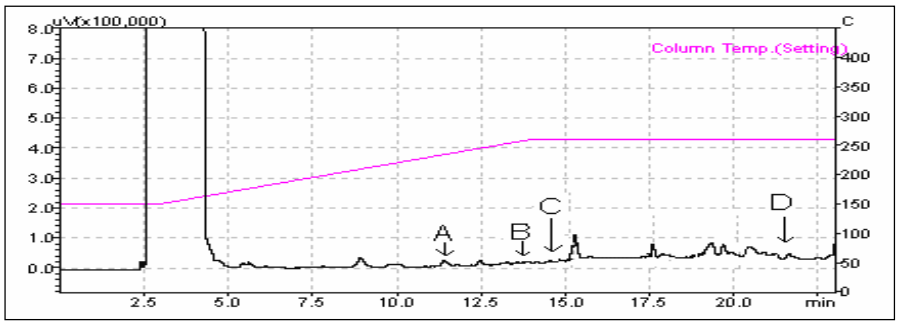


Figure 3.24 Sample 2 Ankara Bali

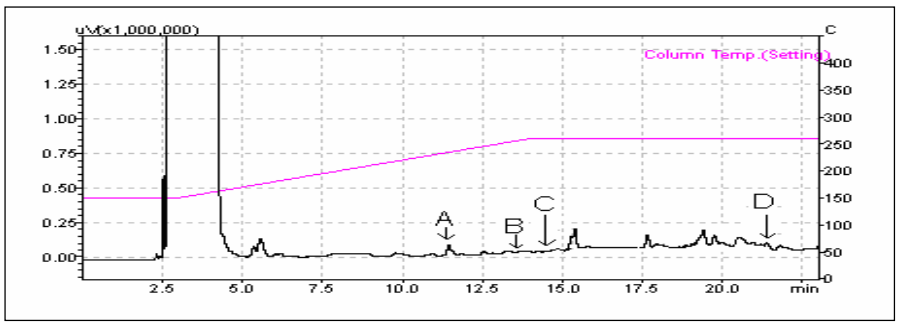


Figure 3.25 Sample 3 Binboğa Bali

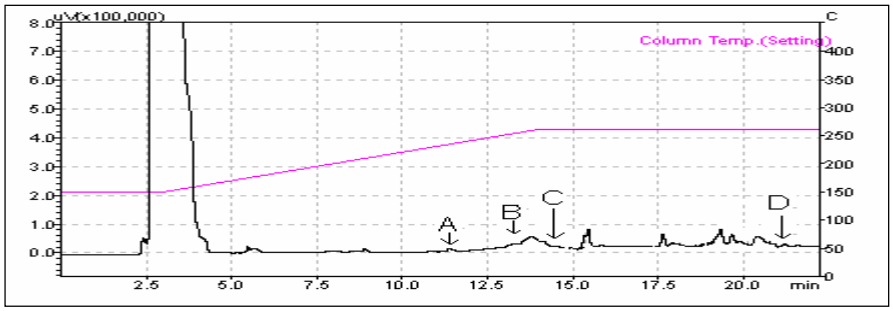


Figure 3.26 Sample 4 Balparmak Bali

Chapter 4

CONCLUSION

In this study, the investigation of some organophosphorus insecticides in honey sample which is a very complex environmental and biological sample was accomplished. Because of the inherent complex nature of honey, a sophisticated sample preparation technique is crucial for trace analysis of organic substances. Matrix solid-phase dispersion extraction and analyte preconcentration technique because of its characteristic nature of sample matrix destruction and dispersal of analyte on the surface of sorbent had been applied to extract the organophosphorus insecticides from honey samples. Since the selectivity of MSPD depend on the type of dispersal sorbent and eluting solvent used, multi-walled carbon nanotubes in powder form was utilized as the dispersant sorbent. MWCNT was selected as sorbent material, because of its large surface area that rendered abrasive property to fractionate the honey matrix and hexagonal arrays allow the trapping of the insecticides within the layers for better recoveries of analytes.

When these two properties (large surface and hexagonal array) of MWCNT are compared with other sorbents such as silica gel, octasilyl, octadecylsilyl, florisil, diatomaceous earth, sand and alumina, where the characters for good matrix solid-phase dispersion sorbent depend on mesh size and derivatization of the sorbent, MWCNT has greater advantages.

It is supposed that if the MWCNT were derivatized by attaching nonpolar organic compounds on the surface layer, the selectivity of matrix solid-phase dispersion would have been better.

From the calibration curves of the analytes in standard addition method (Section 2.7) it has been shown that the silica gel is not an effective cleanup co-sorbent. A good cleanup sorbent should be able to retain the interferences present in the sample and allow the analyte to permeate through it. To obtain this, mesh size and affinity of the cleanup sorbent towards the sample matrix should be considered.

Flame ionization detector is an universal detector that is very sensitive to hydrocarbons in the range of 10^{-12} to 10^{-15} grams. It has little detection capability to phosphorus, nitrogen, oxygen and sulphur containing compounds; therefore to improve its detection limit, an efficient sample preparation is imperative; hence matrix solid-phase dispersion extraction method was chosen for this purpose.

The efficiency of gas chromatographic separation is dependent upon several conditions, thus optimization of each parameter is necessary. The several conditions that affect also MSPD extraction were optimized. By the optimum conditions of the method developed for the trace analysis of malathion, malaoxon, fenitrothion and isomalathion were studied in spiked honey sample and a detection limit of 7.0 ng/g for malaoxon, isomalathion and malathion, and 33.3 ng/g for fenithrotion were obtained. These values are comparable with the values obtained with the other GC methods given in literature (Table 1.5, GC-FPD LOD<1ng/g, GC-NPD LOD<12ng/g).

Real samples of honey collected from different regions of Turkey were studied, fortunately no detectable amount of insecticide was found in any sample.

Moreover, instead of GC-FID, the other chromatography methods that can be used for this study are gas chromatography combined with Mass Spectrometry, Electron Capture, Nitrogen-Phosphorus (NPD) and Flame Photometry (FPD) detectors and Liquid Chromatography method combined with Mass Spectrometry, UV-Spectrometry and Diode Array UV- detectors.

As a future work, determination of OPPs by using GC combined with NPD which is specific for OPPs may be used and the performance of it may be compared with the method studied to find out preconcentration enhancement of MSPD.

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