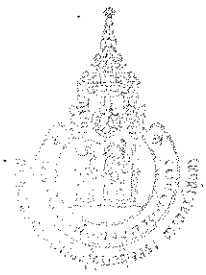

Molecular Imprinted Polymer (MIP) for Pesticide

Sujittra Poorahong

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Analytical Chemistry

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บทคัดย่อ

การวิเคราะห์หาปริมาณคาร์โบฟิวแรนที่ปนเปื้อนในน้ำด้วยเทคนิคแก๊สโครมาโทกราฟีที่มีตัวตรวจวัดชนิดเฟรมไอออไนเซชัน โดยใช้คาปิลลารีคอลัมน์ชนิดอัลตรา 2 (ultra2) ที่มีความยาว 25 เมตร เส้นผ่านศูนย์กลาง 0.53 มิลลิเมตร ความหนาของฟิล์ม 0.32 ไมโครเมตร โมเลกุลลาร์ อิมพรินเต็ด พอลิเมอร์สังเคราะห์ขึ้นเพื่อใช้ในขั้นตอนการเตรียมตัวอย่างเพื่อเป็นตัวดูดซับที่จำเพาะต่อคาร์โบฟิวแรนในเทคนิคตัวดูดซับของแข็ง (solid phase extraction) และโซลิด เฟส ดิสเพอชัน (solid phase dispersion) โดยมีพอลิเมอร์ที่แตกต่างกัน 2 ชนิด ชนิดที่ 1 ใช้อะคริลาไมด์และไดเอทธิลอะมิโนเอทธิลเมทตะไดเลตเป็นมอโนเมอร์ ชนิดที่ 2 กรดเมทตะคลิลิกและไดเอทธิลอะมิโนเอทธิลเมทตะไดเลตเป็นมอโนเมอร์ ผลจากการสังเคราะห์พบว่าให้ค่าการสังเคราะห์ซ้ำที่ดีมีค่าเบี่ยงเบนมาตฐานสัมพัทธ์น้อยกว่า 10 เปอร์เซ็นต์ ศึกษาคุณสมบัติทางกายภาพ (ร้อยละของการบวมตัว ขนาดอนุภาคและความหนาแน่น) ของพอลิเมอร์ และมีความจุของพอลิเมอร์ต่อคาร์โบฟิวแรนของพอลิเมอร์ชนิดที่ 1 และ 2 เท่ากับ 12 และ 11 มิลลิกรัมต่อลิตร ต่อหนึ่งกรัมของพอลิเมอร์ตามลำดับ

ศึกษาสภาวะที่เหมาะสมของการเตรียมตัวอย่างในเทคนิคตัวดูดซับของแข็ง และโซลิด เฟส ดิสเพอชันโดยใช้พอลิเมอร์ที่สังเคราะห์ขึ้นเป็นตัวดูดซับในการสกัดคาร์โบฟิวแรน สภาวะที่เหมาะสมพบว่าพอลิเมอร์มีความจำเพาะต่อคาร์โบฟิวแรนสูงเมื่อเทียบกับสารในกลุ่มคาร์

บาเมตตัวอื่น ๆ (คาร์บาริลและคาร์โบซัลแฟน) และให้ผลการทำซ้ำที่ดี (ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ 6-9 เปอร์เซ็นต์) ช่วงความเป็นเส้นตรงจากเทคนิคตัวดูดซับของแข็ง สำหรับพอลิเมอร์ชนิดที่ 1 เท่ากับ 0.2–200 ไมโครกรัมต่อลิตร และ 2.0–40.0 ไมโครกรัมต่อลิตรสำหรับพอลิเมอร์ชนิดที่ 2 ขีดจำกัดการตรวจวัด เท่ากับ 0.677±0.009 และ 0.36±0.03 ไมโครกรัมต่อลิตรสำหรับพอลิเมอร์ชนิดที่ 1 และ 2 ตามลำดับ สำหรับเทคนิคโซลิด เฟส ดิสเพอชัน ช่วงความเส้นตรงของพอลิเมอร์ชนิดที่ 1 และ 2 อยู่ในช่วง 0.1 – 20.0 และ 0.1 – 20.0 ไมโครกรัมต่อลิตร และขีดจำกัดการตรวจวัดเท่ากับ 0.03±0.001 และ 0.015±0.002 ไมโครกรัมต่อลิตร ทั้งสองเทคนิคให้เปอร์เซ็นต์การได้กลับคืนอยู่ในช่วงที่ยอมรับได้ (EPA, 2001) 80-101 เปอร์เซ็นต์ สำหรับเทคนิคตัวดูดซับของแข็ง และ 71-110 เปอร์เซ็นต์ สำหรับโซลิด เฟส ดิสเพอชันตามลำดับ และให้ค่าเบี่ยงเบนมาตรฐานสัมพันธ์ต่ำกว่า 19 เปอร์เซ็นต์ ที่ความเข้มข้นของคาร์โบฟิวแรน 5.0 และ 20.0 ไมโครกรัมต่อลิตร ทั้งสองเทคนิคได้นำมาประยุกต์ใช้ในการศึกษาเชิงคุณภาพและเชิงปริมาณของคาร์โบฟิวแรนจากตัวอย่างได้แก่น้ำจากอ่างเก็บน้ำและน้ำผิวดิน พบความเข้มข้นของคาร์โบฟิวแรนในน้ำตัวอย่างอยู่ในช่วงไม่สามารถตรวจวัดได้ถึง 80 ไมโครกรัมต่อลิตร

จากผลการวิจัยสามารถสรุปได้ว่าพอลิเมอร์ที่เตรียมขึ้นใช้เป็นตัวดูดซับทั้งสองวิธีสามารถนำไปวิเคราะห์หาปริมาณคาร์โบฟิวแรนในระดับปริมาณน้อยได้ ซึ่งให้ผลการวิเคราะห์ที่ถูกต้องและแม่นยำและมีความไวในการวิเคราะห์สูง เมื่อเปรียบเทียบวิธีการเตรียมตัวอย่างทั้งสองวิธีพบว่าเทคนิคโซลิด เฟส ดิสเพอชันให้ความไวในการวิเคราะห์ที่สูงกว่าเทคนิคตัวดูดซับของแข็ง และให้ขีดจำกัดการตรวจวัดที่ต่ำกว่า อีกทั้งยังใช้ตัวอย่างน้ำปริมาณน้อย และเป็นเทคนิคที่ง่ายใช้เวลาน้อย

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Abstract

Analysis of carbofuran contaminated in water sample by gas chromatography coupled with flame ionization detector (GC-FID) was studied. The optimum conditions of GC-FID were obtained *i.e.* Ultra 2 capillary column: 25 m × 0.53 mm I.D. × 0.32 μm film thickness (5% diphenyl 95% phynylmethylsiloxane) as the analytical column. Molecular imprinted polymer (MIP) was synthesized and used in sample preparation step as selective sorbent for carbofuran in solid phase extraction (SPE) and solid phase dispersion (SPD). Two MIPs were synthesized by using two functional monomers, acrylamide and 2-(diethylamino) ethylmethacrylate (DAM) for MIP 1 and methacrylic acid and DAM for MIP 2 via non-covalent method. Good reproducibility for the synthesis of MIP was obtained with % RSD less than 10. Physical properties of MIP and non-MIP which are % swelling, particle size, bulk density and binding capacity were investigated. The binding capacity of MIP 1 and MIP 2 were studied and the results showed that the maximum capacity of MIP 1 and MIP 2 were 12 and 11 mg L^{-1} g^{-1} of dry polymer, respectively.

Optimum conditions of molecular imprinted solid phase extraction (MISPE) and molecular imprinted solid phase dispersion (MISPD) were investigated and used to extract carbofuran. When compared to two carbamate pesticides, *i.e.*

carbaryl and carbosulfan, which have similar structure to carbofuran, the results showed the highest selectivity for carbofuran. Both techniques provided high repeatability, % RSD 6-9. For MISPE, linear dynamic range for MISPE 1 was 0.2-200 μg L^{-1} and 2.0-40.0 μg L^{-1} for MISPE 2. Limits of detection (LOD) was 0.677±0.009 and 0.36±0.03 μg L^{-1} for MISPE 1 and MISPE 2, respectively. Linear dynamic range between 0.1-20.0 and 0.1-20.0 μg L^{-1} were obtained for MISPD 1 and MISPD 2 and LOD was 0.031±0.001 and 0.015±0.002 μg L^{-1}, respectively. High recoveries were obtained in acceptable level (EPA, 2001), *i.e.* 80-101 % for MISPE and 71-110 % for MISPD. The RSD was lower than 19 % at spiked concentration 5.0 μg L^{-1} and 20.0 μg L^{-1} for both techniques. The proposed methods were applied to analyze carbofuran real samples *i.e.* water from a reservoir and a number of well water samples. The concentrations of carbofuran in sample were in the range from non detectable to 80 μg L^{-1}.

In conclusion, the proposed methods are excellent to use for determination of carbofuran in the trace level. The analysis methods are reliable, precise and high sensitive. MISPD method has more advantages than MISPE *i.e.* higher sensitivity and lower LOD, consume smaller amount of sample. It is a simple and fast sample preparation technique.

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The completion of this thesis would be impossible without the help of many people, whom I would like to thank.

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My parents, brother, sister, grandmother and best friend for their loves, understanding and encouragement.

And finally, friendship of members in the Trace Analysis and Biosensor Research Center who helped me in innumerable ways during these years.

Sujittra Poorahong

The Relevant of the research Work to Thailand

The purpose of this Master of Science Thesis in Analytical Chemistry is to synthesize and evaluate the performance of carbofuran MIP sorbent. This is useful to apply as the sorbent in sample preparation techniques for the analysis of trace carbofuran in water.

These techniques can be applied for quantitative analysis of trace amount of target analyte by several government and private sectors in Thailand which are, the Ministry of Public Health, the Ministry of Environment and Natural Resource and the Ministry of Education.

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List of Abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
AIBN	α,'α-azobisisobutyronitrile
CAR/PDMS	Carboxen/polydimethylsiloxane
CHO	Choline oxidase
CL	Chemiluminescence
CW/DVB	Carbowax/divinylbenze
CW/TPR	Carbowax/templated resin
DAM	2-(diethylamino) ethylmethacrylate
EGDMA	Ethylene glycol dimethacrylate
EPA	Environmental Protection Agency
ESI	Electrospray ionization
EU	European Union
FIA	Flow-injection analysis
FTD	Flame thermionic detection
GC	Gas chromatography
GC-FID	Gas chromatography - flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GLC	Gas liquid chromatography
HETP	Height equivalent to a theoretical plate
HF-LPME	Hollow fiber-liquid phase microextraction
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography-Ultraviolet detection
HS	Headspace

List of Abbreviations (Continued)

IF	Imprinting factor
IUPAC	The international union of pure and applied chemistry
K_D	Dissociation constant
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase microextraction
MCLs	Maximum contaminant levels
MIP	Molecular imprinted polymer
MISPD	Molecular imprinted solid phase dispersion
MISPE	Molecular imprinted solid phase extraction
MSPD	Matrix solid phase dispersion
OEHHA	The Office of Environmental Health Hazard Protection Agency
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/ divinylbenze
R^2	The coefficient of determination
RSD	Relative standard deviation
SD	Standard deviation
SPD	Solid phase dispersion
SPE	Solid phase extraction
SPME	Solid phase microextraction
UV	Ultraviolet

CHAPTER 1

Introduction

1.1 Background and Rationale

Nowadays, the consumption of fruits and vegetables is greatly increase due to humans concern on their health (Roitner-Schobesberger *et al.*, 2008). Therefore, high quality products without disease and insect are demanded by the national and international market leading to the widely used of pesticides, 2.5 million tones per year over the world (Moral *et al.*, 2008). Pesticides are used by directly apply over plants fields or soils. However it has been estimated that only 0.1 % of the pesticide applied to crops actually reaches the target pest, the rest enters the environment gratuitously, contaminating soil, water and air, increasing a serious environmental problem (Arias-Estevez *et al.*, 2008) as shown in Figure 1.1. Some groups of the pesticides have high acute toxicity when exposed to high dose even when they have low environmental persistence such as carbamate and organophosphate pesticides (EPA, 2007; Soler *et al.*, 2006). Moreover, when accumulated for a long period of time they can also have some effect on human health as well as to the environment. The range of these adverse health effects includes developmental and reproductive effects, neurotoxicity, dysfunction of the immune and endocrine systems, lung damage, birth defects, and cancer (Mansour, 2004). Due to their toxicity, the European Union (EU) has included the pesticides in their list of priority pollutants and the European Union Directive on drinking water quality (98/83/EC) established a maximum allowed concentration of 0.1 $\mu g\ L^{-1}$ for individual pesticides and of 0.5 $\mu g\ L^{-1}$ for total pesticides including their metabolites in drinking water (EU, 1998).

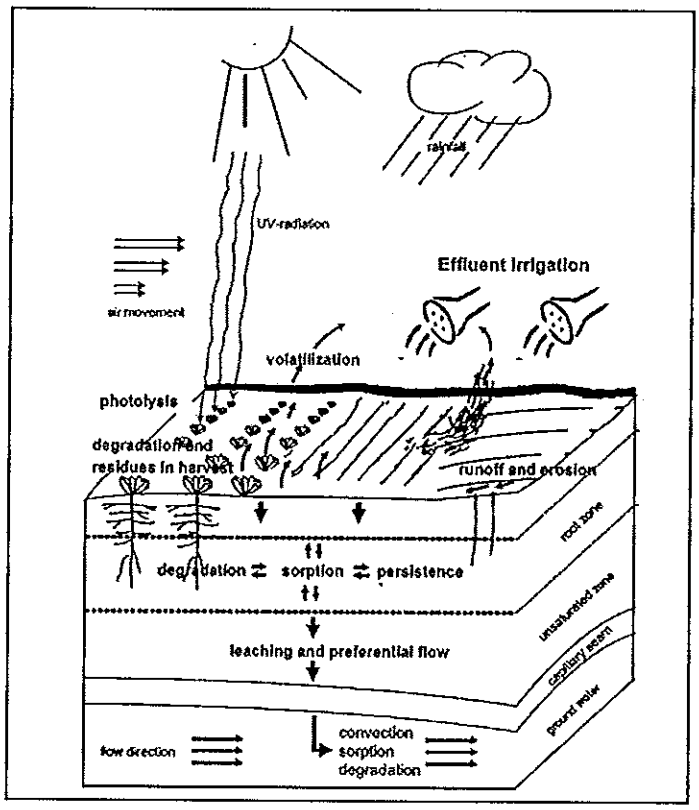


Figure 1.1 The pathways of a pesticide applied to a crop (Muller *et al.*, 2007).

The term pesticides include insecticides, herbicides, fungicides and various other substances used to control pests. Carbamate pesticides are one of pesticides affect the nervous system by inhibition the acetylcholinesterase (AChE) that regulates acetylcholine, a neurotransmitter which the same mechanism toxicity action as the organophosphate pesticides, except their effects is more reversible of their toxicity (EPA, 2007). Pesticides in this group are increasingly used due to their broad spectrum of biological activity (Loewenstein *et al.*, 1993; Soler *et al.*, 2006). Carbofuran is one of carbamate pesticides which agriculturist frequently used to control insects due to its broad-spectrum (Huertas-Pérez *et al.*, 2005). This is also the case in Thailand (Aqueous Solutions, 2008). This pesticide is known to cause nausea, dizziness, confusion, and, at high exposures, respiratory paralysis and death. There has a report on the used of carbofuran that left the residues at trace level in water and soil sample in Thailand (Thapinta and Hudak, 2000). Therefore, the determination of carbofuran contaminated in waters to ensure that human is free from potentially toxic substance is necessary.

The contamination in of carbofuran environmental sample is generally in the low concentration (Nogueira *et al.*, 2003), therefore the development of highly sensitive, selective, rapid and reliable analytical methods is needed. The analysis of trace carbofuran contaminated in water samples are usually carried out by gas chromatography (GC) and high performance liquid chromatography (HPLC), using different detection systems such as nitrogen–phosphorus and flame ionization detector (Cook *et al.*, 1969; Lee and Westcott, 1983; Santos Delgado *et al.*, 2001) in GC and diode array detector (López-Blanco *et al.*, 2002) and fluorescence detector (Sánchez-Brunete *et al.*, 2003) in HPLC technique. Chromatographic methods are generally employed because they can discriminate individual pesticides belonging to the same family. Furthermore, the highly selective detector such as gas chromatography-mass spectrometry (GC-MS) (Maloschik *et al.*, 2007; Zhang and Lee, 2006) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Nogueira *et al.*, 2004; Rodrigues *et al.*, 2007; Soler *et al.*, 2006) can confirm the analytes.

Between the chromatographic techniques, HPLC usually generates organic solvents, used as mobile phase, which requires treatment after the analysis. Chromatography coupled with mass spectrometry offer more accuracy but the instrument and the analysis is more expensive and need expertise. Other method was chemiluminescence with flow injection analysis was also reported for the analysis of carbofuran in water (Huertas-Perez *et al.*, 2005). However, there is the lack of selectivity typically found in such systems *i.e.* many pesticides exhibit similar luminescence properties (Sánchez-Barragan *et al.*, 2007). Therefore, biosensor was developed as the alternative method (Albareda-Sirvent *et al.*, 2001; Bucur *et al.*, 2006; Jin *et al.*, 2004; Palchetti *et al.*, 1997; Suwansa-ard *et al.*, 2005; Tanimoto de Albuquerque and Ferreira, 2007). Although some of the biosensors can be selective but the biological element was not stable ot stable (Huertas-Perez *et al.*, 2005). The disadvantages of the methods have made, gas chromatography coupled with flame ionization detector the most used method for the analysis of carbofuran contaminated in water samples. Normally direct determination of environmental samples cannot be analyzed without some preliminary sample preparation, because contaminants are too low concentration and the matrix is rather complex (Huertas-Pérez *et al.*, 2005). Thus

the high efficient of sample technique that included extraction, preconcentration and interference eliminated are required (Ahmed, 2001; Picó *et al.*, 2007).

Different sample preparation methods have been applied to the determination of pesticides in environmental sample, such as liquid liquid extraction (LLE) with difference solvent (Leong *et al.*, 2007), solid phase extraction (SPE) including on-line and off-line (Albanis *et al.*, 1998; D'Archivio *et al.*, 2007; Fernández *et al.*, 2000; Gonzalez-Rodriguez *et al.*,; Rodrigues *et al.*, 2007; Santos Delgado *et al.*, 2001; Wang *et al.*, 2007), matrix solid dispersion (MSPD) (Fernandez *et al.*, 2000; Valenzuela *et al.*, 1999) and solid phase microextraction (SPME) (López-Blanco *et al.*, 2002). The disadvantages of LLE were tedious, time consuming and requires large amounts of high purity organic solvents, which are expensive, toxic and cause other problems to the environment (Shariati-Feizabadi *et al.*, 2003). In addition some time this method required more than one time and with different solvent in extraction step to obtained high recovery (Leong *et al.*, 2007). SPME allows a rapid and solvent-free extraction, but this technique has some disadvantage such as memory effect, degradation of fibers with increased using time and the fiber is quite expensive (Khalili Zanjani *et al.*, 2007). The advantages of SPE are the reduction of the time required, can handle small samples and requires small volumes of solvent (Huck and Bonn, 2000). There are several types of SPE sorbents available (Hennion, 1999; Masquè *et al.*, 1998). The discrimination between the target compounds and the matrix component to a degree depends on the selectivity of the solid phase. These conventional sorbents are not selective enough to achieve a complete separation. Therefore new type of high selectivity sorbent molecular imprinted polymer (MIP) was introduced.

The aims of this work are to study and develop the solid sorbent by molecular imprinted polymer as solid phase extraction and solid phase dispersion sorbent for qualitative and quantitative analysis of carbamate pesticide contaminated in water. The analysis was performed by gas chromatography coupled with flame ionization detector.

1.2 Carbamate pesticides

Carbamate pesticides are *N*-substituted esters of carbamic acid. They can be classified into nine groups, namely: *N*-methylcarbamates, aminophenyl *N*-methylcarbamates, oxime *N*-methylcarbamates, *N,N*-dimethylcarbamates, *N*-phenylcarmates, benzimidazole carbamates, thiocarbamates, dithiocarbamates and ethylenebisdithiocarbamates. They are mainly used in agriculture, as insecticides, fungicides, herbicides, nematocides, or sprout inhibitors (Nollet, 2006). In addition, they are used as biocides for industrial or other applications and in household products. Thus, these chemicals are part of the large group of synthetic pesticides that have been developed, produced, and used on a large scale in the last 40 years. They were commercial used since the 1950s and the general formula is shown in Figure 1.2.

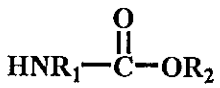


Figure 1.2 General formular of carbamate pesticide where R_2 is an aromatic or aliphatic moiety. Three main classes of carbamate pesticides are classified: (a) carbamate insecticides; R_1 is a methyl group
(b) carbamate herbicides; R_1 is an aromatic moiety
(c) carbamate fungicides; R_1 is a benzimidazole moiety (Nollet, 2006)

Carbamate pesticides are broad spectrum. They affect insects by virtue of their ability to inhibit acetylcholinesterase (AChE) in the nervous system. AChE catalyses the hydrolysis reaction of the neurotransmitter acetylcholine (ACh). ACh is the synaptic mediator of nerve impulses in the nervous system of mammals and insects. The mechanism of toxicity is showed in Figure 1.3. The pesticide bound at serine hydroxyl group of AChE making it inactive. This leads to the accumulation of acetylcholine at the synapse causing hyperexcitation. The insects receiving the pesticides are anxious, tremble and paralysed. This reaction is called carbamylation (Bloomquist, 1999). This reaction is reversible generally within hours, although the time to recovery is chemical-dependent (EPA, 2003).

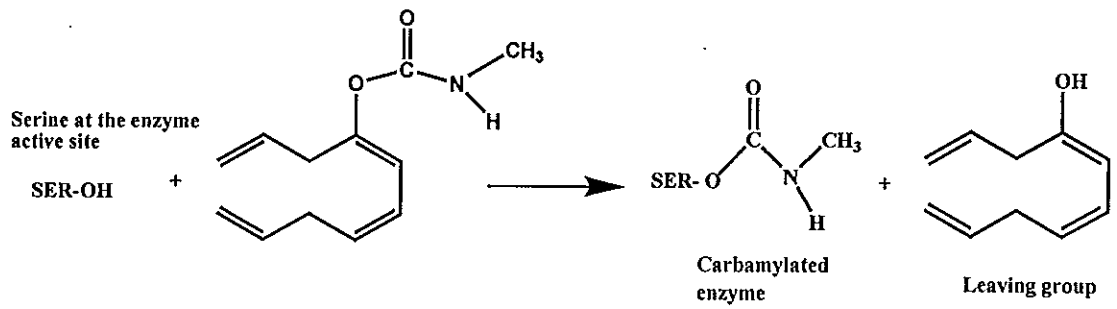


Figure 1.3 Inhibition scheme of AChE by carbamate pesticides (Bloomquist, 1999; Jin *et al.*, 2004).

The acute toxicity for human who are exposed to carbamate pesticides were headache, nausea and dizziness. Anxiety and restlessness are prominent. Worsening may result in muscle twitching, weakness, tremor, incoordination, vomiting, abdominal cramps and diarrhea. Often prominent are sweating, salivation, tearing, rhinorrhea and bronchorrhea. Blurred or dark vision and excessive contraction of the pupil of the eye may also be seen. Tightness in the chest, wheezing and productive cough may progress to frank pulmonary edema. Bradycardia may progress to sinus arrest, or tachycardia and hypertension. Confusion, bizarre behavior, and toxic psychosis may occur. In severe poisonings, toxic myocardiopathy, unconsciousness, incontinence, convulsions, respiratory depression and sometimes death may be seen. Repeated absorption, but not enough to cause acute poisoning may result in persistent anorexia, weakness, and malaise (EPA, 2001a).

1.3 Carbofuran

Common Name: Carbofuran

Chemical Name: 2,3-dihydro-2,2-dimethyl-7-benzofuranyl-*N* methylcarbamate

Chemical Family: *N*-methyl Carbamate

Empirical Formula: $C_{12}H_{15}NO_3$

Molecular weight: 221.3

Trade Names: Furadan

Carbofuran (Figure 1.4) is an odorless, white, crystalline solid with a melting point range of 150-154 °C. It is slightly soluble in water, and is highly soluble in *N*-methyl-2-pyrrolidone, dimethylformamide, dimethyl sulfoxide, acetone, acetonitrile, dichlormethane, cyclohexanone, benzene, and xylene (EPA, 2006). It is used to control soil and leave-feeding insects and nematodes (Bacigalupo *et al.*, 2006). Carbofuran products are used worldwide for control of insects on a wide variety of fruit, vegetables, forage, cotton and other crops including bananas, coffee beans, grapes, potatoes, corn, rice, sugarcane, and wheat (Huertas-Pérez *et al.*, 2005). Carbofuran can be spayed on plants and soils or seedling dip.

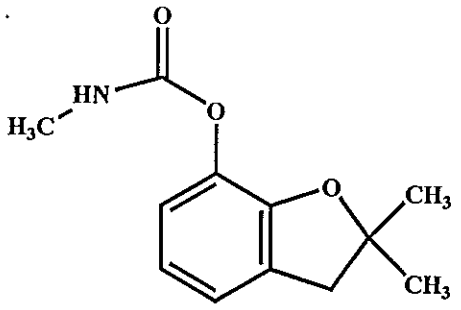


Figure 1.4 Chemical structure of carbofuran.

The current US Environmental Protection Agency (EPA) maximum contaminant levels (MCLs) for carbofuran in drinking water is 40 μg L^{-1}. However, the current California MCL is 18 μg L^{-1} and the Office of Environmental Health

Hazard Protection Agency (OEHHA) of the California Environmental Protection Agency proposes a public health goal of 1.7 μg L^{-1}. The European Union has set a maximum concentration for the sum of admissible concentration of 0.5 μg L^{-1} for the sum of all pesticides and 0.1 μg L^{-1} for an individual compound in drinking water (López-Blanco *et al.*, 2002).

1.4 Analytical method

Accurate and sensitive analytical methods are required for reliable environmental control analyzed. Gas chromatography (GC) and high performance liquid chromatography (HPLC) are currently the most flexible and sensitive method for analysis. The alternative techniques in growing use for the determination of carbamate pesticides in samples are biosensors, chemiluminescence and post column derivatization coupled with fluorescence detection.

1.4.1 Gas chromatography (GC)

Carbamate pesticides in environmental sample have been long determined by GC. Normally, capillary column with various detectors were used. Many types of capillary column with nonpolar stationary phase were used which are HP-1 (100 % dimethylsiloxane) (Santos and Galceran, 2002), HP-5 and HP-5 MS (5% phenyl 95 % methylpolysiloxane) (Elflein *et al.*, 2003; Lambropoulou and Albanis, 2005; Liu *et al.*, 2006) and DB-5, the same stationary phase with HP-5 (Jin *et al.*, 2006; Zhang and Lee, 2006).

For detector of GC, the nitrogen phosphorous detector was usually used since carbamate pesticides are nitrogen-containing compounds. It provides high sensitivity and selectivity when compared to HPLC (Lambropoulou *et al.*, 2002; Sánchez-Brunete *et al.*, 1998; Santos Delgado *et al.*, 2001). Lambropoulou and Albanis (2005) used flame thermionic detection (FTD) to detect many insecticides from water samples. Before analysis the sample was extracted by liquid phase microextraction. The analytes were separated by DB-5 column, 30 m×0.32 mm I.D. provided high resolution of the chromatograms of nine analytes (Lambropoulou and

Albanis, 2005). Flame ionization detector was also used for determination seven pesticides in powdered potato samples. The analytes were previously extracted with a light petroleum– dichloromethane (1:1 v/v) mixture and preconcentred by solid-phase extraction through a C18 cartridge. This technique gave high percentage recoveries with low percentage standard deviation (Santos Delgado *et al.*, 2001).

Recently, the mass selective detector is favoring the detection system of choice for GC by virtue of its high selectivity. GC coupled to MS is the mode common choice for analysis of pesticide in many samples. They are increasingly used due to the efficiency of GC separation, the qualitative information and high sensitivity provided by MS (Nollet, 2006). Many publications reported the used of GC-MS for determination of pesticides in water samples (Liu *et al.*, 2006; Maloschik *et al.*, 2007; Zhang *et al.*, 2006). In all cases, detections were carrier out in positive mode and single monitoring was performed in SIM mode.

1.4.2 High performance liquid chromatography (HPLC) and liquid chromatography (LC)

HPLC is being extensively used for pesticide compounds when the analytes are low volatility, thermally unstable or polar for GC separation (Nollet, 2006). The most used reversed phase chromatography is with C18 or C8 columns (Gou *et al.*, 2000; Rodrigues *et al.*, 2007; Sánchez-Brunete *et al.*, 2003; Soriano *et al.*, 1998). Due to its wide scopes of application, long term stability, ease to use, low cost and selectivity (diode array), UV detection has often been used to determine carbamate pesticides (Gou *et al.*, 2000; Soriano *et al.*, 1998). Direct florescence detection is not widely used since most carbamate pesticides possess no native fluorescence. However, the structure of these pesticides contains an *N*-methyl substituted urethane with variations in the ester moiety. The common methylamine functionality allows the detection of compounds via a two-stage postcolumn reaction which is the EPA standard method for determination carbamate pesticide. EPA method 531.2 determines carbamate pesticides by a HPLC system equipped with a reversed phase (C18) column to separate the analytes. After elution from the column, the analytes are hydrolyzed in a postcolumn reaction with NaOH solution at 80 - 100

°C to form methyl amine which subsequently reacts with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (or N,N-dimethyl-2-mercaptoethyl amine) to form a highly fluorescent isoindole which is detected by a fluorescence detector (EPA, 2001b). Many publications used these reaction to detect carbamate pesticides which show a higher sensitivity and selectivity with specific detectors such as fluorescence for determined carbamate pesticides in many real sample (Herrera *et al.*, 2002; Sánchez-Brunete *et al.*, 2003; Stafford and Lin, 1992). However, this method suffers from some drawbacks and limitations. First, it makes use of time-consuming cleanup procedures. Second, co-extracted substances having native fluorescence may interfere with the analysis (Bogialli *et al.*, 2004).

LC-MS has been widely accepted as an advantage choice for the determination of carbamate pesticide from many matrices which is more robust and flexible in the absence of derivatization. Atmospheric pressure sources such as electrospray ionization (ESI), present several advantages as the samples are ionized directly in the liquid phase at a *quasi*-ambient temperature, minimizing the degradation of thermally labile compounds (Nogueira *et al.*, 2003; Nollet, 2006) is commercially available and have been employed for developing methods for determining carbamate residues (Bogialli *et al.*, 2004; Nogueira *et al.*, 2003; Nogueira *et al.*, 2004; Wu *et al.*, 2002; Xu *et al.*, 2007a). Moreover, the major attraction of ESI applied to water analysis is the low LOD usually found. However, LC-MS is aways less sensitive than GC-MS as a result of the need to transfer the analytes from the liquid phase into a high- vacuum gas phase. Other limitations of LC-MS combination include the inability to use nonvolatile buffers, the narrow optimum range for eluent flow rate influence of the proportion of organic modifier on the sensitivity, the narrow choice of ionization methods (Honing *et al.*, 1995). Furthermore, to achieve excellent sensitivity with complex matrix, tandem mass spectrometry (MS/MS) that uses three quadrupoles was used (Goto *et al.*, 2005; Goto *et al.*, 2006; Rodrigues *et al.*, 2007; Soler *et al.*, 2006), because it eliminates interference prior to measurement of ions originated from target compounds. The first quadrupole functions as a mass filter that passes through only ions within a small range of masses. The second one is the collision chamber, where the isolated ions are cleaved and transferred to the third one. The third one filters the results of the cleavage so they can be scanned as the product

ion spectrum (Goto *et al.*, 2006). Table 1.1 summarized the method for the pretreatment and analyses of carbamate pesticides.

Table 1.1 Summary of sample pretreatment and chromatographic detection for carbamate pesticides.

Matrices	Extraction method	Detection method	Derivatization reagent	Chromatographic column/ mobile phase	Recovery	Limit of detection	Reference
environmental water	hollow fiber liquid phase microextraction (HF-LPME)	GC-FTD	-	DB-5 (30m ×0.32mm I.D.)	80-102	1-43 ng L^{-1}	(Lambropoulou and Albanis, 2005)
Kalamas River	SPME	GC-NPD	-	DB-5 (30 m×0.32mm I.D.)	87-110	0.005-0.08 μg L^{-1}	(Lambropoulou *et al.*, 2002)
Powdered potato	LLE (light petroleum - dichloromethane (1:1, v/v) mixture) /SPE (C8)	GC-FID	-	VA-5 (30 m×0.25mm I.D.)	72 -115	50–210 μg kg^{-1}	(Santos and Galceran, 2002)
Rice	LLE (hexane and acetone (4:1, v/v))/ SPE (Florisil)	GC-MS	-	HP-5 MS (30m×0.25mm I.D.	75 -120	0.41 -87 μg kg^{-1}	(Liu *et al.*, 2006)
Water	LPME	GC-MS	Trimethylphenylammonium hydroxide and trimethylsulfonium hydroxide	DB-5 (30m×0.32mm I.D.)	-	0.2 – 0.8 μg L^{-1}	(Zhang and Lee, 2006)
surface water	SPE (CarboPrep-90 and Carbograph)	GC-MS	-	CP-Sil 8 CB 30m×0.25mm I.D.	93 -102	0.001–0.5 μg L^{-1}	(Maloschik *et al.*, 2007)
soil	LLE (methanol)	LC-fluorescence	OPA	C8 (methanol–water)	82-99	1.6-3.7 μg kg^{-1}	(Sánchez-Brunete *et al.*, 2003)
water	SPME	HPLE-UV	-	C18 (50% acetonitrile in water)	97-100	1-15 μg L^{-1}	(Gou *et al.*, 2000)
water and wine	SPME	LC-MS	-	C18 (40:60 acetonitrile:water)	84-110	0.03-0.32 μg L^{-1}	(Wu *et al.*, 2002)

1.4.3 Biosensors

Biosensor is an analytical technique incorporating a biological sensing element with a signal transducer, to give a sensing system specific to the target analyte. Enzyme, antibody, nucleic acid, cell or tissue can all be used as biological sensing element. Transducer can be electrochemical, optical, piezoelectric or calorimetric (Canh, 1993; Eggins, 1996).

Enzymatic biosensors based on inhibition of AChE have been intensively studied, because carbofuran pesticide can inactivate AChE. In the presence of such compounds the rate of choline production is reduced. AChE hydrolyses acetylcholine to choline, according to the following reaction (1.1):

$$\underset{\text{Acetylcholine}}{CH_3COO(CH_2)_2N^+(CH_3)_3} + H_2O \xrightarrow{AChE} \underset{\text{Choline}}{HO(CH_2)_2N^+(CH_3)_3} + \underset{\text{Acetic acid}}{CH_3COO^-} + H^+ \quad (1.1)$$

The reaction can be detected by two systems potentiometric, using pH electrode, to detect the increase of hydrogen ions (Jin *et al.*, 2004), and conductimetric to detect the increase of conductivity from the increase of ion (Bucur *et al.*, 2006; Suwansa-ard *et al.*, 2005). In another work, bienzyme system was studied (Palchetti *et al.*, 1997) using AChE and choline oxidase. Followed reaction of AChE (1.1) choline oxidase (CHO) catalysed the oxidation of choline as in reaction (1.2).

$$\textbf{Choline} + O_2 \xrightarrow{CHO} \textbf{Betain aldehyde} + H_2O_2 \quad (1.2)$$

Hydrogen peroxide was then oxidized on the surface electrode and detected by amperometric detection by screen-printed electrodes.

Besides the used of AChE, tyrosinase has also been employed. Tyrosinase can catalyze catechol to quinoid products. The amperometric detection can be detected by measuring the quantity of catechol consumed or of o-quinone formed in the reaction (Figure 1.5). The addition of the pesticides caused a decrease in the steady state current for o-quinone produced in the enzymatic reaction and this decrease was used to measure the corresponding inhibition processes. The inhibition

response is dependent on the catechol and pesticide concentrations (Besombes *et al.*, 1995; Tanimoto de Albuquerque and Ferreira, 2007).

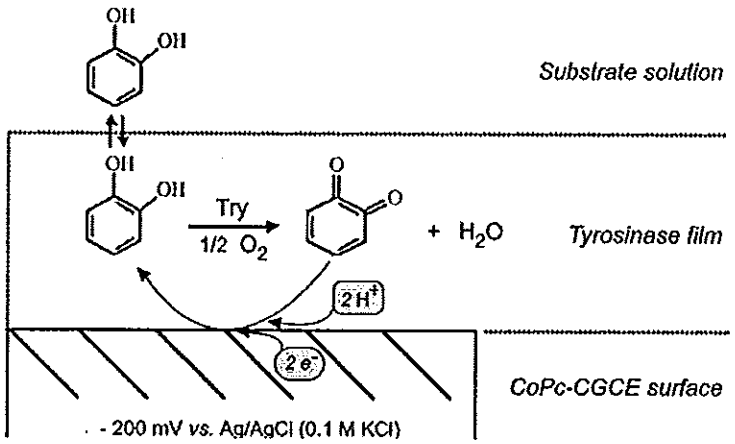


Figure 1.5 Principle of the enzymatic and electrochemical coupled reactions for the detection of catechol as substrate (Tanimoto de Albuquerque and Ferreira, 2007).

Campanella and coworkers (2007) also used bienzyme, first butyrylcholinesterase enzyme was used instead AChE. The reaction is shown in reaction 1.3. Followed reaction of butyrylcholinesterase (1.3) choline oxidase (CHO) catalysed the oxidation of choline (reaction 1.2) (Campanella *et al.*, 2007).

$$\textbf{Butyrylcholine} \xrightarrow{\textbf{butyrylcholinesterase}} \textbf{choline + butyric acid} \qquad (1.3)$$

With carbamate pesticides in the solution, the reaction can be inhibited.

In another approach, Pogačnik and Franko (1999) determined carbamate pesticide by an inhibition photothermal biosensor. The biosensor consists of a cartridge containing immobilized enzyme AChE placed in a flow-injection analysis (FIA) manifold and a photothermal detector based on thermal lens spectrometry. They used acetylthiocholine as the substrate. The determination of enzyme activity was assayed based on two coupled reactions (reaction 1.4 and 1.5):

$$\text{Acetylyhiocholine} \xrightarrow{\text{AChE}} \text{acetic acid + thiocholine} \quad (1.4)$$

$$\text{Thiocholine + 5,5'-dithio-bis-2-nitrobenzoic acis (DTNB)} \longrightarrow \text{2- nitrobenzoate-5-mercaptothiocholine + 5-thio-2-notrobenzaate } (TNB^{2-}) \quad (1.5)$$

The product TNB^{2-} is yellow with an absorption maximum at 410 nm and can be detected optically (Pogačnik and Franko, 1999).

In addition to enzyme, Chouteau and coworkers (2005) used whole cell of *Chlorella vulgaris* microalgae (bioreceptors) as a bi-enzymatic biosensor which is known to be inhibited by carbamates for AChE and heavy metals for alkaline phosphates. They used conductivity as the detection system. Algae were immobilized inside bovine serum albumin membranes reticulated with glutaraldehyde vapors deposited on interdigitated electrodes. The use of micro-organisms for multi-detection can be a good alternative, each living cell containing a large number of enzymes (Chouteau *et al.*, 2005). However, the inhibition signal was obtained after 30 minutes. All enzyme inhibitions were obtained according to Equation 1.1:

$$I(\%) = \frac{I_1 - I_2}{I_1} \times 100 \quad (1.1)$$

Where I % is the degree of inhibition,

I_1 is the current before (without inhibitor)

and I_2 is the current after the incubation in solution with inhibitor (Suwansa-ard *et al.*, 2005; Tanimoto de Albuquerque and Ferreira, 2007).

These techniques were not selective of biological element to substrate because many carbamate and organophosphate pesticide can inhibit the oxidation of enzymes and the biological element was not stable (Huertas-Perez et al., 2005).

1.4.4 Chemiluminescence (CL)

Moris and coworkers (1995) used the enzyme-linked inhibition chemiluminescence (CL) detection of carbofuran pesticide, employing three consecutive enzymatic reactions of AChE (reaction 1.1), choline oxidase (reaction

1.2) and peroxidase (reaction 1.6) which produces photons when luminol is used as a substrate.

$$H_2O_2 + \text{luminol} \xrightarrow{\text{peroxidase}} \text{aminophtalic acid} + H_2O + \text{light} \qquad (1.6)$$

In this case, carbofuran inhibit AChE activity, so the acetylcholine is not hydrolyzed into choline and subsequently it is not oxidized into hydrogen peroxide, blocking the oxidation of luminol and decreasing the CL emission. This method has been reported only for the analysis of tap water at μg L^{-1} level (Moris *et al.*, 1995). The method is highly sensitive but the enzymes employed are very expensive, unstable of enzyme and not selective since AChE and choline oxidase can oxidize both carbamate and organophosphate pesticides (Huertas-Pérez *et al.*, 2005). Huertas-Pérez and coworkers (2003) studied CL reaction from luminol oxidation by potassium permanganate in alkaline medium without catalyst. The detection carried out by the increasing of CL when presence carbofuran pesticide proportional to pesticide concentration. The mechanism of reaction could be as follows: carbofuran is firstly oxidized by permanganate, to produce an intermediate which could subsequently oxidized luminol to form the excited state 3-aminophthalate anion; finally this excited anion decayed to the ground state and produce CL (Huertas-Pérez *et al.*, 2005). This technique was applied for in many real samples (river, ground and tap water and lettuce sample). This technique provided acceptable recovery for routine analysis (60-140%). However many pesticides exhibit similar luminescence properties, explaining the lack of selectivity typically found in such systems (Sánchez-Barragan *et al.*, 2007).

1.5 Sample preparation

Sample preparation is crucial for the analysis of compounds in real samples, since it is the most tedious and time-consuming step and a possible source of imprecision and inaccuracy of the overall analysis (He *et al.*, 2007). The need of high effective, robust and reliable sample preparation, leads to the development of many procedures with the aim of achieving fast, simple and if possible, solvent-free or

solvent-minimized operations. These procedures depend on the polarity of pesticides and on the type of sample matrix. Most of these techniques, both conventional (soxhlet extraction and LLE) and new (SPE, SPME, SFE, MSPD), are used for the analysis of pollutants in many sample (Nollet, 2006; Santos and Galceran, 2002).

1.5.1 Liquid liquid extraction (LLE)

LLE is a widely used technique among the EPA method for preconcentration of pesticides in liquid samples. Nonpolar solvents for the LLE of pesticides include *n*-hexane, benzene and ethyl acetate (Jansson *et al.*, 2004). Mixed solvents have often been used to finely adjust the solvent strength. Acetonitrile/ethyl acetate (1/4, v/v) were used to extract analytes from plasma samples and determine by HPLC-UV. This technique provides high recovery (Zuffa *et al.*, 2004). Also a method based on the extraction by sonification of solid samples placed in small columns using a low volume of methanol was developed for the extraction of six carbamates from soil with recoveries between 82 and 99 % (Sánchez-Brunete *et al.*, 2003). Granby and coworkers (2004) used methanolic ammonium acetate–acetic acid buffer extracted pesticides from vegetables that gave high accuracy. As the analytical procedure does not include any concentration or cleanup steps (Granby *et al.*, 2004).

Although LLE is a simple technique, it has a number of drawbacks including the formation of emulsion, the risk of losses and contamination, the need to used high solvent volume and its difficult automate, which make it a labor-intensive, time consuming and expensive (Nollet, 2006).

1.5.2 Liquid phase microextraction (LPME)

Liquid phase microextraction (LPME) has emerged as an attractive alternative for sample preparation since the principle is the same as LLE. LPME can be performed by using a single drop of solvent or a small length of porous hollow fiber-protected solvent. This novel technique, which is fast and simple, eliminates the disadvantages of LLE such as time consuming operation. It is inexpensive and there is considerable freedom in selecting appropriate solvents for extraction of different

analytes. Since very little solvent is used, there is minimal exposure to toxic organic solvent for the operator. At the same time, LPME combines extraction, concentration and sample introduction in one step. The important feature of the LPME is that almost all of the organic solvent into which the analytes are extracted can be injected into the GC. Normally, hydrophobic membrane which is polypropylene was used to extract the analyte from the sample.

Similar to SPME, there are two modes of LPME sampling: direct-immersion LPME and headspace LPME (HS-LPME). Lambropoulou and Albanis (2005) used the Accurel Q 3/2 polypropylene hollow fiber membrane for direct extraction of nine pesticides in the organophosphorous and carbamate groups. Many parameters of the LLE that affect the extraction efficiency were studied, *i.e.* agitation or stirring rate to increase mass transfer, extraction time, type of extraction solvent and sample volume. This method provided high relative recovery between 80 -102 %. This technique used relative recovery because it is a non-exhaustive extraction procedure, determined as the ratio of the concentrations found in natural and distilled water samples, spiked with the same amount of analytes. When compared the result with the one obtained from SPME, it showed that LODs with HF-LPME lower than SPME (Lambropoulou and Albanis, 2005).

In another development, organic solvent-based hollow fiber protected LPME coupled with on-column derivatization was used for the determination five pesticides in aqueous samples. This method was carried out by withdrawing derivatization reagent into the microsyringe holding the final extractant and injecting the mixture in the GC–MS. On-column derivatization was applied as the derivatization technique to extract carbamates. Both LPME and on-column derivatization are one step procedures, each using only several microliters of reagent with high enrichment factors ranging from 37 to 144 (Zhang and Lee, 2006).

1.5.3 Solid phase microextraction (SPME)

SPME is based on the analyte partitioning between an aqueous sample and a polymeric stationary phase. The absorption dynamics are described mathematically by Equation. 1.2.

$$n = \frac{K_{fs} V_f C_o V_s}{(K_{fs} V_f + V_s)} \quad (1.2)$$

Where n is the moles of analyte absorbed by the stationary phase,

K is the analyte partitioning coefficient between the stationary and the aqueous phase

V_f, V_s is volume the stationary phase of fiber and sample, respectively

and C_o is the initial analyte concentration in the aqueous phase (Pawliszyn, 1999)

Currently, several kinds of SPME fibers are commercially available. In addition to polymeric materials such as polydimethylsiloxane (PDMS), polyacrylate (PA), other fiber coatings based on solid sorbents such as polydimethylsiloxane/ divinylbenze (PDMS/DVB), carboxen/polydimethylsiloxane (CAR/PDMS), carbowax/divinylbenze (CW/DVB), and carbowax/templated resin (CW/TPR) have also been applied (Zeng *et al.*, 2008). There are currently three SPME modes that require either fused-silica fibers or GC capillary columns. Headspace (HS) and direct insertion (DI-SPME) are the two fiber extraction modes, while the GC capillary column mode is referred to as in-tube SPME. Direct SPME is the most common mode for analysis. It is conducted by directly inserting the fiber into the sample matrix (Krutz *et al.*, 2003). Sixty seven compounds from 14 herbicide families have been quantified with DI-SPME. The mode is generally rugged and precise as demonstrated by Boyd-Boland and coworkers (1993) who simultaneously quantified 22 from eight herbicide families: chloroform, acetamides, diphenylether, nitroanilines, uracils, substituted amides, thiocarbamates, triazines and triazoles. The limit of detection (LOD) was obtained between ng and sub-ng L^{-1} (Boyd-Boland *et al.*, 1993).

The HS-SPME mode is adapted for the analysis of volatile analytes. The primary advantage of HS-SPME is the prevention of direct fiber contact with the sample thus lowering background noise (Krutz *et al.*, 2003). Maloschik and coworkers (2007) studied the contamination of ten pesticides in Hugarian surface water by directly extraction using a 65 μm thick CW/DVB fiber. Extraction time was 20 minutes at room temperature with stirring by means of a magnetic stirrer and

subsequently analyzed by GC-MS. They compared the obtainable result with SPE and found that the results obtained from SPE extraction provided high recovery with low percentage relative standard deviation (%RSD) (Maloschik *et al.*, 2007). In addition SPME fiber has many drawbacks, such as fragility of the fiber, nonresistance to high temperature and organic solvents, high cost and short life usability (Zeng *et al.*, 2008).

1.5.4 Matrix solid-phase dispersion (MSPD)

MSPD method is performed by disrupting the sample while dispersing its components into a solid support. MSPD combines sample homogenization with preliminary clean-up of the analytes. The method involves the dispersion of the sample in a solid sorbent such as C18 or C8 (Valenzuela *et al.*, 1999), followed by preliminary purification and the elution of the analytes with a relative small volume of solvent as shown in Figure 1.6. The extractants obtained are generally ready for analysis, but, if necessary, they can easily be subjected to direct extract purification.

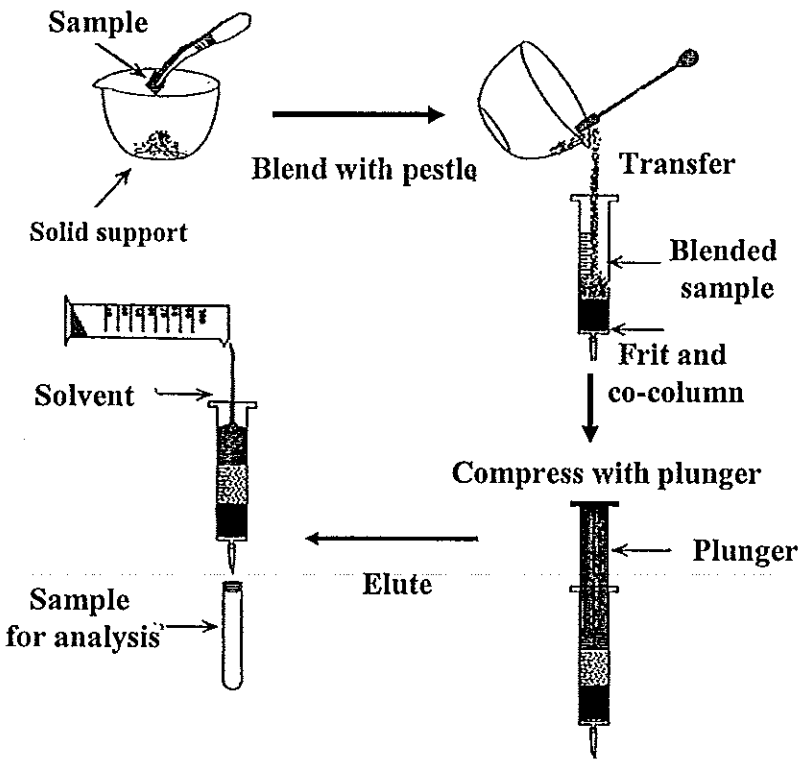


Figure 1.6 Steps in a typical MSPD extraction (Barker, 2007).

MSPD is different from classical SPE in physically and functionally *i.e* (1) sample is completely disruption and dispersal onto very small size particles, providing an enhanced surface area for subsequent extraction of the sample. In SPE sample disruption must be conducted as a separate step in preparing samples and many sample components must be discarded in the process to produce the sample suitable for addition to an SPE column. (2) In SPE the sample is usually absorbed onto the top of the column packing material, not throughout the column as in MSPD. (3) The physical and chemical interactions of the components of the system are greater in MSPD than SPE (Barker, 2007).

1.5.5 Solid phase extraction (SPE)

SPE is a widely used sample-preparation technique for the isolation of selected analytes which based on the partition equilibrium between sorbent and eluting solvent which depended on the affinity of the sorbent (Masquè *et al.*, 1998). The analytes are transferred to the solid phase where they are retained for the duration of the sampling process. The principal goals of SPE are preconcentration, sample clean-up and transfer from the sample matrix to a different solvent. Normally, the SPE sorbents are in the range 50 -60 μm (Poole, 2003). Nowadays, conventional SPE materials are modified silica with C8, C18, CN and other groups, carbon blacks and styrene-divinylbenzene copolymers (PS-DVB) (Masquè *et al.*, 1998). The SPE designs frequency used are in the form of a cartridge, syringe or disk. SPE disks differ from SPE cartridges or syringes, the disk is a membrane loaded with a solid sorbent, whereas the cartridge or syringe contains the sorbent (Figure 1.7).

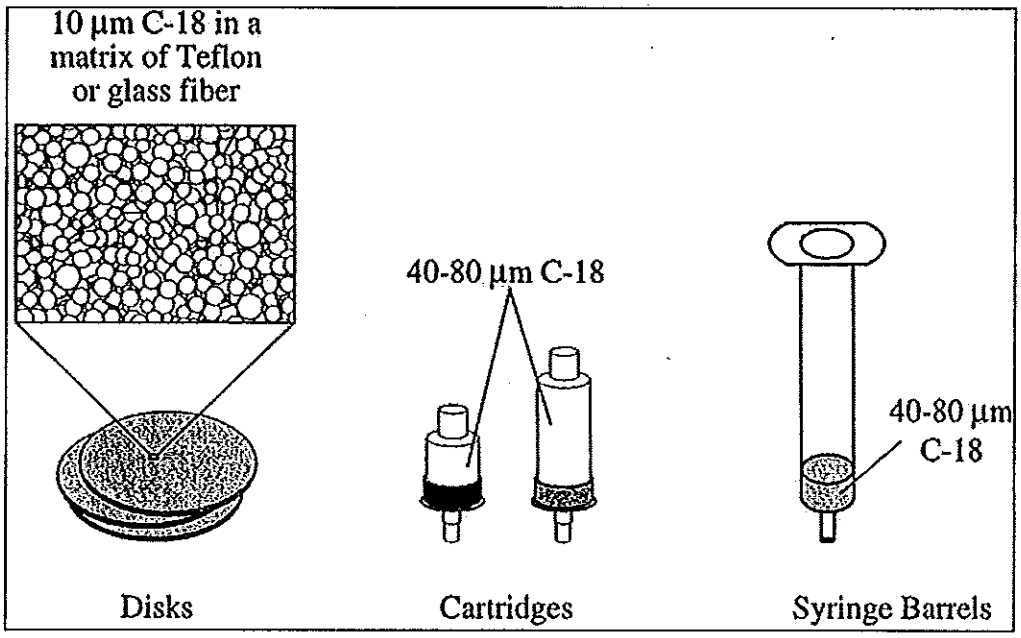


Figure 1.7 Three formats for solid phase extraction, disks, cartridges, and syringe barrels.

Sample processing in solid-phase extraction consists of four steps.

(1) Conditioning: The functional groups of the sorbent are solvated in order to activate them for interaction with analytes. Thus, the sorbent were conditioned with solvent to improve the reproducibility of analyte retention and to reduce the sorbent impurities carry through at the elution stage (Poole, 2003). The most commonly used conditioning for reversed phase is accomplished by passing the solvent with increasing polarity for about three times. First, the mixtures of various solvent were used *i.e* either acetone or acetonitrile, then small volume of methanol and finally, ultra pure passed through the SPE (López-Blanco *et al.*, 2002; Nogueira *et al.*, 2003; Nogueira *et al.*, 2004; Rodrigues *et al.*, 2007). Some of this organic solvent is adsorbed on the surface of the sorbent particles, making the surface more hydrophilic and thus more compatible with a primarily aqueous sample solution. Without such treatment the surface of many common sorbents is hydrophobic and is poorly wetted by the hydrophilic sample solution. The polar liquid flows in small channels through the solid phase without making the necessary close surface contact. The

conditioning step also serves to elute any adsorbed organic close surface contact (Fritz and Macka, 2000).

(2) Sample loading: the sample was passed through the cartridge with a controlled flow rate. Before loading sample, the sample pretreatment should be done, for example sample dilution with low viscosity solvent to reduce sample processing time, remove excessive particle matter by filtration or centrifugation to maintain a constant sample-processing rate, adjust pH to reduce ionization of weak acids and bases for reversed-phase, maintain approximately constant ionic strength for samples and standards when using reversed-phase conditions. Ionic strength is a critical parameter for ion-exchange extraction or deproteination of biofluids that required for acceptable recovery of low-molecular weight analytes for reversed-phase.

(3) Washing: small volume of intermediate strength solvent was passed through the cartridge to elute matrix component (unwanted), since analytes remain immobilized on the sorbent.

(4) Eluting: a small volume of strong solvent was passed through the cartridge to displace all analyte from the sorbent.

Parameters affecting the recovery of this extraction need to be studied, *i.e.* type and volume of eluting and washing step, flow rate of each step and drying time.

The drying step between processing aqueous samples and eluting the retained analyte with a water-miscible organic solvent is also important. The purpose of the drying step is to reduce the volume of water retained by the eluting solvent.

Many reports evaluated the different types of sorbent, Rodrigues and coworkers (2007) compared the extraction recovery of Waters Oasis HLB, Merck LiChrolut EN, Macherey-Nagel C18, Macherey-Nagel C8 and Macherey-Nagel HR-P. They found that only Macherey-Nagel C18 and Macherey-Nagel HR-P provided satisfactory recovery (Rodrigues *et al.*, 2007). Furthermore, Nogueira and coworkers (2004) used three different types of sorbent, Varian-Envi (Bond Elut), Supelco (LC-18) and Waters (Oasis HLB). Both of these can provided satisfactory recovery (Nogueira *et al.*, 2004).

SPE can discriminate between the target compounds and the matrix component to a degree that depends on the selectivity of the solid phase. These conventional sorbents are not selective enough to achieve a complete separation. Therefore, the new types of high selectivity sorbent were introduced which are immunosorbent and molecular imprinted polymer (MIP).

1.5.5.1 Immunosorbent

Immunosorbent is based on the high affinity and selectivity of antigen–antibody. Antibodies form covalent bond with appropriate solid sorbent such as agarose, activated silica and sol–gel (Zhang *et al.*, 2006) and packed in the cartridges. The interactions were carried out by a selective extraction of the target analyte and compounds having a similar structure or metabolites of target compound as showed Figure 1.8 (Pichon, 2007). The target goal of the development of immunosorbent was a one step process for extraction, clean up and preconcentration (Delaunay *et al.*, 2000).

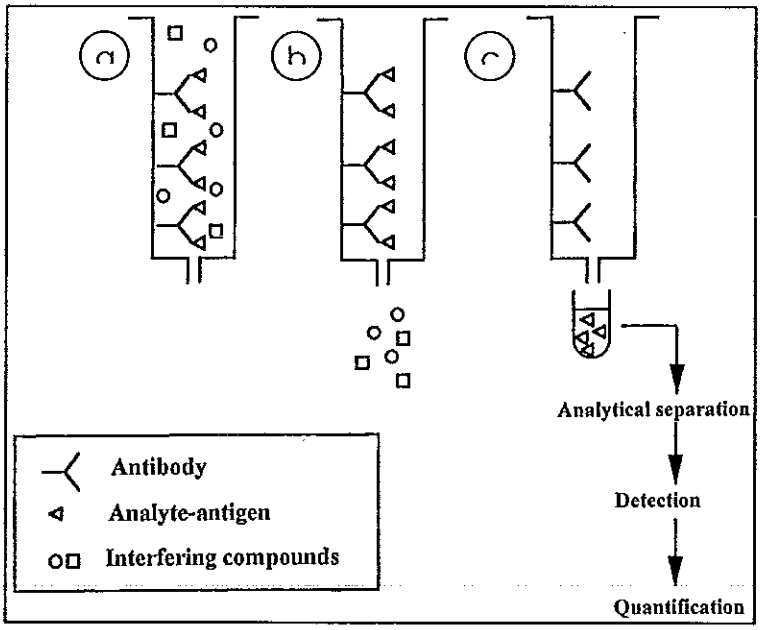


Figure 1.8 The immunosample pretreatment on immunosorbents. (a) percolation of the sample; (b) washing to eliminate the nonretained compounds; (c) elution of compounds retained by the immobilized antibodies (Delaunay *et al.*, 2000).

Many parameters can affect the extraction recovery. Parameters for immunosorbent preparation that can affect the extraction efficiency are type of antibody and solid support and condition of antibody immobilization.

However, the antibody has low stability and environmental tolerance such as pH, temperature, ionic strength and organic solvent and poor inter-batch reproducibility (Hillberg *et al.*, 2005). These drawbacks have led to the recent development of molecular imprinted polymer.

1.5.5.2 Molecular imprinted polymer (MIP)

Molecular imprinted polymers are the crosslinked polymeric materials that exhibit high binding capacity and selectivity against a target molecule (template) purposely present during the synthesis process (García-Calzón and Diaz-García, 2007). In this method, functional monomers were pre-arranged around the template in appropriate porogen. Then the polymerization occurs in the presence of crosslinker to stabilize the arrangement of three dimension polymer matrix. The template was then removed producing a polymer containing a size cavity specific for analyte (Figure 1.9).

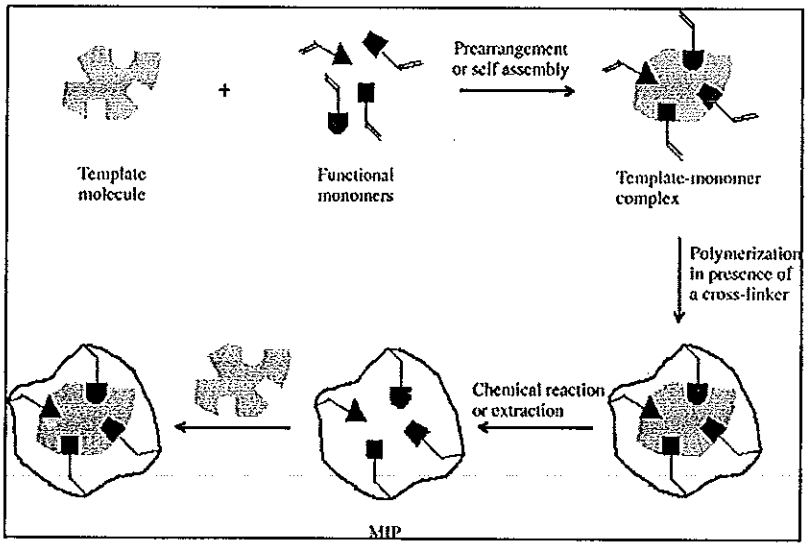


Figure 1.9 Synthesis of molecularly imprinted polymers (MIPs) and its selective recognition to target molecule (He *et al.*, 2007).

The arrangement of the monomers around the template can be done via covalent and non covalent interaction. For the covalent interaction, covalent bond between the template and the functional monomers were necessary prior to polymerization. To remove the template from the polymer matrix after synthesis, it was necessary to cleave the covalent bonds.

In the non covalent procedure, the complexation step was achieved by mixing the template with an appropriate functional monomer, or monomers in a suitable porogen in pre-polymerization step. Normally, an excess of functional monomer relative to the template is usually required to template – functional monomer complex formation. After synthesis, the template is removed from the polymer matrix by washing with an appropriate solvent or a mixture of solvents. The average binding forces of non covalent binding is generally weaker than the one prepared by using covalent methods because non covalent binding is electrostatic, hydrogen bonding, π-π and hydrophobic interactions between the template and the functional monomers (Caro *et al.*, 2006). The basic advantages of non covalent bonding are a higher chance of producing cavities of the desired selectivity, affinity and easy to prepare. The functional monomers and template are simply mixed together and allowed for interact via self assembly and finally easy extraction of the template from the polymer matrix (Cai and Gupta, 2004). In addition some research have demonstrated that the polymer prepared from two functional monomers (cocktail polymerization) improved the recognition capacities compared to polymer that used only single functional monomer (Wei *et al.*, 2005).

The performance of MIP is depending on the initial choice of polymerization ingredients and polymerization conditions that used in polymerization step. These factors consist of nature of template, functional monomer, cosslinker, porogen, method of initiator, polymerization temperature and time (Cormack and Elorza, 2004; Tamayo *et al.*, 2007).

1.6 Synthesis of molecular imprinted polymer

1.6.1 Template

Template is the selected target molecule used in the imprinting procedure. Generally, the analyte was used as the template molecule. It directs the organization of the functional groups pendent to the functional monomers. In free radical polymerization, templates should be chemically inert under the polymerization conditions, thus alternative imprinting strategies may have to be sought if the template can participate in radical reactions or is for any other reason unstable under the polymerization conditions (Cormack and Elorza, 2004). Furthermore the shape of the template may be sufficient to create the necessary steric complementarily for efficient discrimination between two molecules (Sellergren, 1999).

1.6.2 Functional monomer

Functional monomers are responsible for the interaction with the print molecule (template). The ratio of functional monomer to template is clearly an important parameter in the polymerization mixture. Too much functional monomer may contribute to high non-specific binding while too little will result in a poor yield of imprinted sites due to inadequate complexation of the template (Mayes and Whitcombe, 2005).

1.6.3 Crosslinker

In molecular imprinted polymer, crosslinker fulfills three functions. First, it controls the morphology of polymer matrix. Second, it serves to stabilize the imprinted binding site. Finally, it is used to mechanically stabilize the polymer matrix (Cormack and Elorza, 2004). Quite a number of cross-linkers compatible with molecular imprinting are known, many of which are commercially available and a few of which are capable of simultaneously complexing with the template and thus acting as functional monomers (Cai and Gupta, 2004).

1.6.4 Porogen

The solvent serves to bring all the components into the polymerization process and also responsible for creating the pores in macroporous polymers. When increased the volume of porogen the pore volume will also increase (Cai and Gupta, 2004; Wang *et al.*, 2005). The porogen in non covalent binding should be considered such that it simultaneously maximizes the possibility of template, functional monomer complex formation. Normally, this implies that apolar, non-protic solvents, e.g. toluene, are preferred as such solvents stabilize hydrogen bonds, however if hydrophobic forces are being used to drive the complexation then water could well be the solvent of choice (Cormack and Elorza, 2004).

1.6.5 Initiator

Initiator is the substance that induces polymerization. The most widely used method is initiate free radical polymerization. The advantage of free radical polymerization is that it can be performed under mild reaction condition such as room temperature or atmospheric pressure depends on the method of polymerization (photolysis or thermolysis) (Cormack and Elorza, 2004). For example, the azo-initiator, α-α' azobisisobutyronitrile (AIBN) can be decomposed by light or thermal, the mechanism of free radical polymerization of AIBN showed in Figure 1.10. AIBN was cleavage to nitrogen and isobutyronitrile radical (Fried, 2003).

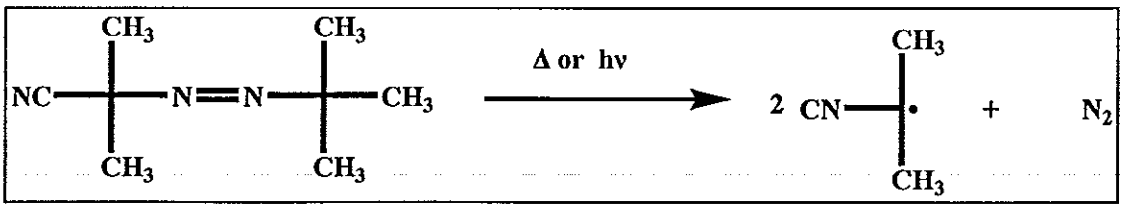


Figure 1.10 The mechanism of free radical polymerization (Fried, 2003).

1.7 Binding characterizations of MIP

1.7.1 Capacity of MIP

One of the key assumptions to the analysis of binding data is that the data are obtained after equilibrium between the template and the imprinted polymer is reached. Usually, this is accomplished by incubation of a known amount of imprinted polymer with different concentrations of the template during a specified period of time necessary to reach equilibrium (García-Calzón and Diaz-García, 2007). The value is obtained by constructing a curve of specific binding versus template concentration shown in Figure 1.11(a). The amount of template bound to the MIPs at equilibrium (Q) increased with the initial concentration of template and reached saturation at higher template concentration (Zhu *et al.*, 2002).

1.7.2 Scatchard analysis

The saturation binding data were further processed with Scatchard equation which is a method of linearizing data from a saturation binding experiment in order to determine binding properties. Scatchard equation is shown in (1.3) (Zhu *et al.*, 2002).

$$\frac{Q}{[template]} = \frac{(Q_{max} - Q)}{K_D} \qquad (1.3)$$

Where Q is the amount of template bound to MIPs at equilibrium

Q_{max} is the apparent maximum number of binding sites

[template] is the free analyte concentration at equilibrium

K_D is dissociation constant

The curve was constructed by Q/[template] (y axis) and Q (x axis). From the curve one binding constant K_D and Q_{max} can be obtained from the slope of the straight line and the intercept of the Scatchard plot as showed in Figure 1.11(b).

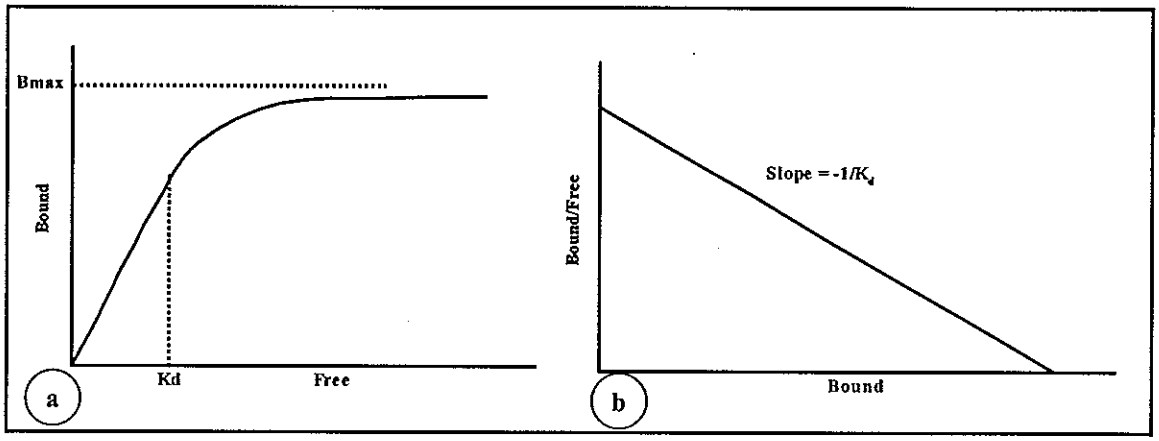


Figure 1.11 (a) the binding isotherm of MIP (b) Scatchard analysis of the binding of the template to imprinted polymer (Xu *et al.*, 2007b).

The use of MIP as sorbent in SPE method is called molecular imprinted solid phase extraction (MISPE). In most cases, MISPEs are used in off-line methods, which are similar to off-line SPE conventional sorbents methods. The main advantages of MISPE are that the whole operation is simple and easy *i.e* various solvents and additives can be used without their influence on the subsequent chromatographic analysis, and consequently a high enrichment factor and selectivity can be obtained (He *et al.*, 2007). On the other hand, the disadvantages of the on-line SPE are the reduced sample throughput, since only small sample volumes can be processed, and lack of versatility of the system. The direct coupling of SPE with GC is more difficult, because it requires effective elimination of traces of water (Picó *et al.*, 2007).

1.8 System performance

1.8.1 Linear dynamic range

Quantitative analysis requires knowing how the response measured that depends on the analyte concentration. This knowledge is obtained using external or internal standardization and formulated into a mathematical expression used for calculating the unknown analyte concentration in real samples. Usually external

calibration curve was used (Lambropoulou and Albanis, 2005; Sánchez-Brunete *et al.*, 2008; Sánchez-Brunete *et al.*, 2003; Zhang and Lee, 2006). It is carried out by plotting between response (y axis) and analyte concentration (x axis). The results should not show a significant deviation from linearity, which is taken to mean that the correlation coefficient, $r > 0.99$. The calibration equation is presented below,

$$y = bx + a \tag{1.4}$$

Where y is measured response; *e.g.* absorbance, peak height or area

x is concentration

b is slope of the calibration curve = sensitivity

and a is intercept

The specified range is normally derived from the linearity studies. The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical method has suitable levels of precision, accuracy and linearity (Australian Pesticides & Veterinary Medicines Authority, 2004; EU, 2000).

1.8.2 Limit of detection (LOD)

Limit of detection is the smallest concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty detected, but not necessarily quantities as an exact value (Cinquina *et al.*, 2003). LOD applies to the instrument tested under the optimum conditions and assumes that the sensitivity is constant. According to the EU Commission Decision 93/256/EEC, LOD must be calculated as the concentration response corresponding to three times the signal-to-noise as showed in Figure 1.12 (Nogueira *et al.*, 2004).

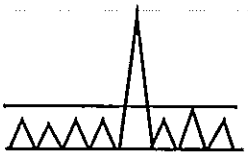


Figure 1.12 A signal-to-noise (S/N) ratio of three times (Bruce *et al.*, 1998).

For IUPAC definition, blank response should be measured 20 times and standard deviation value of the blank response was obtained. Mean value of blank response, $\overline{X_B}$ and standard deviation (S_B) were calculated using these following Equations;

$$\overline{X_B} = \frac{\sum_{j=1}^{n_B} X_{Bj}}{n_b} \tag{1.5}$$

$$S_b = \sqrt{\frac{\sum_{j=1}^{n_B} (X_{Bj} - \overline{X_B})^2}{n_B - 1}} \tag{1.6}$$

In defining the smallest detectable signal (X_L), IUPAC states that

$$X_L = \overline{X_B} + kS_B \tag{1.7}$$

Where k is a numerical factor chosen in accordance with the confidence level desired and the accepted value is 3 at the confidence level of 99.7 %

S_B is the standard deviation for 20 times of injections

C_L was then obtained as a function of X_L

$$C_L = \frac{(X_L - \overline{X_B})}{m} \tag{1.8}$$

Where m is the analytical sensitivity (slope of the calibration curve)

C_L is the smallest concentration that can be detected with reasonable certainty for a given analytical procedure

Because the mean blank reading, $\overline{X_B}$ is not always 0 the signal must be background corrected. Equation 1.9 was obtained after substitution Equation 1.7 into 1.8.

$$C_L = \frac{kS_B}{m} \tag{1.9}$$

Where C_L is the limit of detection

S_B is the standard deviation of blank response (20 times)

m is the slope of the calibration curve

and k is the numberical factor chosen according to the confidence level, $k = 3$ or 3σ limit of detection, corresponds to confidence level of about 99.7 % (Long and Wineforder, 1983)

1.8.3 Limit of quantification (LOQ)

The limit of quantification is the lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the optimum experimental conditions. It is a parameter of quantitative assays for low levels of compounds in sample matrices (Bruce *et al.*, 1998; Long and Wineforder, 1983). From the EU Commission Decision 93/256/EEC, LOQ can be calculated from ten times the signal-to-noise and IUPAC definition, can be calculated from Equation 1.9 with $k = 10$.

1.8.4 Precision

All measurements contain some random error or noise that cannot be removed. This can be evaluated by repeated measurements of the same sample (n = 3-6) and by calculating the standard deviation (sd) and relative standard deviation (RSD), following Equation 1.10 and 1.11, respectively (Bruce *et al.*, 1998).

$$sd = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}} \qquad (1.10)$$

$$RSD = \frac{sd}{\bar{x}} \qquad (1.11)$$

Where n-1 is degree of freedom

$\bar{x}$ is mean

The parameter measurements depend on the technique used. In chromatographic technique, retention time and peak response should be measured (Bruce *et al.*, 1998; Swartz and Krull, 1997). In case of retention time and peak

response % RSD ≤ 1 and 4 is desirable, respectively (Center for Drug Evaluation and Research (CDER), 1994).

1.8.5 Accuracy

The accuracy is a term indicated how close the experiment value to the true value (Center for Drug Evaluation and Research (CDER), 1994). The accuracy of an analytical method may be determined by many ways, such as analyzing a sample of known concentration and comparing the measured value to the true value.

1.8.6 Sensitivity

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. Sensitivity can be expressed as the slope of the linear regression calibration curve, and it is measured at the same time as the linearity tests (Bruce *et al.*, 1998).

1.8.7 Matrix effect

The sample matrix may contain compounds which interfere with the measurement performed by the selected detector without causing a signal that is visible in the specificity test. The interfering compounds may strengthen or reduce the signal, and the magnitude of the effect may also depend on the concentration (Bruce *et al.*, 1998). Matrix effect can be carried out by comparing the slopes on standard addition curves. This can be done by preparing two sample sets. One set includes the sample matrix spiked with various concentration levels of analyte and the other set does not include the sample matrix. The results of these samples should be plotted as a function of analyte addition concentration in the same graph. If the slopes of these two linear curves are the same, it refer that matrix have not been affected. In addition to performing a statistical analysis of matrix tests, the chemical significance of the result should be checked.

1.9 Objectives

This work focuses on the synthesis of carbofuran MIPs sorbent to be used in solid phase extraction and solid phase dispersion for qualitative and quantitative analysis of carbofuran.

1.10 Benefits

It is expected the proposed method will provide high selective, high enrichment factor, simple and fast sample preparation technique that can be apply for analysis of carbofuran in real sample.

CHAPTER 2

Experimental

2.1 Chemical and materials

2.1.1 Standard chemicals

- Carbofuran ($C_{12}H_{15}NO_3$, purity 98 %, Aldrich, Aldrich Chemical Company, USA)
- Carbosulfan ($C_{20}H_{32}N_2O_3S$, purity 96 %, Supelco, Bellefont, PA, USA)
- Carbaryl ($C_{12}H_{11}NO_2$, purity 96 %, Supelco, Bellefont, PA, USA)

2.1.2 Molecular imprinted polymer preparation

- Acrylamide (purity 99 %, Sigma, MO, USA)
- 2-(diethylamino) ethylmethacrylate (purity 99 %, Aldrich Chemical Company, USA)
- Methacrylic acid (purity 99 %, Sigma, MO, USA)
- Ethylene glycol dimethacrylate (purity 99 %, Aldrich Chemical Company, USA)
- α,α′- azoisobutyronitrile (purity 97 %, VWR International LTD., Lulterworth, England)
- Benzoyl peroxide (purity 98 %, $C_{12}H_{15}NO_3$: Sigma, MO, USA)

2.1.3 General chemicals and solvents

All chemicals and reagents were analytical grade.

- Methanol (CH_3OH: LAB-SCAN, Thailand)
- Acetic acid (CH_3COOH: J.T. Baker, USA)
- Acetonitrile (CH_3CN: LAB-SCAN, Thailand

- Acetone (CH_3COCH_3: Merck, Germany)
- Cyclohexane (C_6H_{12}: Merck, Germany)
- Toluene ($C_6H_5CH_3$: LAB-SCAN, Thailand)
- n-Hexane ($CH_3(CH_2)_4CH_3$: Merck, Germany)
- Dichloromethane (CH_2C_{l2}: Merck, Germany)
- Ultra pure water (water was de-ionized with reverse osmosis system and purified with a Maxima ultrapure water instrument to obtain the resistively of 18.2 MΩ, ELGA, England)

2.1.4 Samples

Water samples were collected from Prince of Songkhla Univesity water reservoir. Well waters were form Thambon Bang Riang, Kuan Nieng district, Songkhla and Phom Ki Ri district, Nakorn Si Thammarat. The samples were filtered through GF/F microfiber filter twice in order to remove particulates that can block the binding site of MIP.

2.2 Instruments and apparatus

2.2.1 Gas chromatography – flame ionization detector (GC-FID)

- Gas chromatograph model 6890 series coupled with flame ionization detector (Agilent technology, USA)
- ULTRA-2 capillary column: 25 m × 0.53 mm I.D. × 0.32 μm film thickness of 5% phenyl 95% dimethylsiloxane (Agilent technology, USA)
- Computer system model VL vectra (Hewlette Packard, USA)
- Chemstation software (Agilent technology, USA)
- High purity helium carrier gas (purity 99.995 %, TIG, Thailand)
- Oxidant gas (air zero grade) (purity 99.995 %, TIG, Thailand)
- High purity hydrogen gas (purity 99.995 %, TIG, Thailand)
- High purity nitrogen carrier gas (purity 99.995 %, TIG, Thailand)

- Flow meter (Agilent technology, USA)

2.2.2 Spectrophotometer

- 8452A diode array spectrophotometer (Hewlette Packard, USA)
- Quartz cuvette (Starna Scientific, England)

2.2.3 Microscope

- Inverted microscope (Olympus, CK40, Tokyo, Japan)

2.2.4 Apparatus

- Syringe 10 µL (Agilent technology, USA)
- Polypropylene cartridge 3.00 mL (Terumo, Philippines)
- Microcentrifuge tube 2.00 mL (Eppendrof, Germany)
- Analytical balance AB204-S (Metter Toledo, Switzerland)
- Ultrasonic bath AS7240AT (Automatic Science, Japan)
- Evaporating rotation (EYELA, Japan)
- Vortex Genic-2 (Sciencetific Industries, USA)
- Glass microfiber filter GF/F (Whatman, England)
- SPE vacuum manifold (Altec, USA)
- Vacuum pump (Gast manufacturing., USA)
- Wrist action shaker, model 75 (Burrell coproration, USA)
- Microliter pipette 10 µL, 100 µL, 200 µL, 1000 µL, 5000 µL (Eppendorf, Germany)
- Amber vial 2 mL with polypropylene screw cap and red rubber septa (Agilent technology, USA)
- 11-mm crimper and 11-mm decrimper (Agilent technology, USA)
- Headspace vial 25 mL (Shimazu, Japan) with propylene rubber and aluminium crimp cap
- General glassware such as beakers, volumetric flask, round bottom flask, cylinder and test tube

2.3 Standard solution

Standard solution is a solution of accurately known concentration, prepared using standard substances in one of several ways (IUPAC, 1997). Standard solution must be prevented from the contamination that come from the chemicals and glassware (EPA, 2001). All glassware were washed with detergent, rinsed with ultra pure water, hexane and acetone then dried at 140 °C for at least 4 hours.

2.3.1 Carbofuran, carbosulfan and carbaryl standard stock solution

A 1,000 mg L^{-1}, carbofuran standard stock solution was prepared in acetonitrile. Two additional carbamate pesticides, carbosulfan and carbaryl, which were used to test the selectivity of synthesized MIP were prepared in chloroform at a concentration of 1,000 mg L^{-1}. They were immediately transferred into glass bottles and stored in the dark at 4 °C. The standard stock solution was checked periodically by gas chromatography to ensure the stability, *i.e*, signs of degradation or evaporation, especially just prior of standard calibration preparation.

2.3.2 Carbofuran standard working solution

Standard working solution used in the optimization of GC conditions was prepared by diluting the 1,000 mg L^{-1} standard stock solution with acetonitrile to obtain a concentration of 2.00 mg L^{-1}. Sample preparation procedures were optimized by spiked 100 µL of standard, 40.00 mg L^{-1}, to 100 mL of ultra pure water and 100 µL, 4.00 mg L^{-1} to 1.00 mL of ultra pure water to obtain 40.0 µg L^{-1} and 4.0 µg L^{-1} and used as the sample in MISPE and MISPD, respectively.

2.4 Optimization of GC-FID conditions

Parameters of GC-FID (Figure 2.1), *i.e* flow rates of carrier gas, fuel gas (H_2) and oxidant gas (air), temperature of injector, column and detector were optimized in order to achieve the high efficiency such as highest response, best

separation of the chromatogram and short analysis time (Grob and Barry, 2004). Optimization was carried out by varied only a single parameter while kept other parameters constant (Reedy, 1997). In these studies 1 μL of the mixture between carbofuran (analyte) and carbaryl (test selectivity) working standard solution (2 mg L^{-1}) was injected to GC-FID, five replications for each value. When the optimum condition was obtained, it was used to optimize the next parameter. The starting operating conditions of GC-FID are shown in Table 2.1.

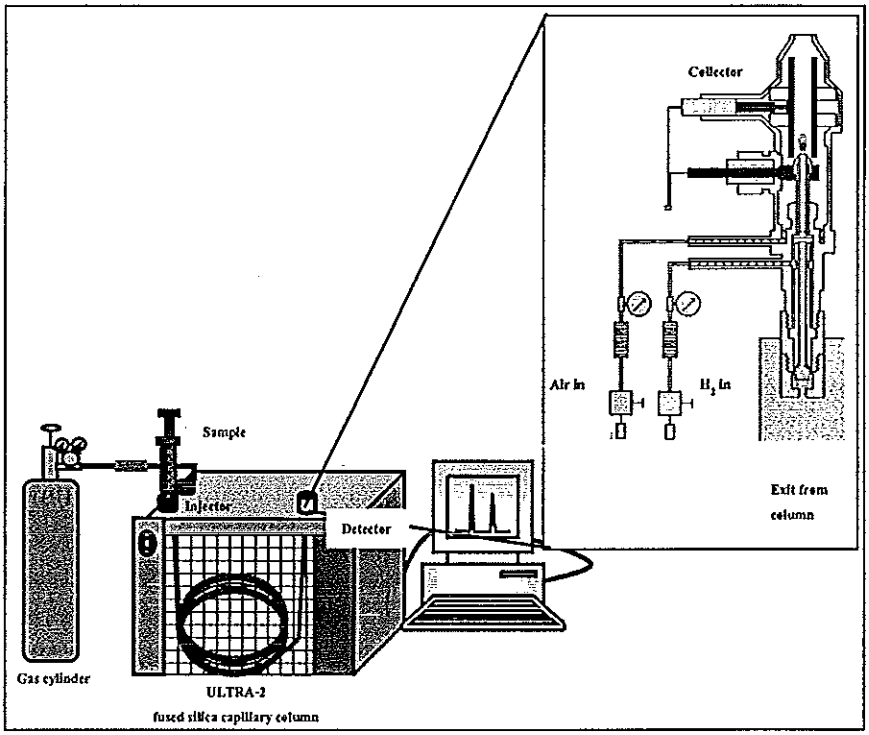


Figure 2.1 Schematic diagram of gas chromatography-flame ionization detector (GC-FID) (Grob and Barry, 2004).

Table 2.1 Starting GC-FID conditions (Agilent Technology 1995).

Parameter	Condition
Carrier gas (He) flow rate (mL min^{-1})	1.8
Fuel (H_2) flow rate (mL min^{-1})	30
Oxidant (air) flow rate (mL min^{-1})	300
Make up (N_2) flow rate (mL min^{-1})	30
Column temperature program	
- Initial temperature (°C)	90
- Initial holding time (min)	0
- Ramp rate (°C min^{-1})	3
- Final temperature (°C)	140
- Final holding time (min)	0
Injector temperature (°C)	210
Detector temperature(°C)	280

2.4.1 Carrier gas flow rate

The most important parameter to be optimized in gas chromatography was carrier gas flow rate (Reedy, 1997). To achieve the highest sensitivity, the flow rate was studied at 1.8, 2.1, 2.4, 2.7, 3.0 and 3.3 mL min^{-1}. Theoretical plate number (N) and height equivalent to a theoretical plate (HETP) was calculated from the retention time, peak height and peak area of the analyte obtained from the chromatogram. A van Deemter graph was then plotted between HETP and carrier gas flow rate. The optimum carrier gas flow rate was the one that gave the lowest HETP.

2.4.2 Column temperature program

Since the boiling points of carbofuran, carbaryl and carbosulfan are quite closed, their chromatographic peaks can not be separated by isothermal temperature. Therefore, column temperature program was applied by optimizing initial temperature, initial holding time, ramp rate, final temperature and final holding time (Table 2.2). The optimum column temperature program was optimized to obtain short analysis time, the best separation and symmetry chromatogram.

Table 2.2 Studied values of column program temperature.

Step	Parameter	Studied value
1	Initial temperature (°C)	90, 100, 110 and 120
2	Initial time (minute)	0, 0.5, 1.0, 1.5 and 2.0
3	Ramp rate (°C min^{-1})	3, 5, 10, 15, 20, 25, 30 and 35
4	Final temperature (°C)	160, 170, 180, 190, 200, 210 and 220
5	Final time (minute)	3, 4, 5 and 6

2.4.3 Injector temperature

The optimum injector temperature was investigated by varied the temperature at 260, 270, 280, 290 and 300 °C. The temperature that provided the highest peak area was chosen as the optimum injector temperature.

2.4.4 Detector temperature

The responses of various detector temperature, 270, 280, 290 and 300 °C were investigated to obtain the temperature that provided the highest response.

2.4.5 Fuel (H_2) flow rate

The process of flame ionization detector involved ionization mechanism. The heat energy was produced from reaction between fuel gas (hydrogen) and oxidant (air). When the organic compounds reach the flame, charged species were formed and collected at the electrode. In order to obtain the highest oxidizing compound hydrogen gas flow rate was investigated at 20, 30, 40, and 50 mL min^{-1}. The flow rate of air (oxidant) was set at 300 mL min^{-1}, as recommended in the Operating Manual Agilent 6890 Series Gas Chromatography (Agilent Technology 1995).

2.4.6 Oxidant (air) flow rate

The oxidant gas flow rate was investigated at 200, 300, 400 and 500 mL min^{-1}. Peak areas were compared and the optimum flow rate was determined from the highest response.

2.5 System performance of GC-FID

The GC-FID system performance was validated by the linear dynamic range, limit of detection and instrument precision.

2.5.1 Linearity and range

Determination of linearity was carried out for carbofuran at concentration levels between 0.5 mg L^{-1} and 700 mg L^{-1}, at 25 mg L^{-1} interval between 0.5-150 mg L^{-1} and 50 mg L^{-1} between 150-700 L^{-1}. One microliter of standard carbofuran was injected to GC-FID at the optimum conditions. Linearity was determined from the curve between responses (y axis) and concentrations of the analyte (x axis) when the coefficient of determination (R^2) was greater than 0.99. Since the value of intercept and slope has some random error, therefore the considerations of these values are importance. The standard deviation of the slope and

the intercept can be calculated by Equation. 2.1 and 2.2, respectively (Miller and Miller, 2000).

$$S_b = \frac{S_{y/x}}{\sqrt{\sum_i (x_i - \bar{x}_i)^2}} \tag{2.1}$$

$$S_a = S_{y/x} \sqrt{\frac{\sum_i x_i^2}{n \sum_i (x_i - \bar{x}_i)^2}} \tag{2.2}$$

Where S_b is standard deviation of the slope

S_a is standard deviation of the intercept

$S_{y/x}$ is the random error in the y-direction Equation 2.3

$$S_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}} \tag{2.3}$$

Equation 2.3 utilizes the y-residuals, where $\hat{y}_i$ value are the points on the calculated regression line corresponding to the individual x value ('fitted' y value) as shown in Figure 2.2.

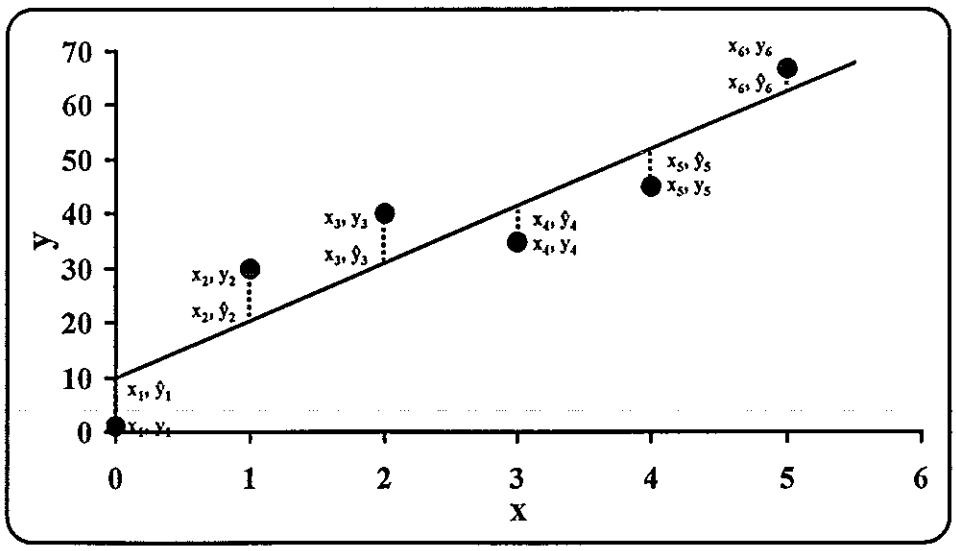


Figure 2.2 The y residue of regression line (Miller and Miller, 2000).

2.5.2 Limit of detection (LOD)

The limit of detection is the lowest concentration of the analyte in the sample which can be detected with reasonable statistical certainty but not necessarily quantity (Cinquina *et al.*, 2003). In this study, LOD was determined base on IUPAC definition. Blank response was measured 20 times and standard deviation value of the maximum average from blank response was obtained. LOD was calculated from Equation 1.9.

2.5.3 Limit of quantitative (LOQ)

Limit of quantification is defined as the lowest concentration of the analyte in the sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method (Swartz and Krull, 1997). It can be applied to analytical procedures that exhibit baseline noise. In this study, LOQ was determined base on IUPAC definition with $k = 10$ in Equation 1.9 (Long and Wineforder, 1983).

2.5.4 Instrument precision

All measurements contain some random error or noise that cannot be removed, the standard solution of carbofuran was repeated injections (n = 3-6) and calculated the standard deviation (SD) and relative standard deviation (RSD) of the measurement parameters, retention time and response (peak area or peak height) from gas chromatography (Bruce *et al.*, 1998). The acceptable value of % RSD for retention time is 1 % and peak area is 4 % (Synder and Kirkland, 1979).

2.6 Molecular imprinted polymers preparation

The molecular imprinted polymer for carbofuran was synthesized and used as the sorbent in sample preparation techniques (solid phase extraction and solid

phase dispersion). After that the extractant was analyzed by GC-FID. The summarized procedures show in Figure 2.3.

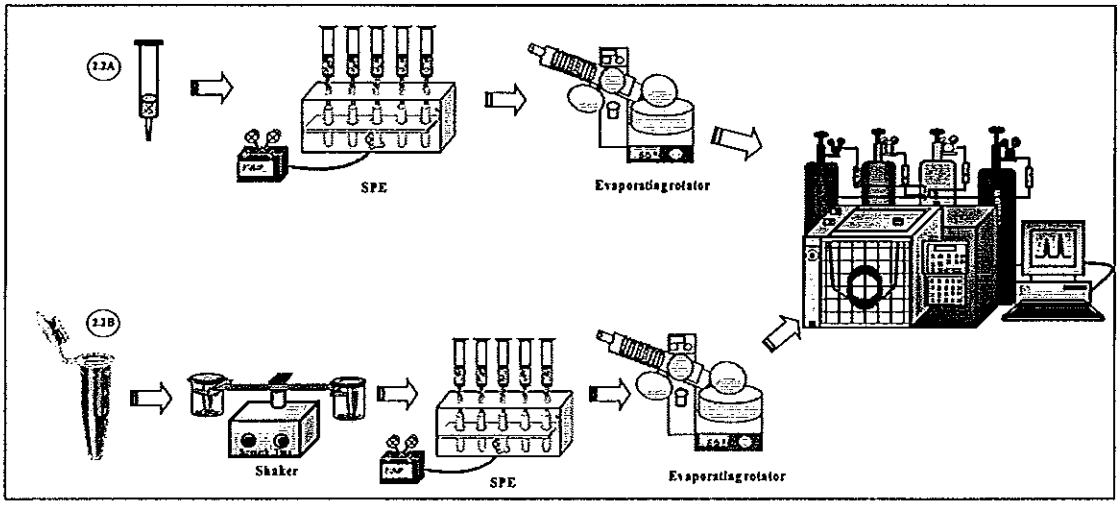


Figure 2.3 The diagram of sample preparation processes and analysis system for determination carbofuran contaminated in waters consist of A. solid phase extraction B. solid phase dispersion and analysis system.

In this work cocktail polymer with two functional monomers were used to prepare the MIP in order to improve the recognition property. Two MIPs were synthesized using two functional monomers, acrylamide and 2-(diethylamino) ethylmethacrylate (DAM) for MIP 1 and methacrylic acid and DAM for MIP 2. A control (non-MIP) was also synthesized by the same procedure, but without template (carbofuran). Other components, *i.e.* crosslinker (EGDMA), porogen (acetone) and initiator (α,α′-azobisisobutyronitrile: AIBN) were added with the same amount. All components were mixed in a vial (Figure 2.4A). Then the mixture was sonicated and purged with nitrogen gas five minute to eliminate oxygen that can suppress free radical polymerizations (Figure 2.4B) (Cormack and Elorza, 2004). The mixture was then thermally polymerized at optimum temperature (Figure 2.4C). When the optimum polymerization time was reached, the synthesized polymers were grinded and sieved (Figure 2.4D). Template was then removed by soxhlet extraction in 100 mL of methanol/acetic acid solution (90/10, v/v) for 1 hour to obtain the polymer

containing a functional group and size cavity specific to analyte (Figure 2.4E). The wash out template in the extractant was measured by UV detection. The extractant was collected every hour until the response of template was constant and minimize. After that the imprinted polymer was stirred in 30 mL of methanol/acetic acid solution (90/10, v/v) for 1 hour to assure that no template was remained in the MIP. Finally the MIPs were dried at room temperature. Parameters affecting the binding properties of molecular imprinted polymers were optimized, *i.e.* amount of functional monomer, crosslinker, porogen, polymerization temperature and time (Piletsky *et al.*, 2004; Wei *et al.*, 2005).

The binding property towards carbofuran of each varied parameter was studied by batch adsorption as shown in Figure 2.5. MIP was suspended in 100 mL ultra pure water containing 1000 µL, 1000 mg L^{-1} of carbofuran. The MIP was incubated at excess time (24 hours) to obtain equilibrium (García-Calzón and Diaz-García, 2007). The imprinted polymers were filtered and the filtrated was determined with UV-vis spectroscopy at 280 nm (Figure 2.4G). The optimum conditions were determined by considering the highest binding capacity. When an optimum condition was obtained, it was used to optimize the next parameter. The starting synthesized conditions are shown in Table 2.3 and the polymerization process was allowed at 70 °C for 24 hours.

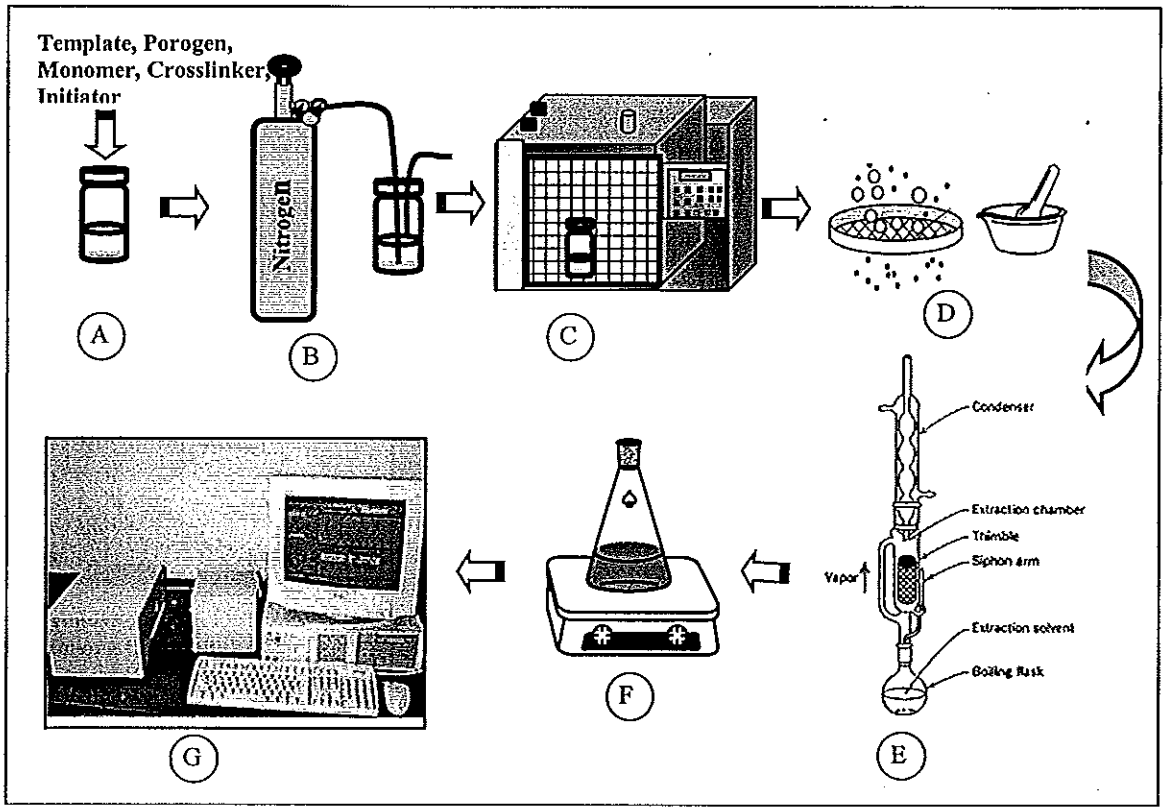


Figure 2.4 The scheme of molecular imprinted polymer synthesis

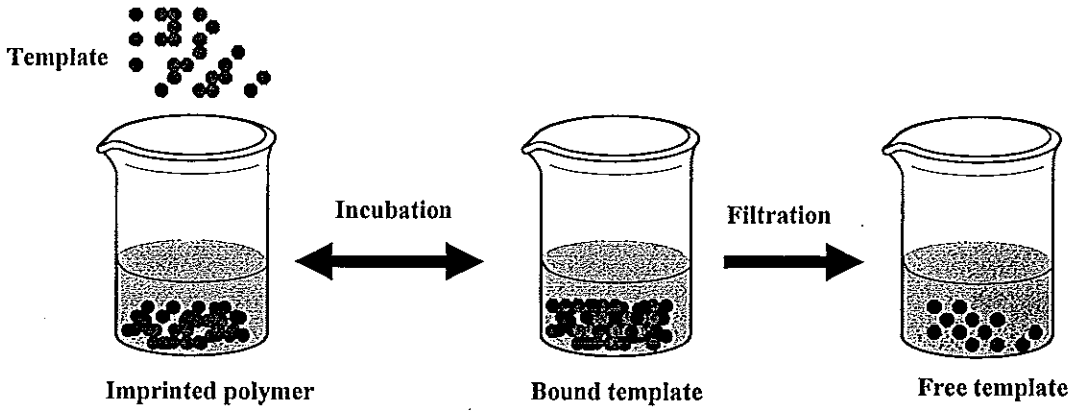


Figure 2.5 Batch rebinding procedures (Wei *et al.*, 2005).

Table 2.3 Conditions for the preparation of MIPs and non-MIPs.

Conditions	MIP 1	non-MIP1	MIP 2	non-MIP 2
Template (carbofuran, mol)	0.038	-	0.038	-
functional monomer				
- DAM (mol)	0.302	0.302	0.302	0.302
- Acrylamide (mol)	0.302	0.302	-	-
- Methacrylic acid (mol)	-	-	0.302	0.302
Crosslinker (EGDMA, mol)	1.985	1.985	1.985	1.985
Porogen (acetone, mol)	0.016	0.016	0.016	0.016
Innitiator (benzoyl peroxide, mol)	0.019	0.019	0.019	0.019

Parameters affecting recognition capacity of MIPs were optimized, for both MIPs.

2.6.1 Ratio of functional monomers

Functional monomers were responsible for the binding interactions in the imprinted binding sites. Quality and quantity of MIP binding sites is a direct function of the mechanisms of functional monomer (Karim *et al.*, 2005). The ratio of functional monomers was optimized to ensure that copolymerisation is feasible when two or more functional monomers are used simultaneously (Cormack and Elorza, 2004). The ratios of acrylamide or methacrylic acid and DAM (MIP 1 or MIP 2) were varied at 1:1, 1:2, 1:3 and 2:1 while maintaining a fixed amount of template.

2.6.2 Amount of crosslinker

The crosslinker was important for controlling the morphology of the polymer matrix and served as the stabilizer at the imprinted binding site. The optimum amount of crosslinker was studied by varied percentage amount at 78, 83,

88, 93 and 98 % mol of mixture. The optimum condition was the minimum amount of crosslinker necessary to form a rigid enough polymer network that provided the maximum binding capacity.

2.6.3 Type of porogen

Since, the porogen plays an important role in the formation of the porous structure of MIP, which can affect binding and selectivity of MIP (Spivak, 2005). Four types of hydrophilic porogen were studied *i.e* cyclohexane, acetonitrile, toluene and acetone. The porogen type led to the highest binding capacity was selected.

2.6.4 Concentration of porogen

Because of the nature and level of porogen, it can be used to control the morphology and the total pore volume, the porogen concentration was studied at 0.010, 0.015, 0.030, 0.050 and 0.100 mol. The optimum concentration of porogen is the one that could provide the highest binding capacity.

2.6.5 Type of initiator

In this work, benzoylperoxide and α,α′-azobisisobutyronitrile (AIBN) that can initiate free radical polymerization was studied. A more suitable initiator was considered by the higher binding capacity.

2.6.6 Concentration of initiator

Since the initiator was used to induce polymerization, therefore, its amount should be investigated. The concentration of initiator was varied at 0.006, 0.009, 0.019, 0.038 and 0.057 mol.

2.6.7 Polymerization temperature

Free radical polymerization can be initiated with an initiator, either by photochemical homolysis below room temperature or thermochemically at 60 °C or higher. Thermochemical polymerization was operated in this work and the temperature was studied at 50, 60, 70, 80 and 90 °C. The temperature that provided the highest binding capacity was the optimum polymerized temperature.

2.6.8 Optimum time for template removal (soxhlet extraction time)

After the MIP was ground and sieved, the template was removed by soxhlet extraction. The removal time of 2, 4, 6, 8 and 10 hours was studied final to final the time that the template was completely removed from the imprinted polymer.

2.7 Characterizations of MIPs

2.7.1 Particle size measurement

The average size of MIP particles was measured under an optical microscope. About 200 particles were captured in the images and their average diameter was calculated. All measurements were made at 20 °C (Piletsky *et al.*, 2004).

2.7.2 Swelling measurement

Dry MIP (1 mL, 25-100 μm) was placed in a glass cylinder and the initial volume (V_{dry}) and weight were recorded. This gave the apparent dry density of the polymer. Excess acetonitrile was added and agitated to remove air bubbles. The swelling was expressed as percentage change in volume (mL) per mg of polymer after 12 hours due to the maximum time used in sample preparation step (Sellergren *et al.*, 1997). The measurement was done for three batches.

2.7.3 Bulk density

Each of the three batches of dry polymer (1 mL, 25-100 μm) was placed in a glass cylinder and weighed. The apparent dry density of the polymer was calculated in the unit of gram per milliliter (Piletsky *et al.*, 2004).

2.7.4 Capacity of imprinted (MIP) and non-imprinted (non-MIP) polymer

Three batches of 100 mg of either MIP or non-MIP mixed with 100 mL acetonitrile containing various concentrations of carbofuran, 0.5, 1.0, 3.0, 5.0, 7.0, 8.0, 10.0, 11.0, 12.0 and 13.0 mg L^{-1} were used to study the capacity. They were stirred and incubated for 24 hours at room temperature. After that the mixtures were filtered, the filtrates were measured by UV spectroscopy at λ_{max} 280 nm. Then the graph was constructed between amount of carbofuran bound with MIP per gram of dry polymer (y axis) and amount of standard spiked (x axis). The capacity was obtained at the concentration that provided a constant response (García-Calzón and Diaz-García, 2007).

2.7.5 Scatchard analysis

Scatchard analysis was used to estimate the binding parameters of the MIP. Each of the standard carbofuran, 0.5, 1.0, 3.0, 5.0, 7.0, 8.0, 10.0, 11.0, 12.0 and 13.0 mg L^{-1}, was spiked into 100 mL of acetonitrile containing 100 mg of MIP or non-MIP. The mixtures were stirred at 200 rpm 25 °C for 24 hour, then filtered and the filtrates were analyzed for carbofuran by UV spectroscopy at the maximum wavelength of 280 nm. The amount bounds of carbofuran on the MIPs were quantified from the unbound fractions. The values of amount bound were plotted against the concentrations of carbofuran. The average data of three replications were recorded. From Scatchard curve one can obtain the apparent maximum number of binding sites and dissociation constant (Ikegami *et al.*, 2004; Zhu *et al.*, 2002).

2.7.6 Imprinting factor

The imprinting factor was the term that expressed the effect of the imprinted process. It was the ratio of amount of analyte bound with MIP to non-MIP (Cai and Gupta, 2004; Xu *et al.*, 2007). It can be determined by spiked different concentrations of carbofuran in to 20 mL of ultrapure water containing 1.00 g of MIP or non-MIP sorbent, each value was done for three replicates. At equilibrium time, each batch was filtered and the concentration of carbofuran that can bind with MIP or non-MIP sorbent was determined.

2.7.7 Reproducibility of synthesized MIP

To ensure batch-to-batch reproducibility, results from three different batches of synthesized MIP were compared, by considered the percentage relative standard deviation of capacity property.

2.8 Sample preparation step

The synthesized MIPs were used as sorbent in solid phase extraction and solid phase dispersion techniques to determine carbofuran contamination in water samples. Parameters affecting the extraction efficiency of both techniques were studied.

2.8.1 Molecular imprinted solid phase extraction (MISPE)

Molecular imprinted solid phase extraction cartridge was prepared by using 3 mL polyethylene cartridge. Dry MIP, 100 mg, was place between two pieces of GF/F microfiber filter (0.9 cm ID) (Figure 2.6). For SPE method, MIP cartridges were arranged on the manifold in a closed-valve position as shown in Figure 2.7. The cartridges were conditioned by 10 mL of acetronitrile and ultra pure water to activate the sorbent. Then 100 mL of ultra pure water was spiked with 100 μL, 40.00 mg L^{-1}, of standard carbofuran solution and applied slowly. The cartridges were allowed to

dry under vacuum. Other compounds that adsorbed on the MIP were washed by washing solvent in order to eliminate non specific binding. Then carbofuran was eluted by eluting solvent. The eluent was transferred into a round bottom flask and evaporated to dryness at 50 °C. The residue was reconstituted with 500 μL of acetonitrile. One microliter was injected into GC-FID at the optimum conditions. The same procedure was performed on both MIP 1 and MIP 2.

Molecular imprinted solid phase extraction (MISPE) parameters were optimized *i.e* flow rate of sample loading, type and volume of washing and type, volume and flow rate of eluting solvent. Each parameter was repeated five times and five replications of each extractant were analyzed by GC-FID. The optimum conditions of MISPE were determined by considering the highest recovery, consumed small amount of organic solvent and short sample preparation time. The optimization was done by varied a single parameter while kept other parameters constant. The starting operation conditions for the optimization of MISPE which for MIPs (MIP1 and MIP2) are showed in Table 2.4. When the optimum value of one parameter was obtained it was used in the optimization of the next parameter.

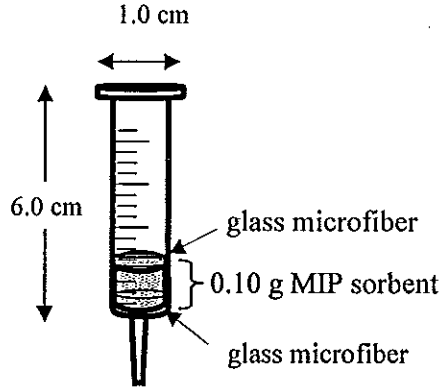


Figure 2.6 Packed MIP cartridge.

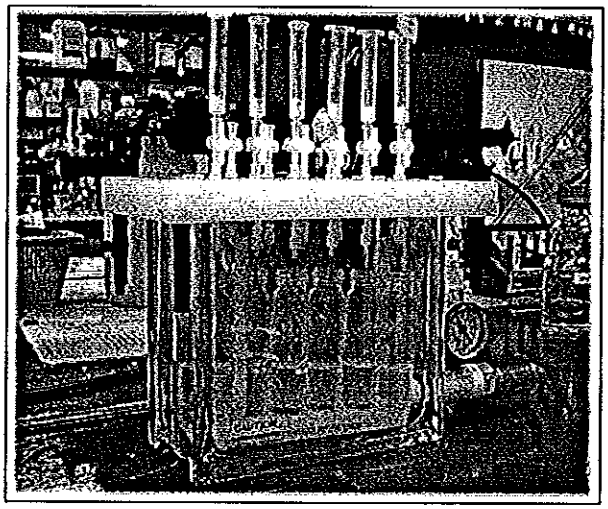


Figure 2.7 Solid phase extraction system used for clean up sample consists of cartridge packed with MIP or non-MIP, manifold and vacuum pump.

Table 2.4 The starting operation conditions for the optimization of MISPE.

Parameter	Operation condition
Flow rate of loading sample (mL)	0.5
Eluting step	
- Type	methanol
- Flow rate (mL min^{-1})	0.5
- Volume (mL)	10
Washing step	
- Type	hexane
- Flow rate (mL min^{-1})	0.5
- Volume (mL)	10

2.8.1.1 Flow rate of sample loading

The sample flow rate was optimized to achieve the maximum efficiency. The flow rate was varied at 0.2, 0.5, 0.8 and 1.0 mL min^{-1} and the optimum was obtained at the highest response flow rate.

2.8.1.2 Drying time

Since the elution solvent is water-immiscible, it was important to remove the residue water from the sorbent prior to analyte elution. The drying time was varied at 0, 2.5, 5.0, 7.5, 10.0 and 12.0 minutes. The time that provided the highest response was then selected as the optimum.

2.8.1.3 Type of eluting solvent

The eluting solvent should be strong in order to obtain the high selectivity of the analysis. Three eluting solvents selected from literatures were studied *i.e.* methanol (Jiang *et al.*, 2008; Sánchez-Barragan *et al.*, 2007; Schirmer and Meisel, 2006; Tang *et al.*, 2008), 9:1 (v/v) methanol - acetic acid (Baggiani *et al.*, 2007; Pascale *et al.*, 2008) and acetonitrile (Le Moullec *et al.*, 2007). The eluting solvent that provided the highest extraction efficiency was selected.

2.8.1.4 Volume of eluting solvent

Volume of the optimum eluting solvent was varied at 1.0, 1.5, 2.0, 2.5 and 3.0 mL. The smallest volume that provided the highest response was then selected as optimum condition.

2.8.1.5 Flow rate of eluting solvent

The flow rate of eluting solvent through SPE cartridge was optimized for the maximum extraction efficiency. The flow rate was varied at 0.2, 0.5, 0.8 and 1.0 mL min^{-1}. The flow rate that gave the highest response was selected as optimum eluting flow rate.

2.8.1.6 Type of washing solvent

Four organic solvents *i.e* hexane, cyclohexane, toluene and dichloromethane were investigated as washing solvent. These solvents were selected from the polarity of carbofuran (analyte; water solubility 0.07 g in 100 mL) and carbaryl (water solubility 0.004 g in 100 mL) (Mackay *et al.*, 2006) that used for the selectivity tested. Ultra pure water (100 mL) spiked with 100 µL, 40.00 mg L^{-1}, of standard mixture solution was passed through the cartridge, then washed with washing solvent. The optimum washing solvent should be able to remove carbaryl, expected to have non specific binding with MIP, while the analyte must remain bound with the sorbent. The solvent that provided the maximum response of the analyte and lowest response of matrix was selected as optimum washing solvent.

2.8.1.7 Volume of washing solvent

The volume of washing should be enough to remove the non specific binding from MIP while the analyte would remain bound. The volume was studied at 2.5, 5.0, 10.0, 15.0 and 20.0 mL and 1.5, 2.0, 5.0, 10.0 and 15.0 mL for MISPE 1 and 2, respectively. The minimum solvent volume that provided the lowest response of carbaryl was selected as the optimum condition.

2.8.1.8 Breakthrough volume

Breakthrough volume, loading capacity of sorbent, is an important parameter for adsorption phenomena. To obtain a high enrichment factor, a large volume of sample should be treated. Breakthrough volume was determined by passing increasing volume (25–200 mL) of water sample spiked with three concentration levels (100, 200 and 300 µg L^{-1}) of analyte (carbofuran) through the cartridge packed with 200 mg MIP. The fortified water samples were eluted, evaporated almost to dryness and the residue dissolved in 500 µL of acetonitrile. The breakthrough curve, a plot between volume and recovery, was obtained by using GC-FID to monitor the peak area of the analyte. Breakthrough was considered to occur when reach the

concentration that provide the response constant (Carneiro *et al.*, 2000; Li *et al.*, 2007).

2.8.1.9 Selectivity

Selectivity is the method ability to discriminate between different substrates (analyte and interference) and the main objective of this work was to improve the selectivity of sample clean up. To test selectivity 40, 70, 100 and 200 μg L^{-1} of carbofuran and the other two carbamate pesticides, carbosulfan and carbaryl, were passed through the cartridge. Then followed through the steps as described in section 2.8.1. Finally, one microliter was injected into GC-FID at the optimum conditions. The graph was plotted between the response and concentration of spiked standard. The selectivity was identified by the slope of obtained from each standard.

2.8.1.10 Reusability

Reusability is an important issue when developing sorbent since this would help to reduce cost. Reusability of a cartridge of MISPE was investigated by spiking 100 μL, 40 mg L^{-1}, of standard carbofuran to ultra pure water and extracted with optimum conditions where the eluent was analyzed with GC-FID. The process was repeated with the same cartridge. The reusability was obtained by considering percentage response from each extraction of the same cartridge.

2.8.1.11 Repeatability

Repeatability is expressed as the precision under the same operating conditions. To test the repeatability, five replicates of the proposed method were carried out and extracted under optimum conditions of MISPE and analyzed under optimum conditions of GC-FID. Each replication was injected five times. The responses of five replications were obtained and % RSD of response was used to consider the repeatability.

2.8.2 Molecular imprinted solid phase dispersion (MISPD)

The synthesized MIPs were used as sorbent for another extraction technique, *i.e.* solid phase dispersion. One hundred milligrams of MIP and 1,000 μL of spiked DI water (40.0 μg L^{-1}) were added into a 2 mL microcentrifuge tube. The mixture was agitated to obtain the formation of a dispersion phase. The microcentrifuge tube was immediately placed into a 20 mL beaker and held in place with plasticine. The beaker was put on a shaker at 270 oscillation min^{-1}. When the optimum shaking time was reached, the mixture was filtered through a cartridge plugged with GF/F glass microfiber filter that arranged on the manifold in order to manipulate the flow rate (Figure 2.1B). Then washed the sorbent with washing solvent in order to eliminate non specific binding. Carbofuran was eluted by eluting solvent. The eluent was then transferred into a round bottom flask and evaporated to dryness at 50 °C. The residue was reconstituted with 300 μL of acetonitrile where 1 μL was injected into GC-FID at the optimum conditions. The same procedure was applied to MIP 2. Parameters affecting extraction efficiency were optimized. These are shaking time and strength, volume of washing and eluting solvent. Type and flow rate of washing and eluting solvent was set at the optimum values of MISPE system as described in sections 2.8.1.6 – 2.8.1.7. Each parameter was repeated five times and five replications of each extractant were analyzed by GC-FID. The optimum conditions of SPD were determined by considering the highest recovery, consumed small amount of organic solvent and short sample preparation time. The optimization was done by varied a single parameter while kept other parameters constant. The starting operation conditions for optimization of solid phase dispersion for both MIPs are shown in Table 2.5 (the conditions of washing and eluting steps were the optimum condition of SPE technique) and the optimization range are the same. When the optimum value of one parameter was obtained it was used in the optimization of the next parameter.

Table 2.5 Starting operation conditions for the optimization of solid phase dispersion.

Parameter	Operating condition
Shaker strength (oscillation min^{-1})	231
Shaker time (min)	5
Washing step	
- Type	hexane
- Flow rate (mL min^{-1})	0.2
- Volume (mL)	10
Eluting step	
- Type	methanol
- Flow rate (mL min^{-1})	0.2
- Volume (mL)	10

2.8.2.1 Shaker strength and time

Shaking the mixture is a way to keep the MIP dispersed in the sample and thus, increase the opportunity of the analyte to come into contact with its binding site. Therefore, the strength of the shaker and shaking time are important and these two parameters were varied together. The optimum values are those given the highest extraction efficiency and short extraction time. Shaker strength was studied at 193, 231, 270, 308 and 347 oscillation min^{-1} while the shaker time was at 5, 10, 15, 20, 25 and 30 minutes.

2.8.2.2 Washing solvent volume

The volume of washing solvent should be enough to wash all interferences that are non specifically bound with MIP. The volume was investigated at 0.5, 1.0, 2.5, 5.0 and 10 mL. The minimum solvent volume that provided the lowest response of interference was selected as the optimum value.

2.8.2.3 Eluting solvent volume

In order to achieve the highest response and at the same time use minimal solvent, volume of the optimum eluting solvent was studied at 1.0, 2.5, 5.0 and 10 mL. The smallest volume that provided the highest response was then selected.

2.8.2.4 Repeatability

To test the repeatability of the proposed methods, five replicates at the extraction were studied under optimum condition of MISPD and analyzed under optimum conditions of GC-FID. Each replication was injected five times. The responses of five replications were obtained and the % RSD of response was used to considering the repeatability.

2.9 Method validation

Method validation is the process to test that the proposed method was acceptable for its intended purpose. For this work the applicability of the developed method was tested following the accepted criteria for analytical method validation, including linear dynamic range, accuracy, precision, limit of detection, limit of quantification and matrix effect. Method validations were estimated based on the analyses of spiked water samples and data were evaluated by statistical method.

2.9.1 Linear dynamic range and calibration curve

The study was carried out by using standard solution of carbofuran at concentration levels between 0.2 and 600 $\mu g\ L^{-1}$ for solid phase extraction and 0.05 – 40 $\mu g\ L^{-1}$ for solid phase dispersion. Each point was repeated five times and five replications of each extractant were analyzed by GC-FID. The average values were used to constructed calibration curves by plotting the corresponding peak area versus analyte concentration. Linear range are the concentration range that expect to found in real sample which coefficient of determination (R^2) greater than 0.99.

2.9.2 Limit of detection and limit of quantification (LOD and LOQ)

LOD and LOQ were established using IUPAC method. Blank was prepared by passing ultra pure water through the sorbent cartridge and followed the proposed procedures of SPE or SPD. Twenty blank samples were studied with five replications for each sample and analyzed by GC-FID. The average maximum from blank responses were obtained and used to calculate standard deviation value. LOD and LOQ were obtained following Equation 1.9.

2.9.3 Precision

The precision of the proposed method was tested by repeatedly five analyzing the spiked sample samples five times. Precision was determined by considering the % RSD of peak area and retention time of the spiked water sample at two different concentrations (low and high concentration).

2.9.4 Accuracy

The accuracy of the methods was expressed as the recoveries of spiked analytes in sample. Recovery is carried out by comparing the response obtained from spiked water sample and standard injection at three different concentrations (2, 20 and 40 μg L^{-1}) which were extracted following the proposed methods. Percentage recovery (%R) is calculated followed Equation 2.5. The acceptable percentage recovery is 70 -120 % by US EPA.

$$\%R = [\frac{(CF - CU)}{CA}] \times 100 \qquad (2.5)$$

When CF is the concentration of analyte measured in the sample or spiked sample

CU is the concentration of analyte in sample blank

and CA is the concentration of analyte added in the sample (EU, 2007)

2.9.5 Matrix effect

The matrix of environmental samples can greatly affect the analyte signals, enhance background noises or suppress the analyte responses. The matrix effects may result as positive or negative responses that can greatly affect the method reproducibility and accuracy (EU, 2000; EU, 2007). To estimate the matrix effect, slope of each matrix-matched standard calibration curves were compared to ultra pure spiked standard calibration curves and confirmed percentage different by using two-way anova (R software). The matrix effect is indicated of when the slopes of the two calibration curves were significantly difference.

2.10 Qualitative and quantitative analysis of carbofuran in water samples

2.10.1 Qualitative analysis

Qualitative analysis was carried out by comparing the retention time (t_R) of carbofuran obtained from the chromatogram of an unknown sample to the one of standard solution (Grob and Barry, 2004) under the same operating conditions.

2.10.2 Quantitative analysis

Quantitative analysis of carbofuran contaminated in water sample was based on the response of chromatographic peak that was proportional to the amount of analyte. Two analytical techniques were used, matrix-matched calibration curve to calculate the concentration of analyte from the peak area and standard addition when the response was non detectable (ND) or the concentration of unknown was lower than the LOQ (Swartz and Krull, 1997).

2.10.2.1 Matrix match calibration curve

In SPE technique, matrix match calibration curve was carried out by spiking standard carbofuran in 100 mL of water samples to give the final concentration of 0.2 – 600 μg L^{-1} and 2.0 – 500 μg L^{-1} for MIP 1 and 2, respectively. For SPD technique, standard carbofuran was spiked in 1.00 mL of water samples to the final concentration of 0.05 – 40.0 μg L^{-1} for both MIPs. The water samples spiked with standard carbofuran then went through sample preparation steps of each technique described in section 2.8.1 and 2.8.2 and the extractants were analyzed with GC-FID under optimum conditions. Unspiked water sample with standard carbofuran was also tested. Five replications were carried out for each concentration. The matrix match calibration curves were obtained by plotting peak area, after subtracted by the concentration of the analyte in the unspiked sample, *versus* concentration of standard carbofuran. The response from the chromatogram was used to calculate the concentration of carbofuran in the unknown samples from the matrix match calibration curve.

2.10.2.2 Standard addition method

Standard addition was performed in the water sample that showed non detectable response or when the obtained concentration was lower than the LOQ. This method was carried out by spiked standard to give the final concentrations of 0.5, 2.5, 5.0, 7.5 and 10.0 μg L^{-1} in 100 mL water sample for SPE and 0.1, 1.0, 2.5, 5.0 and 7.5 μg L^{-1} into 1 mL in SPD. The spiking was done before the sample preparation steps. The spiked and unspiked samples were then analyzed by GC-FID under optimum conditions. The experiment was done in five replicates. The results were plotted between peak area (y axis) and added concentration of carbofuran (x axis). The original concentration is determine by extrapolated the regression line to the x axis at y = 0 (Grob and Barry, 2004). The negative intercept on x axis corresponds to the amount of analyte in water sample as shown in Figure 2.8. Since the intercept and slope of the regression line has some error, thus, the standard deviation of the extrapolated x value (Sx_E) is calculated by Equation 2.6.

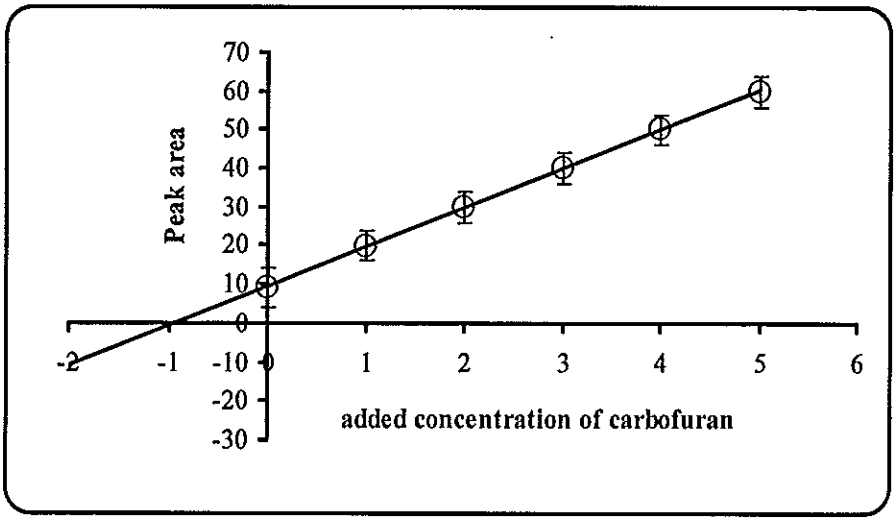


Figure 2.8 The method of standard addition (Miller and Miller, 2000).

$$Sx_E = \frac{S_{y/x}}{b}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{b^2 \sum_i (x_i - \bar{x})^2}} \qquad (2.6)$$

Where Sx_E is the standard deviation of the extrapolated x value and

B is the slope of the least squares line which can be calculated from Equation 2.7

$$b = \frac{\sum_i \{(x_i - \bar{x})(y_i - \bar{y})\}}{\sum_i (x_i - \bar{x})^2} \qquad (2.7)$$

CHAPTER 3

Results and discussion

Trace analysis of carbofuran pesticide contamination in water sample was carried out by gas chromatography-flame ionization detector. In this study, ULTRA-2 capillary column: 25 m × 0.53 mm I.D. × 0.32 μm film thickness of 5% phenyl 95% dimethylsiloxane is used as analytical column and helium gas as the carrier gas. The molecular imprinted polymer for carbofuran was prepared and used as the sorbent for the sample preparation step, either as solid phase extraction (MISPE) or solid phase dispersion (MISPD) technique. The optimum conditions of GC-FID, MISPE and MISPD were investigated and applied to the real water samples.

3.1 Optimization of GC-FID conditions for carbofuran

The analytical method for carbofuran in water must be selective and sensitive, due to the very low concentration and high interference of matrices. The determination was carried out by gas chromatography coupled with flame ionization detector (GC-FID) with purify helium as carrier gas. The detection system, FID, consisted of a small hydrogen-air diffusion flame burning at the end of a jet; the eluted components from the column are directed with carrier gas and make up gas flow. When the organic components reach the flame, electrically charged species are formed and collected at the electrode producing an increase in current proportional to the amount of carbon in the flame. The resulting current is amplified by an electrometer (Grob and Barry, 2004).

In order to achieve specific goals such as higher speed of analysis or improved peak to peak resolution, optimization of a number of important variables and their interactions must be done (Grob and Barry, 2004). GC-FID conditions were optimized, *i.e,* carrier, oxidant (air) and fuel (hydrogen) gas flow rates, column

temperature program, injector and detector temperatures. The optimum conditions were obtained by considering the best resolution, highest response and less analysis time. In this studied only 1 μL was injected because at larger injection when the sample vaporizes, the vapor may overflow the inlet and degrade the chromatograph (Agilent Technology 1995).

3.1.1 Carrier gas flow rate

Column efficiency and peak resolution is affected by carrier gas flow rate, each column will have an optimum flow rate for the carrier gas used for the analysis (Scott, 1998). The optimum carrier gas flow rate was obtained from the van Deemter plot, that considered the relationship between the height equivalent to a theoretical plate (HETP) and carrier gas flow rate (Figure 3.1). The van Deemter equation expresses the extent to which a component band spreads as it passed through the column in terms of physical constants and mass transfer effect as shown in Equation 3.1 (Grob and Barry, 2004).

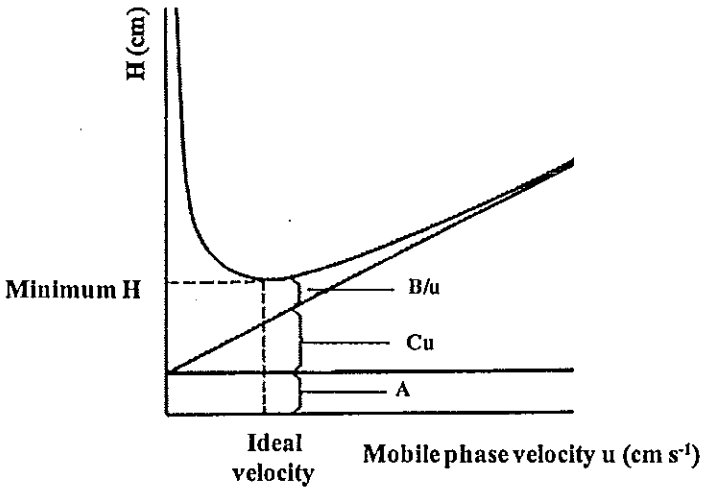


Figure 3.1 The van Deemter plot.

$$\text{HETP (H)} = A + \frac{B}{u} + Cu \quad (3.1)$$

Where A is the eddy diffusion term; a constant that accounts for the effect of "eddy" diffusion column

B is longitudinal or ordinary diffusion term; a constant that accounts for the molecular diffusion of the vapor in the direction of the column axis

C is resistance to mass transfer term; a constant proportional to the resistance of the column packing to mass transfer of solute through it

and u is the linear velocity of carrier gas

From this equation; the first term, the eddy diffusion term (A), represents the distance a flowing stream of the vapor moves before its direction is changed by the column packing. Because of the multitude of different routes molecules can travel through the column, different molecules will arrive at the outlet of the column at different times. A is independent of carrier gas velocity, since this path length is independent of carrier gas velocity. Eddy diffusion is proportional only to the average particle diameter *(dp)* of the solid support and a constant (A) related to the geometry of the support particles and how uniformly they are packed. The molecular diffusional term, B is a function of the diffusion coefficient of the solute in the gas phase *(Dg)* and the time spent in the column. Since B is dependent on the time the solute resides in the column, it must be dependent on carrier gas flow rate. The resistance to mass transfer, C is a function of physical process of crossing the gas-liquid phase boundary and also within the liquid phase to the gas phase. This term can be represented as the composite of the resistance to mass transfer in the mobile phase (C_g) and that in the stationary phase (C_l), therefore, $C = C_g + C_l$. From equation 3.1, at low u, the B term is large but quickly diminishes with increasing u and Cg to lesser extent, C_l, then dominates. The smallest values of H is H_{min}, at with u is optimum, u_{opt}. The greater u_{opt}, the faster sample can be analyzed (Baugh, 1993).

In this work, 25 m × 0.53 mm I.D. × 0.32 μm film thickness capillary column (megabore column) was used for analysis. In this column a liquid phase is coated on fused silica wall with no packing material, therefore, the A term (eddy diffusion) is nonexistent because there is only one flow path. The resistance to mass

transfer, term *C* has greatest effect on band broadening, and its effect in capillary column is controlled by the mass transfer in the gas phase (Cg). Thus, equation 3.1 take a different form for capillary column and this is known as the Golay equation (Equation 3.2) (Grob and Barry, 2004).

$$\text{HETP}\,(H) = \frac{B}{u} + C_g u \tag{3.2}$$

The above equation showed that HETP is proportional to the flow rate of carrier gas (*u*). It is also known that an optimum carrier gas flow rate will give an optimum column resolution with the narrowest HETP (Grob and Barry, 2004).

In practice, the terms *A, B* and *C* in the equation are difficult to obtain. However, the plate theory assumes that the column is divided into a number of zones called theoretical plate (N). The zone thickness or height equivalent to a theoretical plate (HETP) is determined by assuming that there is perfect equilibrium between the gas and liquid phases within each plate. The indication of the column efficiency in the term of HETP is determined by Equation 3.3.

$$HETP = \frac{L}{N} \tag{3.3}$$

Where L is length of column

and N is the number of theoretical plates

The plate number (N) of the column can be calculated from Equation 3.4. If a width of half height ($w_{1/2}$) was used instead of the width (w) at the base, the plate number could be calculated by Equation 3.5.

$$N = 16\left(\frac{t_R}{w}\right)^2 \tag{3.4}$$

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2 \tag{3.5}$$

Where t_R is the retention time of the peak

w is the base peak width

and $w_{1/2}$ is the width at half height

Since the megabore column was used, sharp and less tailing peaks were obtained making it difficult to determine the base peak width. Thus, the plate number (N) was calculated directly from the value obtained from a chromatogram as shown in Figure 3.2 by the relation ship

$$N = 2\pi(t_R h / A)^2 \tag{3.6}$$

Where t_R is the retention time

h is integrated peak height

and A is integrated peak area (Grob and Barry, 2004)

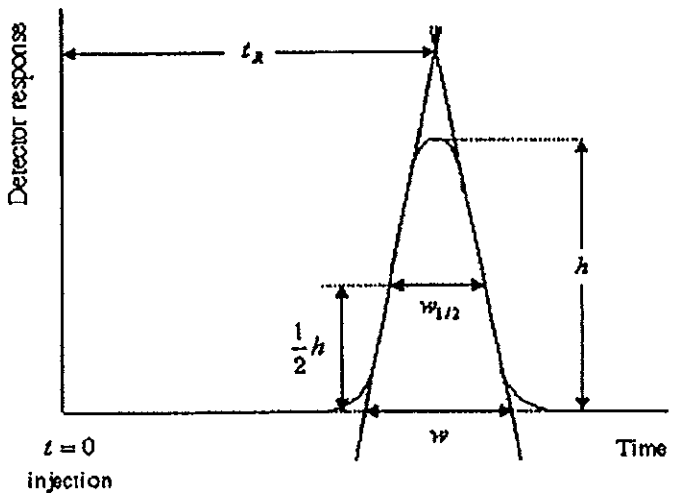


Figure 3.2 Measurement used in calculation total theoretical plate (Grob and Barry, 2004).

N was calculated from Equation 3.6 and substituted in Equation 3.3 with a know L term (column length) to obtain HETP. The van Deemter plot of HETP versus carrier gas flow rate is shown in Figure 3.3. At higher flow rate, the analysis time is reduced (Table 3.1) because sample was swept from the inlet to the column more rapidly and completely, however the theoretical plate number will decrease due to a larger plate height (Cramers and Leclercq, 1999; Poole and Schuette, 1984). The flow rate of 3.0 mL min^{-1} was chosen (*i.e.* lowest HETP) as optimum since it gave the highest *N*.

Table 3.1 The height equivalent to a theoretical plate (HETP) of carbofuran and carbaryl at different carrier gas flow rate.

Flow rate ($mL\ min^{-1}$)	Carbofuran*		Carbaryl*	
	Retention time (minute)	HETP $\times 10^{-2}$ (cm)	Retention time (minute)	HETP $\times 10^{-2}$ (cm)
1.8	6.13	7.1 ± 0.6	10.05	9.3 ± 0.1
2.1	5.57	7.8 ± 0.8	9.43	9.3 ± 0.1
2.4	5.09	7.8 ± 0.5	8.96	7.6 ± 0.1
2.7	4.76	8.4 ± 0.4	8.60	7.6 ± 0.1
3.0	4.48	6.8 ± 0.3	8.32	6.5 ± 0.1
3.3	4.20	7.8 ± 0.4	8.06	5.4 ± 0.1

* 5 replications

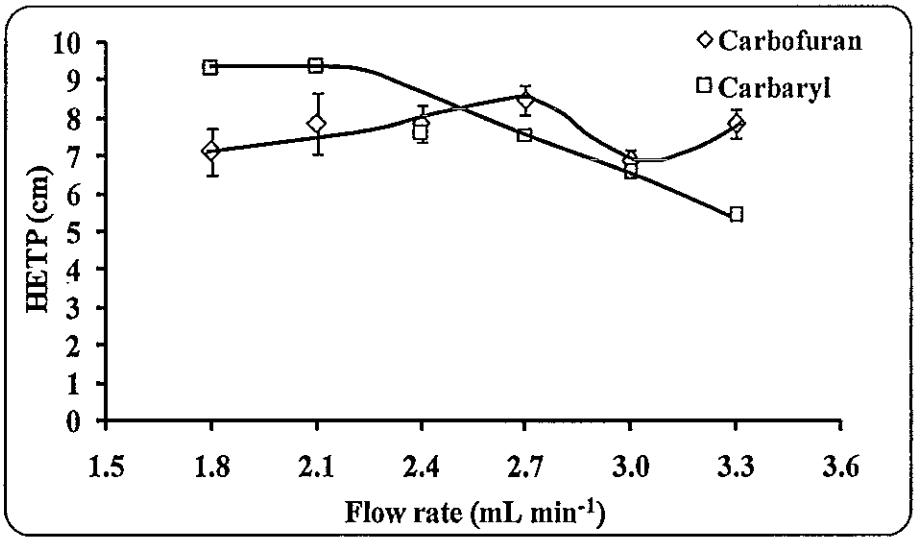


Figure 3.3 The van Deemter plots of carbofuran and carbaryl (n=5).

3.1.2 Column temperature program

The column is the heart of chromatography since it leads to peak resolution and minimizes analysis time. Carbaryl and carbofuran has the same range of polarity and molecular weight (Table 3.2), so the temperature programming is

necessary. To obtain high resolution and minimize analysis time, the optimum program temperature was studied by considered high response and short analysis time. A temperature program consists of a series of changes in the oven temperature and includes isothermal and controlled temperature rise segment that are selected by a mechanical or microprocessor controller (Poole and Schuette, 1984). Initial temperature, holding time of initial temperature, ramp rate, final temperature and holding time of final temperature were optimized as follows.

Step I Initial temperature: The analysis time decreased when the initial temperature increased (Table 3.3). 100 °C provided the highest response for carbaryl, but for carbofuran the response decreased with increase initial temperature (Table 3.3, Figure 3.4). This is probably because in gas liquid chromatography (GLC) the separation occurs due to the interaction between the solute and the stationary liquid phase. On the molecular level the principal intermolecular forces that occur between a solute and a solvent are induction and orientation forces that are generally weak and decrease with increasing temperature and approach zero at very high temperatures when all orientations are equally probable. These forces depended on the polarity, since carbofuran has higher polarity than carbaryl therefore carbofuran is more affected than carbaryl (Poole and Schuette, 1984). When considered the responses carbaryl gave lower signal than carbofuran. To obtain low LOD of both compounds the optimum condition was chosen at the optimum value of carbaryl, thus, 100 °C was chosen.

Table 3.2 Properties of carbofuran and carbaryl (Mackay *et al.*, 2006).

Analyte	**Molecular weight**	**Octanol/Water partition coefficient (log K_{ow})**
carbofuran	221.25	2.32
carbaryl	201.22	2.36

Table 3.3 Effect of initial temperature on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Initial temperature (°C)	Peak area (pA.s)*		Analysis time (minute)
	Carbofuran	Carbaryl	
90	505 ± 21	75 ± 17	33.3
100	446 ± 29	91 ± 12	20.0
110	393 ± 2	79 ± 2	16.7
120	299 ± 27	75 ± 7	13.3

* 5 replications

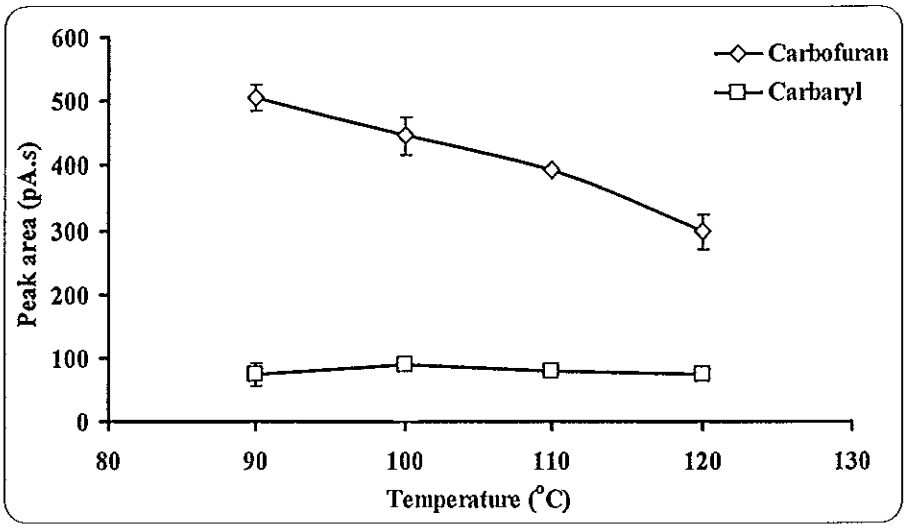


Figure 3.4 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at various initial temperatures (n=5).

Step II - The results of the initial holding time of initial temperature (100 °C) are shown in Table 3.4 and Figure 3.5. The response increased as the holding time increased from 0 – 1.0 minutes, at 1.0, 1.5 and 2.0 minutes the response differed by less than 10 %, thus the shorter holding time, 1.0 minute was chosen.

Table 3.4 Effect of initial holding time on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Initial holding time (min)	Peak area (pA.s)*		Analysis time (minute)
	Carbofuran	Carbaryl	
0.0	651 ± 21	137 ± 10	20.0
0.5	647 ± 26	114 ± 10	20.5
1.0	712 ± 23	154 ± 7	21.0
1.5	723 ± 35	164 ±10	21.5
2.0	720 ± 24	161 ± 7	22.0

* 5 replications

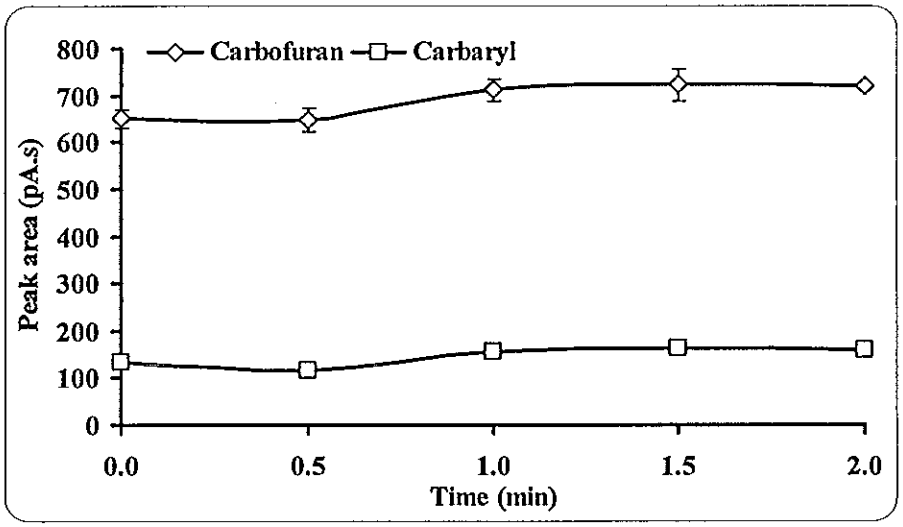


Figure 3.5 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at various initial holding times (n=5).

Step *III*- The effect of temperature ramp rate is shown in Table 3.5 and Figure 3.6. The responses are relatively constant except at 35 °C min^{-1} where the response of carbofuran increased. However at this ramp rate the baseline started to drift. Therefore, 30 °C min^{-1} was selected as optimum ramp rate.

Table 3.5 Effect of ramp rate on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Ramp rate (°C/min)	Peak area (pA.s)*		Analysis time (min)
	Carbofuran	Carbaryl	
3	188 ± 12	33 ± 3	21.0
5	208 ± 4	42 ± 3	13.0
10	205 ± 2	42 ± 3	7.0
15	209 ± 2	37.8 ± 0.6	5.0
20	208 ± 4	42 ± 2	4.0
25	211 ± 8	41 ± 3	3.4
30	212 ± 5	43 ± 2	3.0
35	234 ± 15	36 ± 6	2.7

* 5 Replications

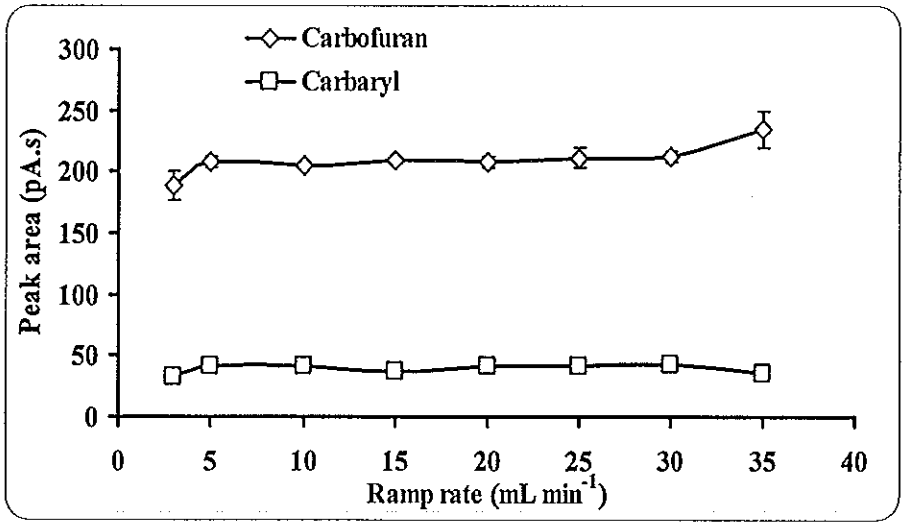


Figure 3.6 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at various ramp rates (n=5).

Step IV- Responses at different final temperature were obtained as shown in Table 3.6 and Figure 3.7. The response increased with final temperature. The highest response is at 210 °C and this was selected as the optimum final temperature.

Table 3.6 Effect of final temperature on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Final temperature (°C)	Peak area (pA.s)*		Analysis time (minute)
	Carbofuran	Carbaryl	
160	98 ± 18	18 ± 4	3.0
170	128 ± 5	29 ± 2	3.3
180	154 ± 8	30 ± 3	3.6
190	159 ± 6	32 ± 2	4.0
200	162 ± 7	33 ± 2	4.3
210	179 ± 9	45 ± 4	4.6
220	166 ± 9	35 ± 4	5.0

* 5 replications

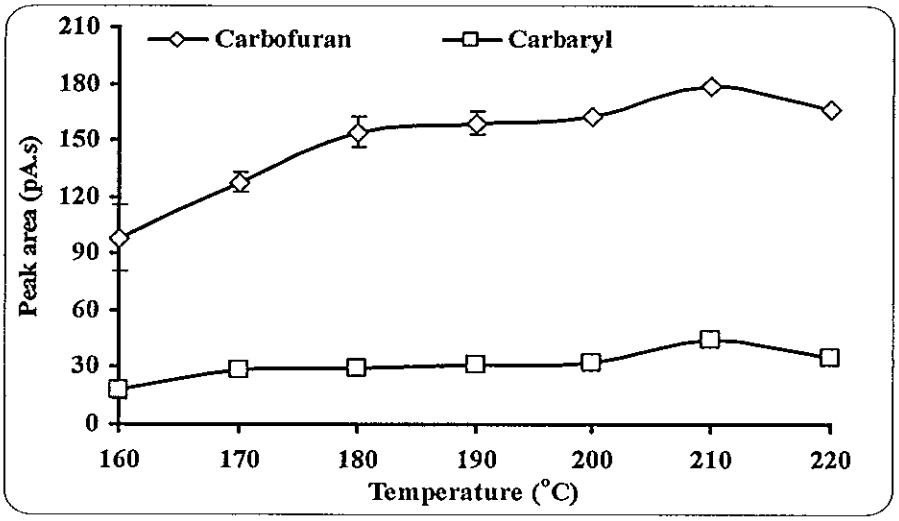


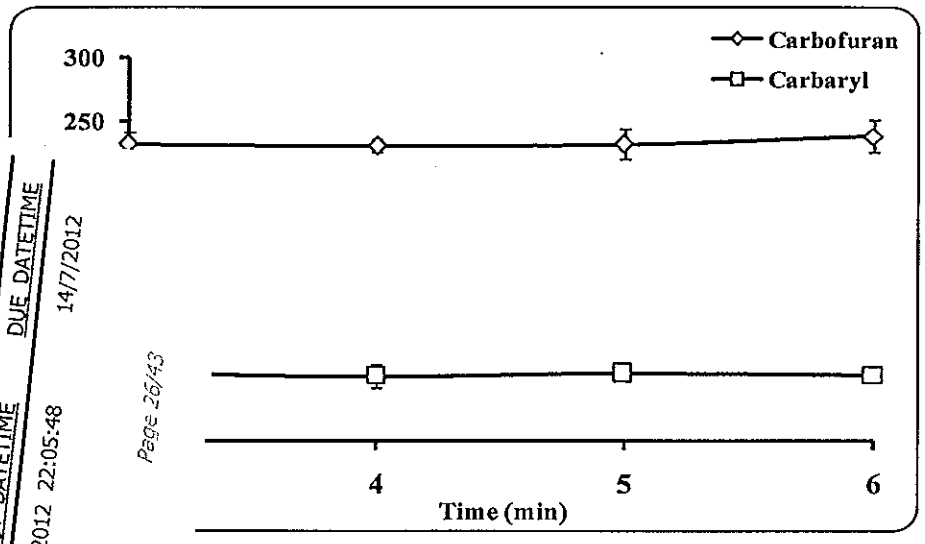
Figure 3.7 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at final temperature (n=5).

Step V- The last step, final holding time was investigated by considering the retention time of the last compound, carbaryl, that was eluted from the column. Although carbaryl was eluted from the column after 1 minute of the final time. 3-6 minutes of the final time was studied to allow the signal go back to the baseline. The result found that it had no effect on the response (Table 3.7 and Figure 3.8). Therefore the shortest time (3 minute) was selected.

Table 3.7 Effect of final holding time on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Final holding time (minute)	Peak area (pA.s)*		Analysis time (minute)
	Carbofuran	Carbaryl	
3	232 ± 10	53 ± 2	7.6
4	231 ± 5	51 ± 9	8.6
5	233 ± 12	54 ± 3	9.6
6	239 ± 13	51 ± 2	10.6

* 5 replications



.e of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard .ng time (n=5).

optimum conditions of the column temperature programming are summarized in Figure 3.9. The total analysis time is 7.7 s indicated that this could minimize the elution time of carbofuran and carbaryl with good resolution, increase the delectability and decrease analysis time.

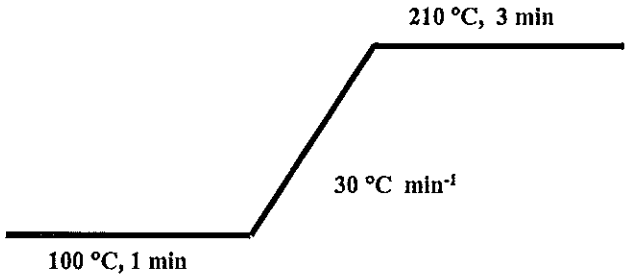


Figure 3.9 Optimum program temperature of column for carbofuran and carbaryl analysis.

3.1.3 Injector temperature

Injector is an important part of the system that performs the physical task of transferring the sample from a syringe into the column of a gas

chromatograph. The injector temperature should be set high enough such that there is enough thermal mass and energy in the inlet to vaporize the injected sample without causing the inlet to cool significantly, but not so high that sample components are decomposed. In this work, the splitless inlet was used (Figure 3.10). It provides a means for improving sensitivity by transferring nearly the entire injected sample into the capillary column rather than venting most of it through the purge vent. In splitless inlet, the purge valve is closed at the moment of injection and remains closed for a period of time (typically 30-60 sec) following the injection. During this period, the sample vapor has no place to go but into the capillary column. When the purge valve is opened, any sample vapor remaining in the inlet is rapidly swept out of the purge valve. Typically, about 95 % of the injected sample reaches the capillary column, with sample overload and peak broadening avoided through a series of complex phenomena, related to flow, thermal and solvent effects (Grob and Barry, 2004). The principal advantages of the injection using splitless inlet are that rapid vaporization of the sample is not required (relatively low injection temperatures can be used to minimize sample degradation), it can minimize band broadening by allowing a uniform migration of the solvent (Reedy, 1997), the analysis of very dilute samples is possible without preconcentration, can improve detection limit and the injection device is easily dismantled for removing in volatile sample components (Poole and Schuette, 1984). It is the most commonly used technique for trace analyses of compounds.

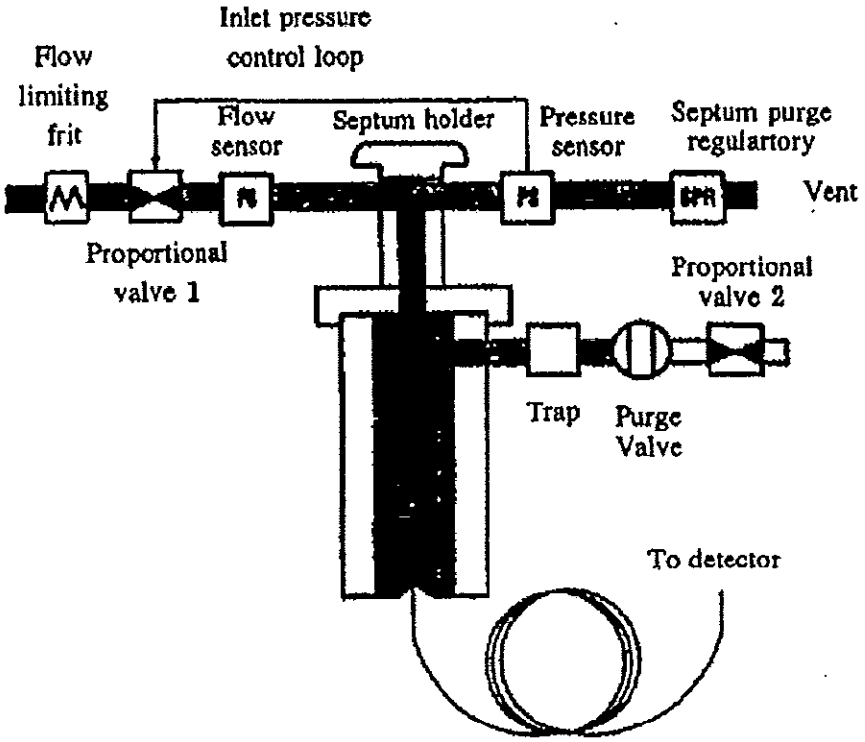


Figure 3.10 Diagram of spiltless inlet (Grob and Barry, 2004).

The optimum injection temperature was studied by varied the injector temperature. The results (Table 3.8 and Figure 3.11) indicate the highest response at 270 °C. After 270 °C, the response tends to be constant. Since lower injection temperature can minimize the sample degradation and for splitless injection, rapid vaporization of the sample is not required (Poole and Schuette, 1984). Therefore, 270 °C was selected to be the optimum injector temperature for splitless mode injection analysis.

Table 3.8 Effect of injector temperature on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Temperature (°C)	Peak area (pA.s)*	
	Carbofuran	Carbaryl
260	234 ± 3	56 ± 3
270	247 ± 7	56 ± 3
280	241 ± 7	58 ± 4
290	241 ± 12	60 ± 6
300	243 ± 13	59 ± 3

* 5 replications

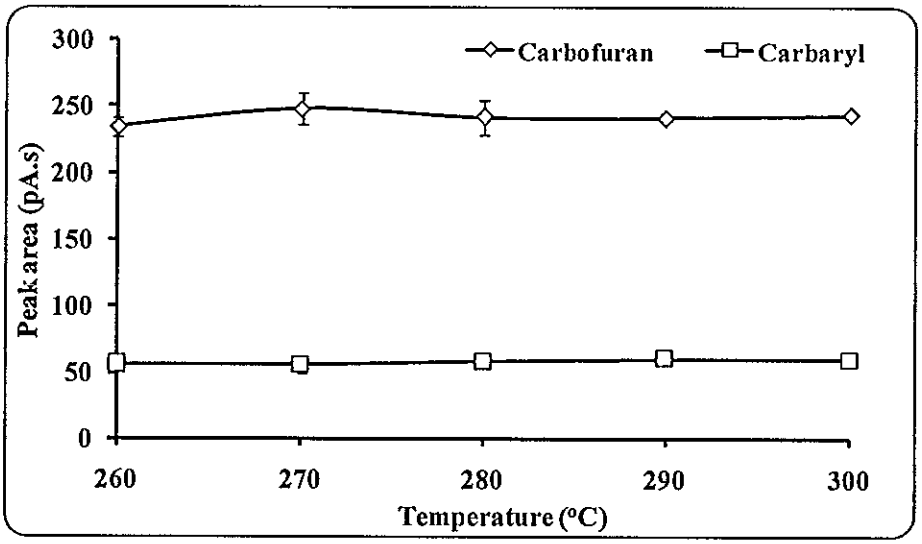


Figure 3.11 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at various injector temperatures (n=5).

3.1.4 Detector temperature

The optimum detector temperature was selected by considering the effect of the temperature on selectivity, analysis time and life time of the stationary phase. The detector temperature is always set above 100 ° C to prevent moisture water formation in the combustion process inside the detector (Grob and Barry, 2004). The

responses from various detector temperatures are as shown in Table 3.9 and Figure 3.12 where 280 °C provides the highest response.

Table 3.9 Effect of detector temperature on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Temperature (°C)	Peak area (pA.s)*	
	Carbofuran	Carbaryl
270	216 ± 25	64 ± 1
280	240 ± 13	66 ± 1
290	236 ± 4	65 ± 3
300	235 ± 5	64 ± 1

* 5 Replications

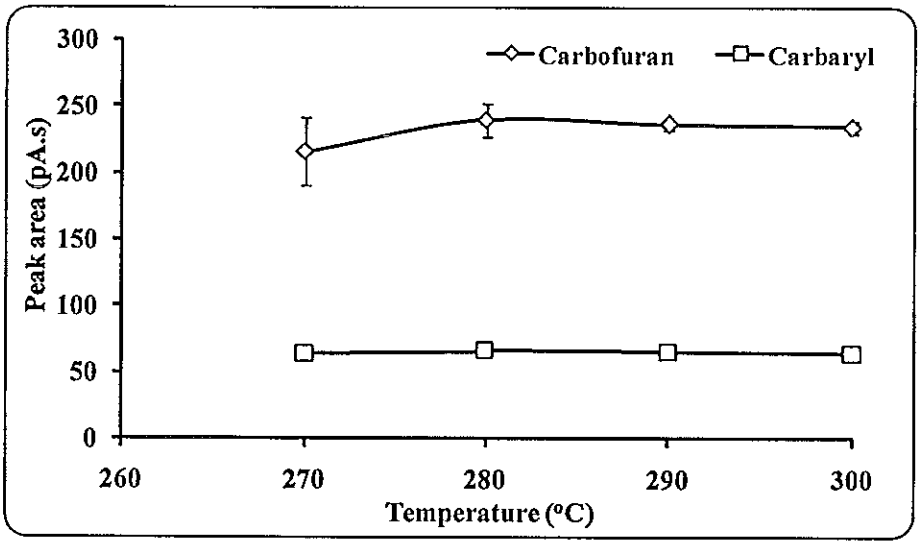


Figure 3.12 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at various detector temperatures (n=5).

3.1.5 Fuel (H_2) flow rate

The FID detector operates in a hydrogen-rich mode and uses oxidant (air) to support combustion. Therefore, it requires fuel (hydrogen, H_2) and oxidant

(air) to make the flame and flow rates of hydrogen and air can significantly influence the detector sensitivity and the noise level. The effect of fuel gas flow rate is shown in Table 3.10 and Figure 3.13. The response increased with hydrogen gas flow rate until 40 mL min^{-1} and became constant. Therefore, 40 mL min^{-1} was selected as optimum hydrogen gas flow rate.

Table 3.10 Effect of hydrogen gas flow rate on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Flow rate (mL min^{-1})	Peak area (pA.s)*	
	Carbofuran	Carbaryl
20	173 ± 4	49 ± 4
30	245 ± 15	67 ± 4
40	268 ± 13	79 ± 10
50	260 ± 2	71 ± 4

* 5 replications

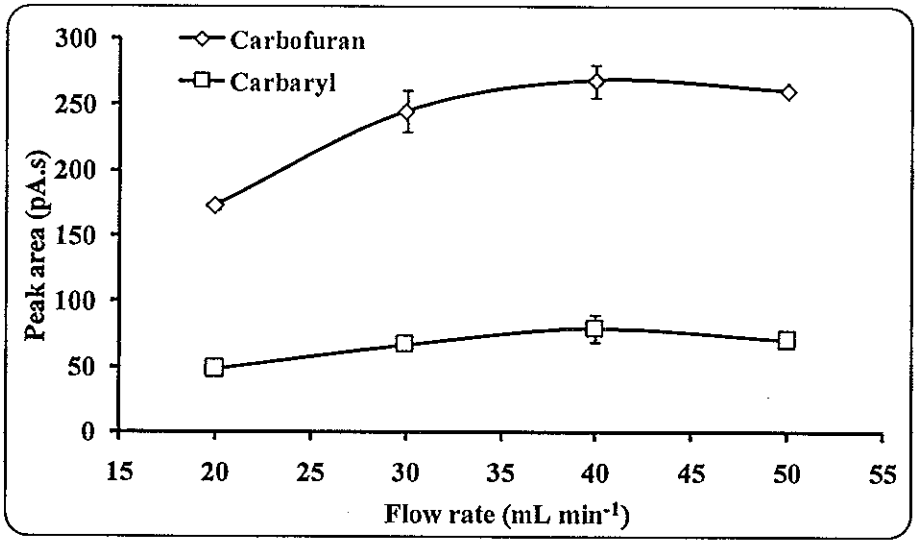


Figure 3.13 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at various hydrogen gas flow rate (n=5).

3.1.6 Oxidant gas (air) flow rate

In this study, air was used as an oxidant for FID. Typical flow rates of air are 300 -500 mL min^{-1} (Grob and Barry, 2004). Table 3.11 and Figure 3.14 show the effect of air flow rate where 300 mL min^{-1} gave the highest response, thus this flow rate was chosen as the optimum condition. The optimum fuel and air flow rate is 1:7.5, this ratio agreed with most GC-FID system (Poole and Schuette, 1984).

Table 3.11 Effect of oxidant gas flow rate on the response and analysis time of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution.

Flow rate (mL min^{-1})	Peak area (pA.s)*	
	Carbofuran	Carbaryl
200	347 ± 9	70 ± 12
300	357 ± 3	90 ± 7
400	350 ± 10	89 ± 6
500	350 ± 13	83 ± 7

* 5 replications

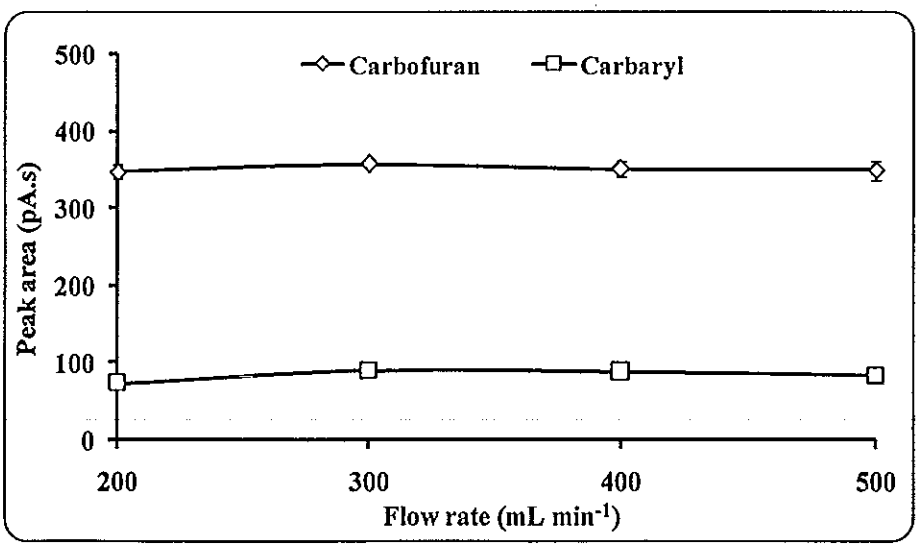


Figure 3.14 Response of 2.00 mg L^{-1} mixture of carbofuran and carbaryl standard solution at various oxidant gas flow rate (n=5).

In addition, a makeup gas is also required to carry the analyte in the column to the detector zone. It is added to the stream just before the analyte enter the detector. FID response is greater with nitrogen as makeup gas (Grob and Barry, 2004). In this study, the flow rate of nitrogen was applied at 30 mL min^{-1} as recommended by the manufacturer (Agilent Technology 1995).

3.1.7 Summary of optimum conditions of GC-FID

The optimum conditions for carbofuran and carbaryl analysis on capillary column (ULTRA-2: 25 m × 0.53 mm I.D. × 0.32 μm film thickness of 5% phenyl 95% dimethylsiloxane) with flame ionization detector are summarized in Table 3.12. The chromatogram obtained using these conditions is shown in Figure 3.15.

Table 3.12 Optimum conditions of sample preparation procedure.

Parameter	Studied range	Optimum conditions
Carrier gas flow rate (mL min^{-1})	1.8 - 3.3	3.0
Oxidant gas flow rate (mL min^{-1})	200 - 500	300
Hydrogen gas flow rate (mL min^{-1})	20 - 50	40
Injector temp (°C)	260 - 300	270
Detector temp (°C)	270 - 300	280
Program temperature		
Initial temperature (°C)	90 - 120	100
Initial holding time (min)	0 - 1.5	1
Ramp rate (°C min^{-1})	3 - 35	30
Final temperature (°C)	160 - 220	210
Final holding time (min)	3 - 6	3

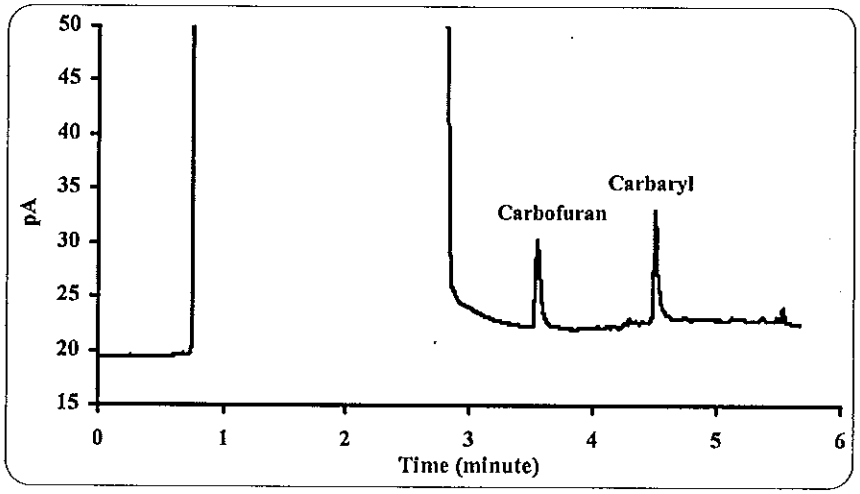


Figure 3.15 Chromatogram of carbofuran and carbaryl at the optimum conditions.

3.2 System performance of GC-FID

The GC-FID system performance was validated on the following parameters: linear dynamic range, limit of detection and instrument precision.

3.2.1 Linearity and range

The linearity is the ability of analytical procedure to produce test results which are proportional to the concentration (amount) of analyte in samples within a given concentration range. The dynamic range was investigated by serial dilutions of a stock standard solution. Linearity is achieved when the coefficient of the determination (R^2) is greater than 0.99. The slope of the regression line will provide the sensitivity of the regression (Bruce *et al.*, 1998). Figure 3.16 shows the response of carbofuran and carbaryl at various concentrations. The system provided a wide linear dynamic range from 0.5 – 600 mg L^{-1} and 0.5 -550 mg L^{-1} for carbofuran and carbaryl, respectively, with R^2 greater than 0.99 and relative standard deviations (RSD) lower than 4 %.

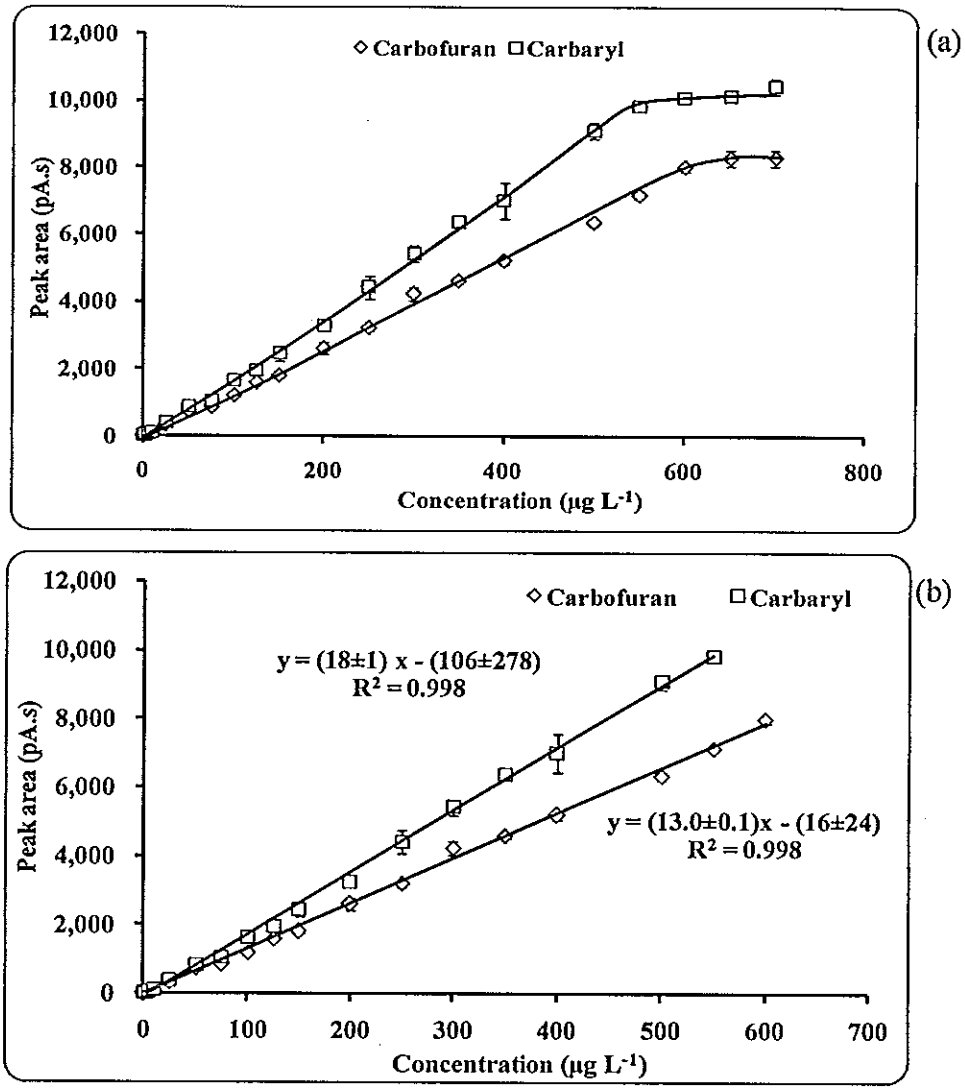


Figure 3.16 (a) Studied concentration range (b) Linear dynamic range of carbofuran and carbaryl obtained from direct injections (n=5).

3.2.2 Limit of detection (LOD) and limit of quantitative (LOQ)

The limit of detection is determined using IUPAC method from the peak area of 20 blank injections (Long and Winefordner, 1983) (Table 3.13). (See 2.5.2 and 2.5.3) and the results are shown in Table 3.14.

Table 3.13 The maximum response of 20 blank injections.

injection times	Maximum response (pA.s)
1	2.70
2	3.90
3	1.80
4	2.50
5	2.50
6	1.20
7	2.40
8	2.70
9	2.00
10	4.50
11	2.90
12	3.90
13	3.80
14	2.60
15	2.10
16	5.20
17	3.50
18	1.30
19	1.70
20	2.00
Average	**2.76**
sd	**1.07**

Table 3.14 Limit of detection and Limit of quantitative of carbofuran and carbaryl.

Compounds	Limit of detection (LOD) (mg L^{-1})	Limit of quantitative (LOQ) (mg L^{-1})
Carbofuran	0.247 ± 0.002	0.823 ± 0.006
Carbaryl	0.18 ± 0.01	0.59 ± 0.03

3.2.3 Instrument precision

The instrument precision was presented in term of % RSD of retention time and peak area of ten replication injections of standard solution. Mixed standard solution of 2.0 mg L^{-1} carbofuran and carbaryl was injected to the GC-FID system under optimum conditions. The results are shown in Table 3.15. The % RSD of retention time (0.41 and 0.04) and peak area (3.13 and 3.46) were acceptable, *i.e.,* 1 % for retention time and 4 % for peak area (L.R.Synder and Kirkland, 1979).

Table 3.15 % Relative standard deviation of retention time and peak area of carbofuran and carbaryl.

No	Carbofuran		Carbaryl	
	Retention time (min)	**Peak area (pA.s)**	**Retention time (min)**	**Peak area (pA.s)**
1	3.651	234.9	4.496	51.8
2	3.658	226.9	4.499	52.0
3	3.660	248.2	4.499	54.7
4	3.658	226.9	4.500	51.9
5	3.658	223.8	4.500	52.3
6	3.610	234.0	4.501	53.8
7	3.651	227.4	4.499	54.5
8	3.659	226.4	4.499	57.4
9	3.657	237.0	4.499	53.1
10	3.658	230.6	4.495	55.6
Average	3.652	231.6	4.50	53.7
SD	0.02	7.24	0.00	1.86
% RSD	0.41	3.13	0.04	3.46

3.3 Optimization of MIPs synthesized

MIP for carbofuran was synthesized by non-covalent imprinted protocol. Carbofuran containing functional groups that can engage in hydrogen bonding between the carboxylic acid groups of the polymerized methacrylic acid or amine group of acrylamide and a ring nitrogen of the template. The preparation step was described in section 2.6. It should also be mentioned that, as a control in each polymerization, a non-imprinted polymer (non-MIP) is also synthesized in the same

way as the MIP but in absence of the template. To evaluate the imprinting effect, the selectivity of the non-MIP and MIP are then compared.

3.3.1 The ratio of functional monomers

From the literatures, polymer prepared from two functional monomers (cocktail polymerization) can improve the recognition capacities compared to polymer that used only single functional monomer (Cormack and Elorza, 2004; Wei *et al.*, 2005). Therefore, this work used two systems of cocktail polymerization, MIP 1, acrylamide and 2-(diethylamino) ethylmethacrylate (DAM) and methacrylic acid and DAM for MIP 2.

Methacrylic acid has been the most frequently employed monomer, the templates used have been restricted mainly to those able to interact by hydrogen bonding with methacrylic acid. Other monomer, acylamide is also has the structure similar to methacrylic acid. It has amine functional group instead hydroxyl group of methacrylic acid. Based on the different chemical structures and physical properties of these two monomers *i.e.* acrylamide and methacrylic acid, the interactions between the template molecule with individual monomers are different. Therefore the ratio of monomers, acrylamide and DAM or methacrylic acid and DAM were studied as describe in 2.6.1.

Figure 3.17 shows effect of the ratio of functional monomers on binding capacity of carbofuran. For MIP 1, the ratio between acrylamide and DAM of 1:2 and MIP 2, ratio between methacrylic acid and DAM of 1:1 gave the highest binding capacity. These results indicated that at these ratio are shown an excess of acrylamide-DAM and methacrylic acid-DAM relative to carbofuran is required for template-functional monomer complex formation and to maintain its integrity during polymerization (Caro *et al.*, 2006).

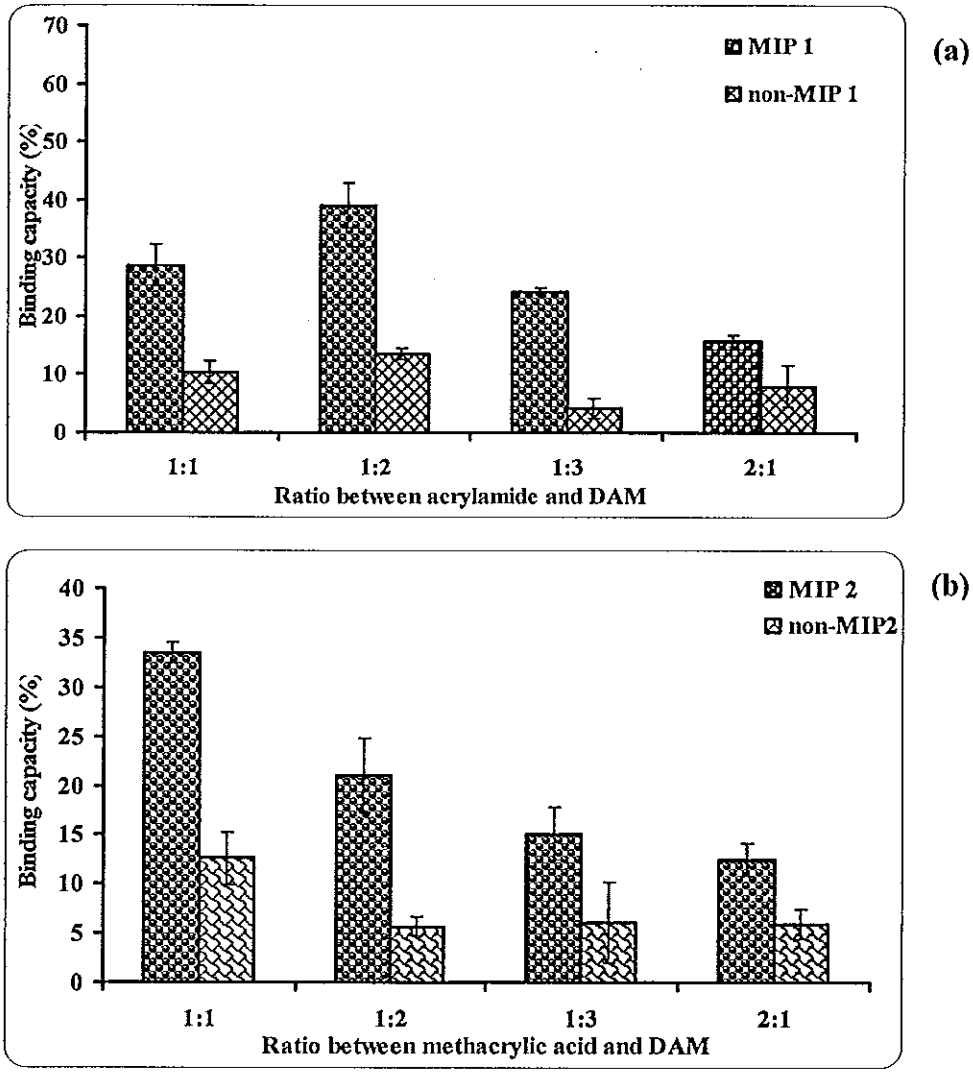


Figure 3.17 Effect of ratio of functional monomer on the binding capacity of 40.00 μg L^{-1} of carbofuran standard solution spiked DI water (n=5). (a) MIP 1: ratio between acrylamide and DAM (b) MIP 2: ratio between methacrylic acid and DAM.

3.3.2 Amount of crosslinker

The quantity of the crosslinker, EGDMA used in MIPs is an important factor to obtain high affinity binding sites. High EGDMA ratios are generally preferred in order to access permanently porous (macroporous) materials and in order to be able to generate materials with adequate mechanical stability. Polymers with crosslinker ratios in excess of 80 % are often the norm (Cormack and Elorza, 2004).

The experiment is described in 2.6.2 and the results are shown in Figure 3.18. The percentage of EGDMA indeed drew a significant influence on the binding capacity of the MIP towards carbofuran and gave the highest binding capacity at 93 % of EGDMA. When the crosslinking density is too low, the effective length between crosslinks will be too large for the imprinting process to be successful. This creates an excessively large cavity and results in a non-specific imprint. An effective length between crosslinks that is too low may result in a network that traps the imprint molecule completely (J. Wizeman and Kofinas, 2001).

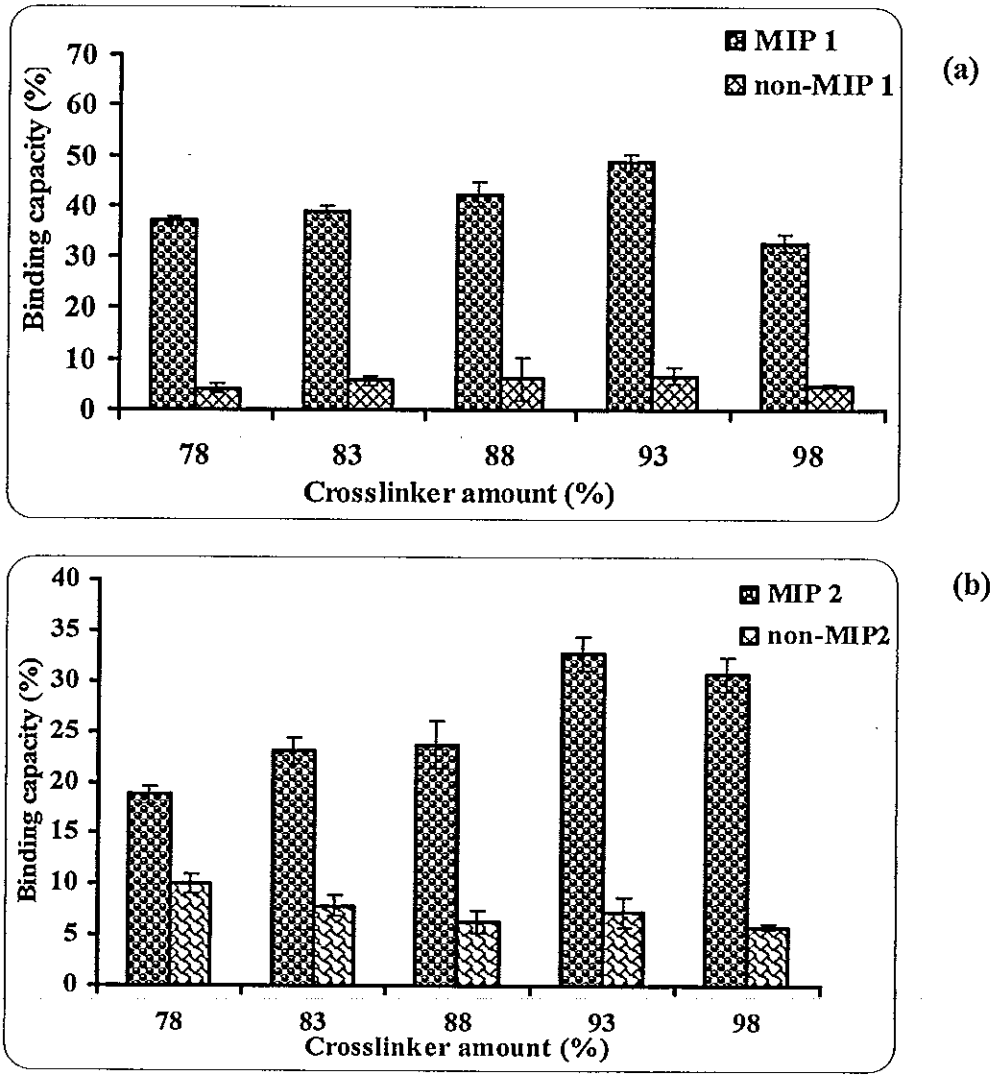


Figure 3.18 Effect of EGDMA amount on the binding capacity of 40.00 μg L^{-1} of carbofuran standard solution spiked DI water (n=5) (a) MIP 1 (b) MIP 2.

3.3.3 Type of porogen

The solvent serves to bring all the components *i.e.* carbofuran, acrylamide and DAM or methacrylic acid and DAM, EGDMA and AIBN into one phase for the polymerization (Cormack and Elorza, 2004). The important role of porogen is controlling the morphology and the total pore volume. Besides its dual roles as a solvent and as a pore forming agent, this suggests that a stronger interaction between the solvent and the template molecule and monomer leads to a reduced interaction between the template molecule and monomer. As a consequence, the complementary structure desired for molecular recognition may not be established. In the preparation of a molecular imprinted media through non-covalent forces, the H-bond is often the major force in the formation of the complementary structure (Dong *et al.*, 2007). Thus, the intermolecular force between the solvent respectively with template molecule and the monomer should be carefully controlled to prevent it from inhibiting the formation of H-bond between the template molecule and monomer. Furthermore, the strength of the H-bond between the template molecule and the monomer was greatly affected by the solvent (Dong *et al.*, 2007). Figure 3.19 shows the effect of type of porogen (section 2.6.3) on the binding capacity between MIP and carbofuran. The results indicated that toluene gave the highest binding capacity. This is due to the fact that toluene is a non-protic solvent and this helps to stabilize hydrogen bonds that provide high specific surface areas (Cormack and Elorza, 2004).

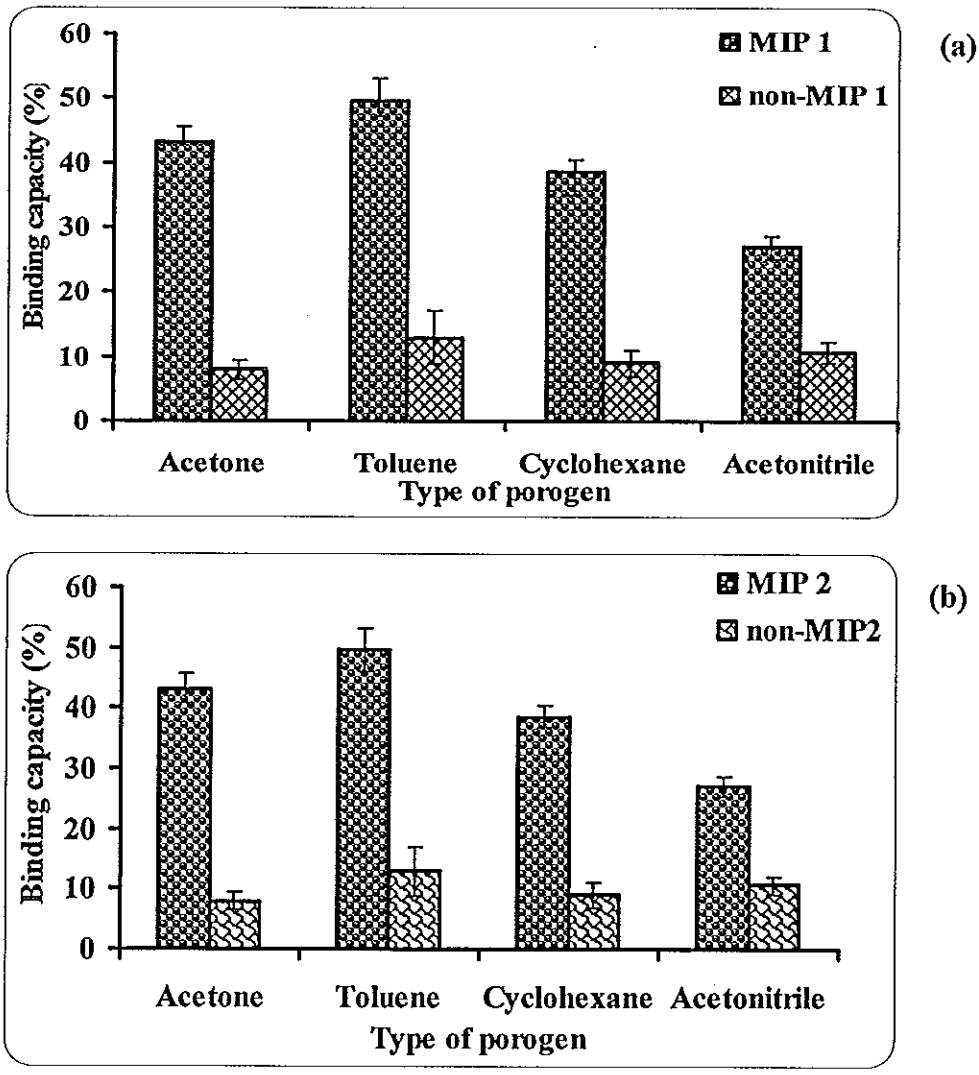


Figure 3.19 Effect of type of porogen on the binding capacity of 40.00 μg L^{-1} of carbofuran standard solution spiked DI water (n=5) (a) MIP 1 (b) MIP 2.

3.3.4 Concentration of porogen

Besides the type, the level of the porogen can also be used to control the morphology and the total pore volume (Cormack and Elorza, 2004). Figure 3.20 shows the effect of concentration of toluene to the binding capacity of carbofuran. Toluene at 0.050 and 0.010 mol gave the highest binding capacity because increasing the volume of toluene can increase the pore volume. However, excess volume of toluene causes the MIP to swell and provided low binding capacity. This can be seen

in the case of MIP 2, when the concentration of porogen increased the binding capacity decreased.

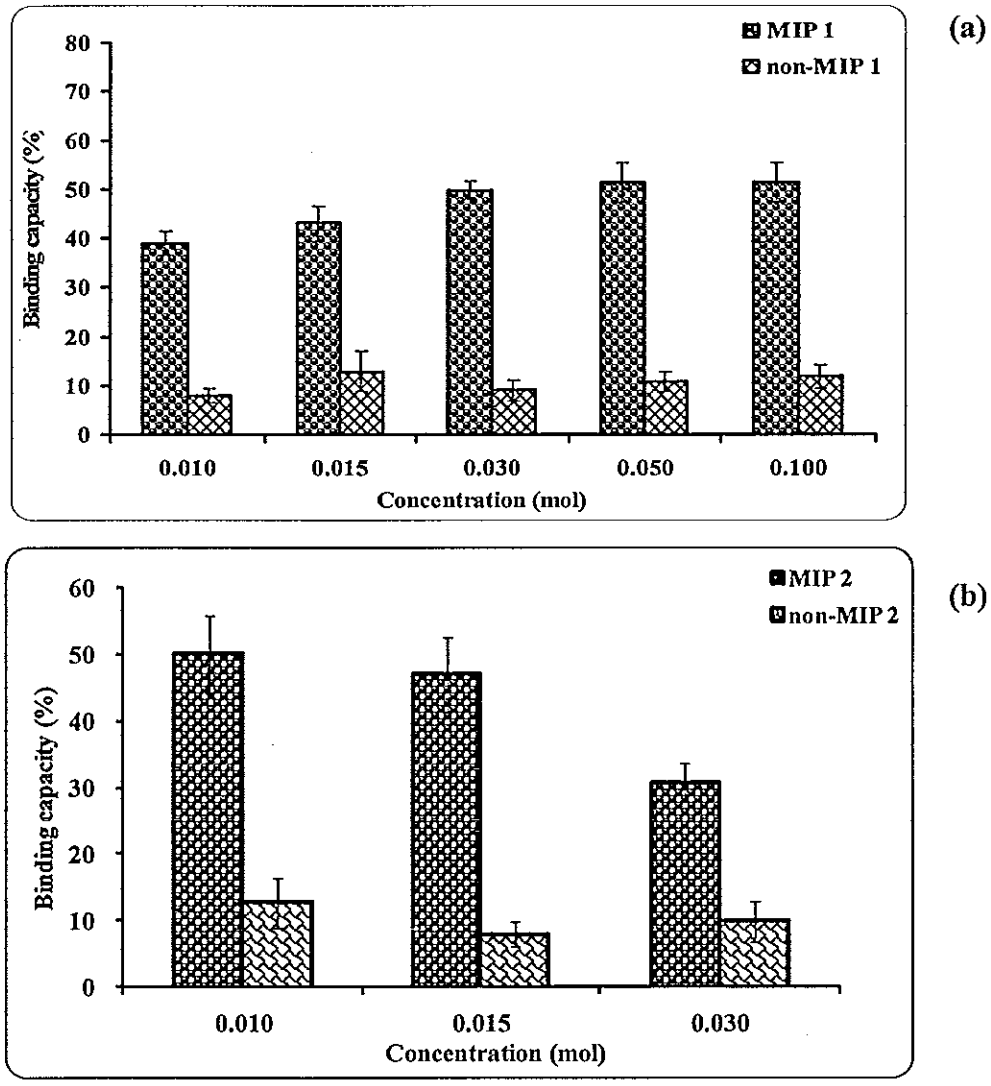
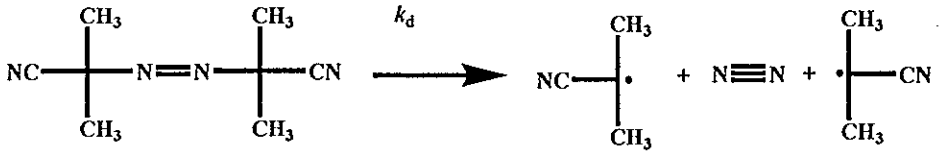


Figure 3.20 Effect of concentration of toluene on the binding capacity of 40.00 μg L^{-1} of carbofuran standard solution spiked DI water (n=5) (a) MIP 1 (b) MIP 2.

3.3.5 Type of initiator

Free radical (or chain growth) polymerization is the most important synthetic method available. It can be performed under mild reaction conditions. Initiators for free-radical polymerizations include any organic compound with a labile

group, such as an azo (-N=N-), disulfide (-S-S-), or peroxide (-O-O-) compound. Normally they are used at low levels compared to the monomer. The rate and mode of decomposition of an initiator to radicals can be triggered and controlled in a number of ways, including heat, light and by chemical/electrochemical means, depending upon its chemical nature. The effect of two different type of initiator (section 2.6.5) *i.e* AIBN and benzoyl peroxide are shown in Figure 3.21. AIBN gave higher binding capacity than benzoyl peroxide. This is probably due to the termination step of polymerization. Normally the mechanism of free radical polymerization is characterized by three distinct stages: (1) initiation of the active monomer (2) propagation or growth of the active (free radical) chain by sequential addition of monomers. Final step is termination of active chain to give the final polymer product (Fried, 2003). An initial free-radical polymerization consists of two steps – dissociation of initiator to from two radical species, followed by addition of a single monomer molecule to the initiating radical (the association step). The dissociation of the AIBN to form two free-radical initiator species can be represented as



(3.7)

Where k_d is the dissociation rate constant

The dissociation rate constant is strongly dependence on temperature; dissociation rate constants for different initiators vary with the nature of the solvent used in solution polymerization. Table 3.16 showed example data of initiator (Fried, 2003). In the termination step, one chain is terminated, but another one initiates. This particular reaction reduces molecular weight and wastes initiator (*i.e.,* an initiator molecule is consumed, but no new chains are begun). Sometimes this process is called induced decomposition of the initiator. It is a common side reaction for the peroxy initiators, but happens less often with the azo initiators (Odian, 1991).

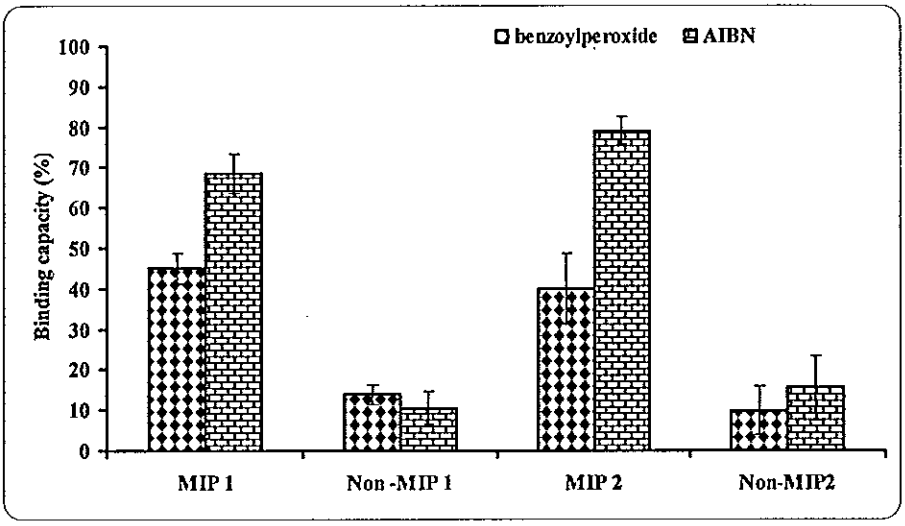


Figure 3.21 Effect of type of initiator on the binding capacity of 40.00 μg L^{-1} of carbofuran standard solution spiked DI water.

Table 3.16 Dissociation Rate Constants for Some Common Initiators in solution (Fried, 2003).

Initiator	Solvent	T (°C)	K_d (s^{-1})
Benzoyl peroxide	benzene	30	4.80×10^{-8}
		70	1.38×10^{-5}
	Toluene	30	4.94×10^{-8}
		70	1.10×10^{-5}
AIBN	benzene	40	5.44×10^{-7}
		70	3.17×10^{-5}
	Toluene	70	4.00×10^{-5}

3.3.6 Concentration of initiator

The amount of initiator is also a key for synthesizing the suitable imprint in the MIPs. Figure 3.22 shows the effect of concentration of initiator where 0.019 mol of initiator (AIBN) gave the highest binding capacity for both MIPs.

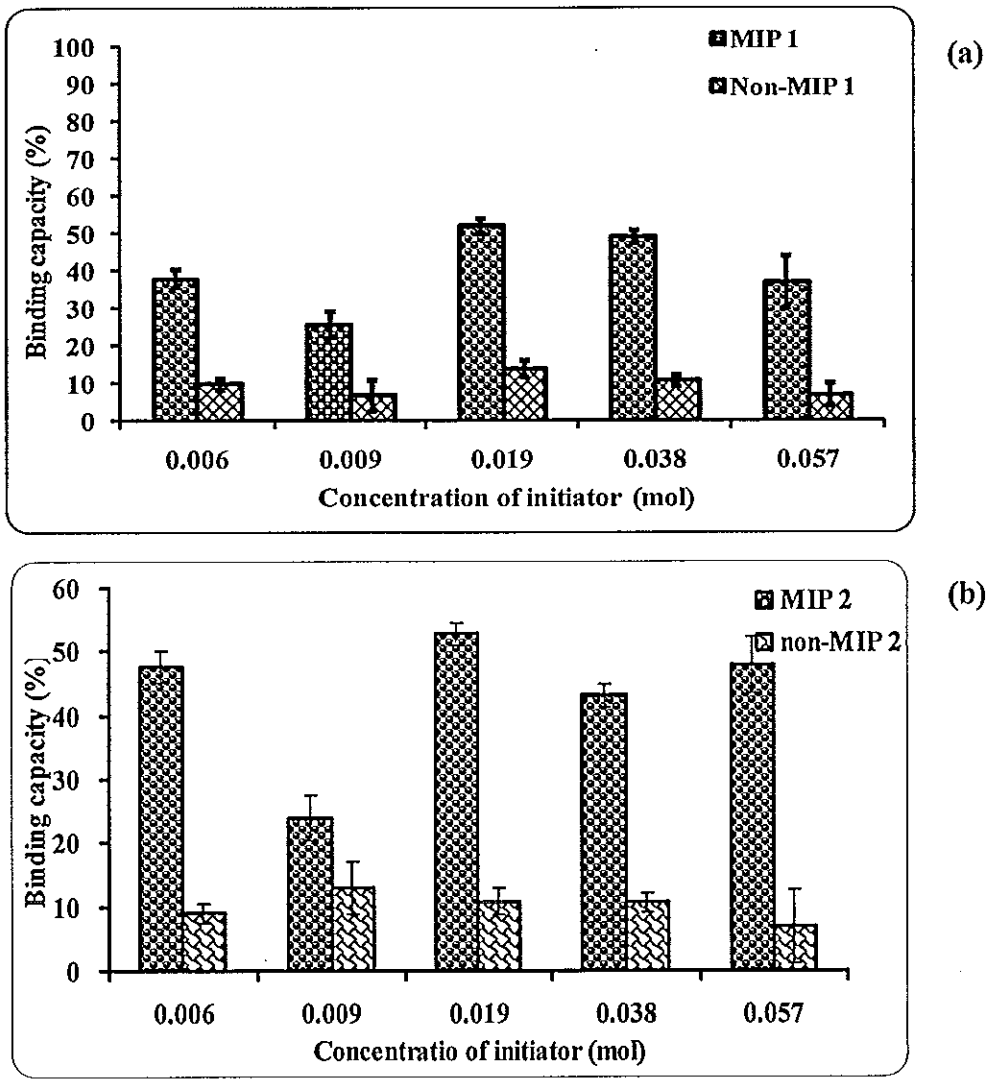


Figure 3.22 Effect of concentration of AIBN on the binding capacity of 40.00 μg L^{-1} of carbofuran standard solution spiked DI water (n=5) (a) MIP 1 (b) MIP 2.

3.3.7 Polymerization temperature

Intermolecular interactions between carbofuran and functional monomers depend strongly on temperature. So it is believed that the polymerization temperature should play an important role on imprint formation; stronger specific interactions should lead to more, well-formed imprint sites (Wei *et al.*, 2005). The effect of polymerization temperature on MIP 1 and 2 were investigated as described in 2.6.7. From the result (Figure 3.23) the highest binding capacity was obtained at 60

°C for both MIPs. This is because AIBN normally starts to cleavage at about 60 °C (Cormack and Elorza, 2004) and the non-covalent interactions are stronger at low temperature (Wei *et al.*, 2005). Therefore when the polymerization temperature increased binding capacity decreased.

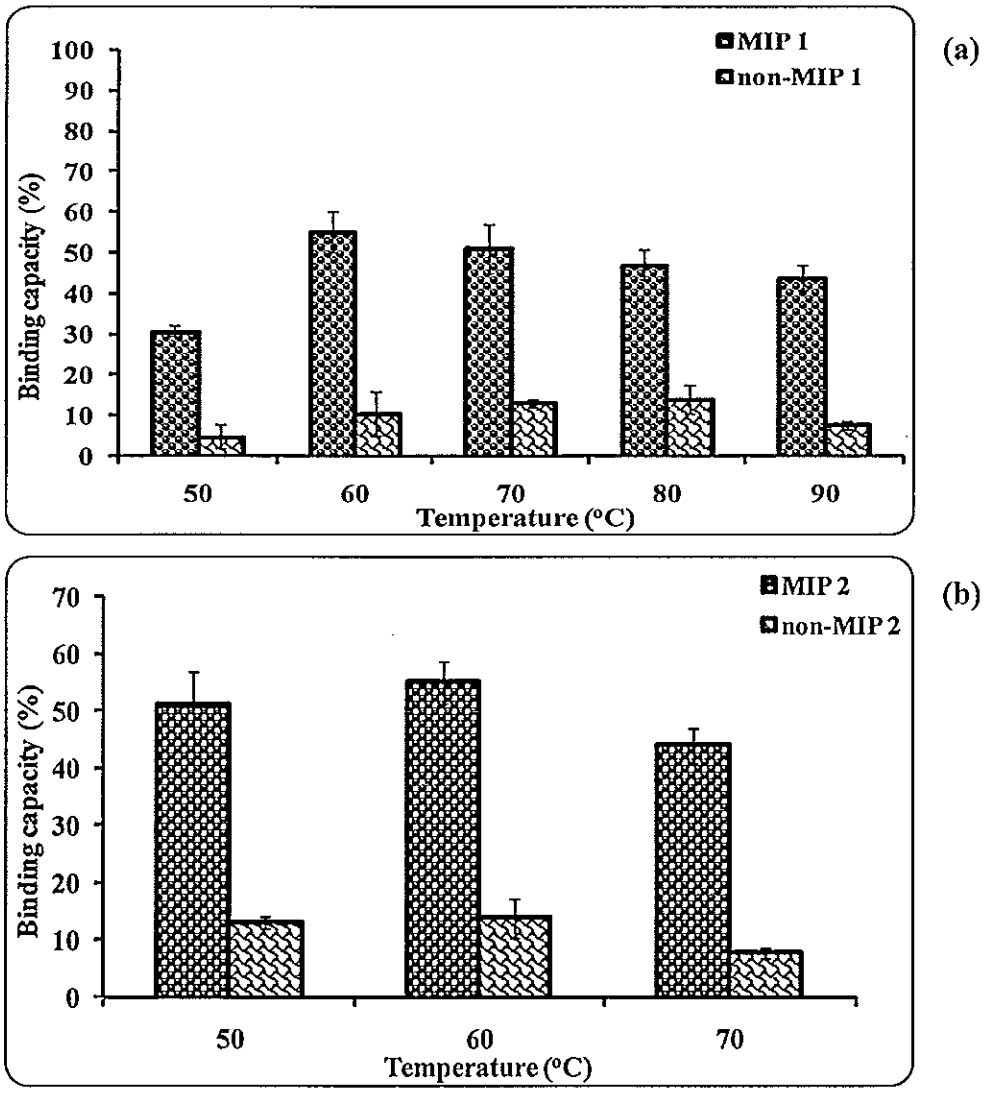


Figure 3.23 Effect of polymerization temperature on the binding capacity of 40.00 μg L^{-1} of carbofuran standard solution spiked DI water (n=5) (a) MIP 1 (b) MIP 2.

3.3.8 Optimum time for template removal (soxhlet extraction time)

In order to remove the imprinted molecule from MIPs after cross-linking, it is necessary to break the non-covalent bond between carbofuran molecule and carboxyl group of the polymer. This was performed by continuous extraction with methanol containing 10 % acetic acid in a soxhlet extractor. Figure 3.24 shows the effect of soxhlet extraction time. After 6 hours the absorbance is constant which means that no more template can be removed from the polymer, therefore, 6 hours was chosen as the time for soxhlet extraction time. To confirm that the template was completely removed from the polymer, sample polymer was stirred with 10 % acetic acid in methanol for 24 hours, no absorbance peak of carbofuran was obtained.

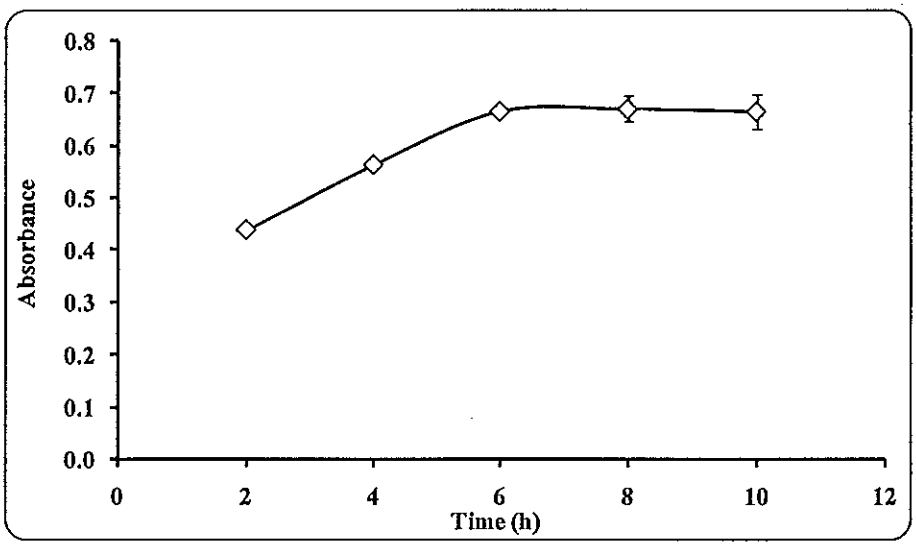


Figure 3.24 The effect soxhlet extraction time to remove carbofuran.

3.4 Characterizations of MIPs

Equilibrium binding experiments were carried out (section 2.7) to evaluate the binding properties of the MIP. For this method, a known mass of carbofuran in solution is added to a vial containing a fixed mass of MIP. Once the system has come to equilibrium, the concentration of free carbofuran in solution is measured and the mass of carbofuran adsorbed to the MIP was calculated.

3.4.1 Particle size measurement

Particle size is an important property of the MIP used in SPE coarse particles tend to channel more readily than fine particles, but confer high permeability and filtering efficiency upon a bed packed with such material. Fine particles have a tendency to be lost through the outer filter. Furthermore, fine particle could cause SPE to clog more readily (Simpson, 2000). The particle sizes of MIP and non-MIP were investigated (2.7.1) and the results are shown in Table 3.17. They were in the range which can be accepted (> 40 μm) for SPE application (Synder and Kirkland, 1979).

3.4.2 Swelling measurement

To evaluate the effect of equilibrated solvent on the binding property of imprinted polymer, the percent swelling of both MIP and non-MIP were measured (2.7.2) in acetonitrile, used as the medium solvent to re-bind the analyte. Swelling was measured by allowing a known volume, particle size and weight of dry polymer equilibrated in the solvent, after the volume of the swollen particles was measured. The volume swelling ration was calculated. Results in Table 3.17 indicated that the percent swelling of MIP did not differ from non-MIP.

3.4.3 Bulk density

The results of bulk density are shown in Table 3.17. Bulk density was displayed as the weight of 1 mL of polymer particles. Since the particle size of MIP and non-MIP were in the same range, this revealed that the property of the polymer did not depend on its particle size. In general the rigidity of imprinted polymer was determined by crosslinker (EGDMA) content. In the synthesis polymer, the equal amount of EGDMA was used to prepared MIP and non-MIP. Therefore, bulk density of MIPs and non-MIPs were not different.

Table 3.17 The physical characterization of imprinted polymer (MIPs and non-MIPs).

Properties	MIP 1	non - MIP 1	MIP 2	non - MIP 2
Swelling (mL mg^{-1})	40 ± 5	47 ± 3	43 ± 3	42 ± 10
Particle size (μm)	93 ± 14	81 ± 15	118 ± 9	70 ± 7
Bulk density (g mL^{-1})	0.361 ± 0.008	0.42 ± 0.02	0.36 ± 0.02	0.38 ± 0.01

3.4.4 Capacity of imprinted (MIP) and non-imprinted (non-MIP) polymer

The polymers were incubated with carbofuran in water for 24 hours at room temperature, and the amount of carbofuran bound to the polymer was plotted as a function of concentration, the results are shown Figure 3.25. The amount of carbofuran bound to the MIPs at equilibrium (Q) increased with concentration of carbofuran and reached saturation at 12 mg L^{-1} and 11 mg L^{-1} for MIP 1 and 2, respectively. When compared to non-MIP the results suggested that the template binding to MIPs is more possibly caused by the specific binding to a limited number of binding sites in the polymer network than by the non-specific adsorption, but the binding amount of carbofuran on MIPs were more than that on non-MIPs in the whole concentration range. This result could be ascribed to the imprinting effect (Matsui *et al.*, 1995).

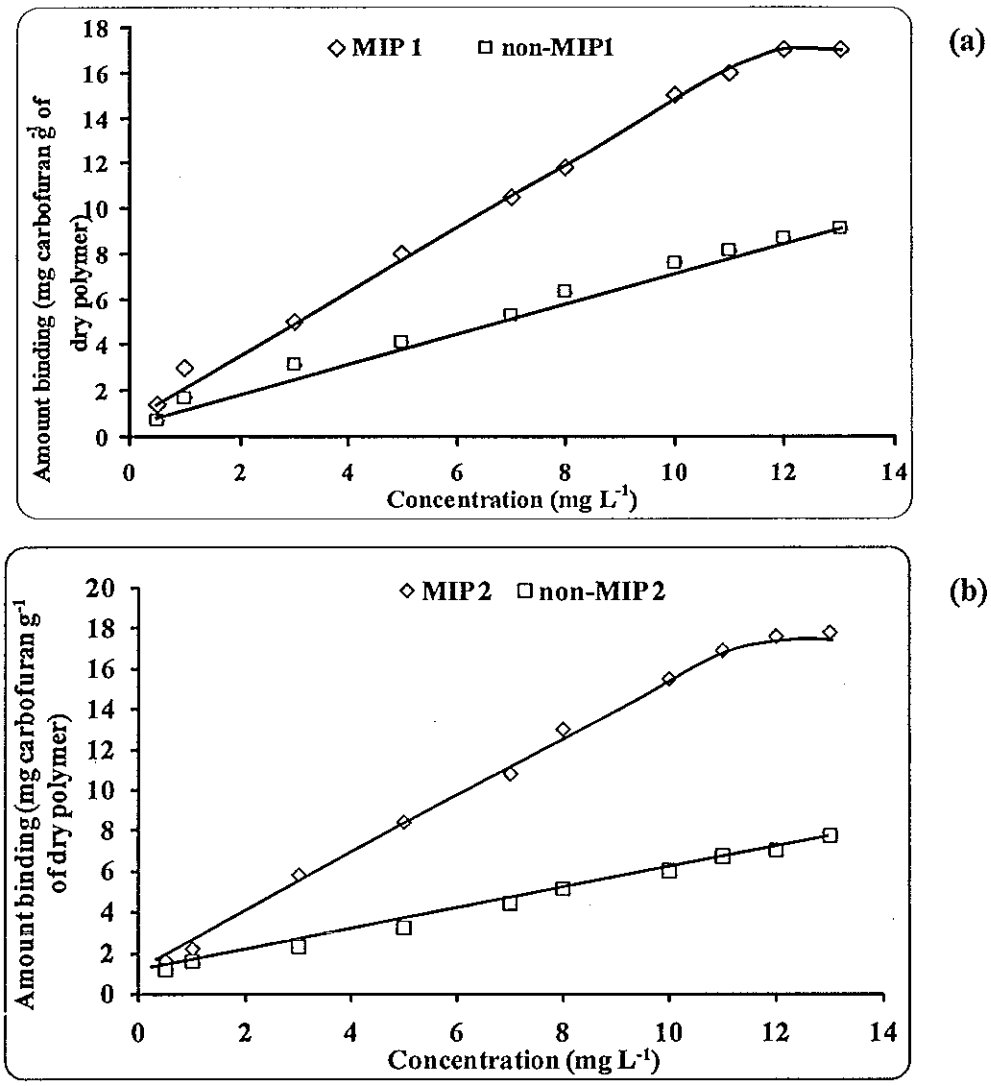


Figure 3.25 Binding isotherm of carbofuran imprinted polymer. MIP: 1 g; volume: 25.0 mL; binding time: 12 hours (a) MIP 1 (b) MIP 2.

3.4.5 Scatchard analysis

The saturation binding data were further processed with Scatchard equation to estimate the binding properties of MIPs. Scatchard equation follows Equation 1.3. The saturation binding data were shown in Table 3.18 and from the data in this table, [bound]/[free] (y axis) and Q (x axis) was plotting (Figures 3.26 and 3.27 for MISPE and MISPD, respectively). The results found that the Scatchard plot of MIP and non-MIP are not single straight line, but consists of two parts with

different slope. This suggested that the binding sites in the MIPs are heterogeneous in respect to the affinity for carbofuran (Xu *et al.*, 2007; Zhu *et al.*, 2002) and indicated that the binding sites in the imprinted polymer could be classified into two distinct groups with specific binding properties. The dissociation constant (K_D) and the apparent maximum number of binding sites (Q_{max}) of the higher and lower affinity binding sites of both MIPs and non-MIPs can be calculated and show in Table 3.19. Most MIPs prepared with non-covalent imprinting approach suffer from a heterogeneous distribution of binding sites (Xu *et al.*, 2007; Yan *et al.*, 2007; Zhu *et al.*, 2002), which is primarily affected by two factors. First, because of amorphous nature of MIPs, the binding sites are not identical, somewhat similar to a polyclonal preparation of antibodies. The sites may reside in domains with different cross-linking density and accessibility. The second effect is the incompleteness of the monomer–template association. In most cases only a part of template associates with the functional monomer to produce selective binding sites due to the major part of the functional monomer existing in a free or dimerized form. The poor yield of binding sites results in a strong dependence of selectivity and binding on sample load (Zhu *et al.*, 2002).

The imprinting sites situated at the surface can be considered as high affinity to carbofuran, so easy diffusion into carbofuran molecule. The lower affinity binding sites were assembly believed that located deeply inside the polymer particle. It was mostly due to the difficult diffusion process of the template to these binding sites. The binding events of the inside imprinting hole can occur after the saturation of the imprinted sites from the template binding at the surface attained. This explanation can be clearly revealed from two slopes in Scatchard analysis which the higher affinity binding sites represented the higher slope at lower template concentration and correspondingly, lower slope of a straight line at high template concentration showed the lower affinity imprinted sites.

Comparing between MIP and non-MIP, the high affinity region of MIP has higher negative slope than non-MIP. It revealed the sensitivity if binding site in the MIP to the carbofuran. Q_{max} of both MIP and non-MIP were produced from the high affinity and low affinity binding site. The amount of high affinity of MIP has less than the amount of low affinity because the imprinting site located at the surface area

of imprinting polymer has less than the inside imprinting hole similar to non-MIP. Comparing between MIP and non-MIP, sites consist of specific binding and non-specific binding whereas non-MIP was obtained from non-specific binding (Xu *et al.*, 2007).

Table 3.18 Saturation binding data of carbofuran various concentrations on MIPs and non-MIPs.

Concentration (mg L^{-1})	MIP 1			non-MIP 1			MIP 2			non-MIP 2		
	Free[a]	Bound[b]	Bound/Free	Free[a]	Bound[b]	Bound/Free	Free[a]	Bound[b]	Bound/Free	Free[a]	Bound[b]	Bound/Free
0.5	10	3	0.25	12	1	0.09	11	2	0.16	11	1	0.11
1.0	21	4	0.19	23	2	0.07	22	3	0.15	23	2	0.07
3.0	70	5	0.07	72	3	0.04	69	6	0.08	73	2	0.03
5.0	117	8	0.07	121	4	0.03	117	8	0.07	122	3	0.03
7.0	165	11	0.06	170	5	0.03	164	11	0.07	171	4	0.03
8.0	188	12	0.06	194	6	0.03	187	13	0.07	195	5	0.03
10.0	235	15	0.06	242	8	0.03	235	16	0.07	244	6	0.02
11.0	259	16	0.06	267	8	0.03	258	17	0.07	268	7	0.02
12.0	283	17	0.06	291	9	0.03	282	18	0.06	293	7	0.02
13.0	308	17	0.06	316	9	0.03	307	18	0.06	317	8	0.02

[a] The free carbofuran at equilibrium [carbofuran]

[b] The amount of template bound to MIPs at equilibrium (Q)

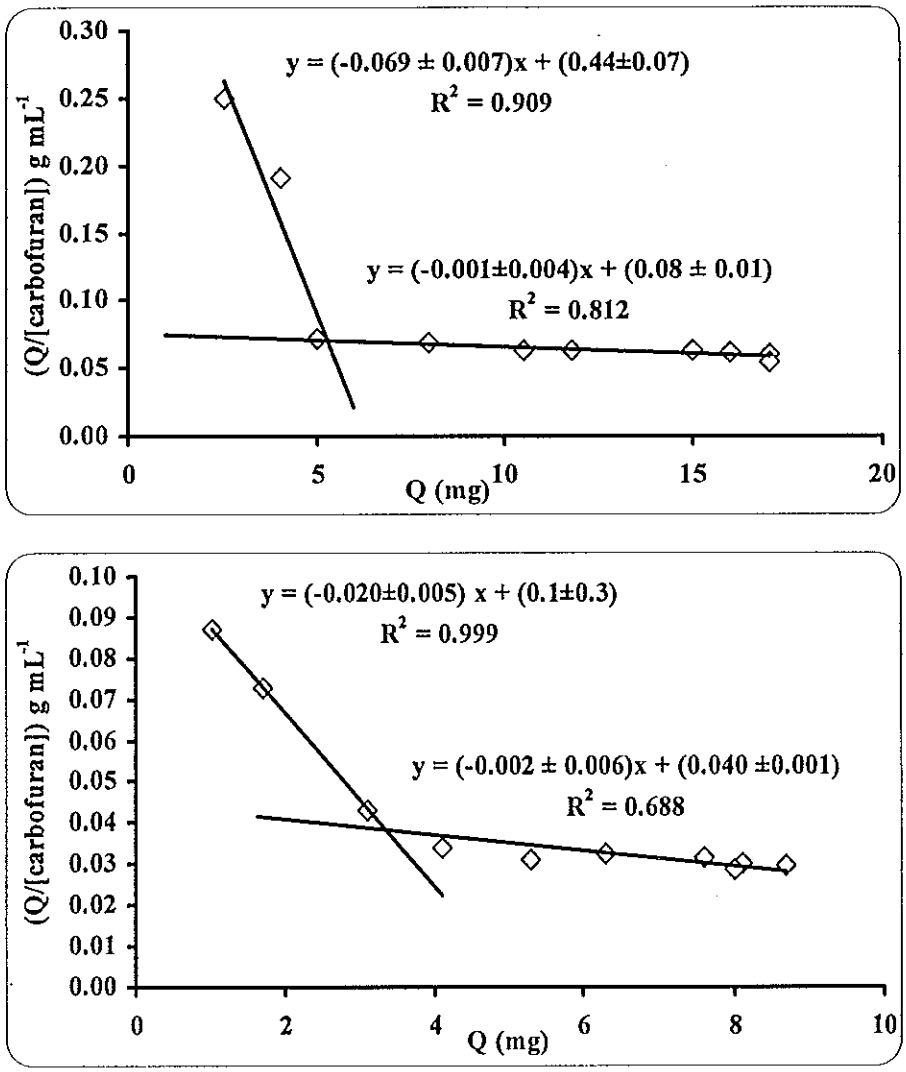
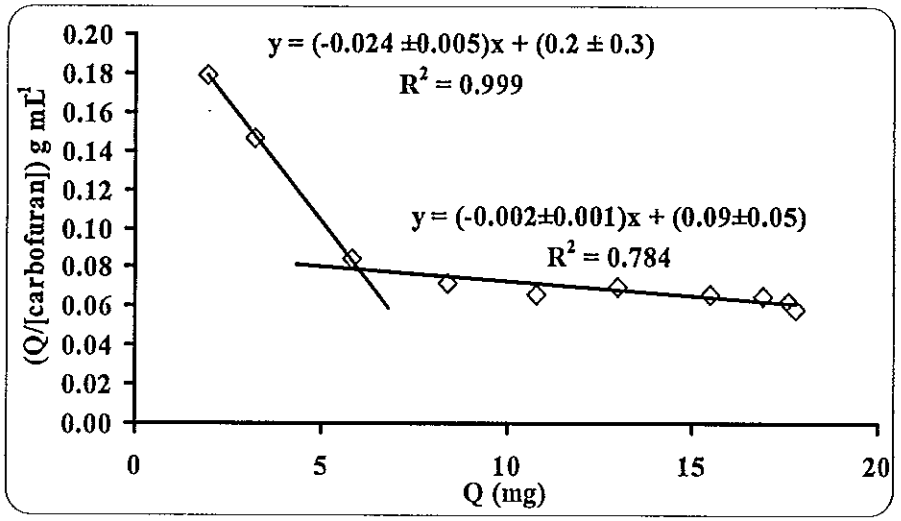


Figure 3.26 Scatchard plot of the binding of carbofuran. Q: bound carbofuran; [carbofuran]: concentration of free carbofuran (n=5) (a) MIP 1 (b) non-MIP1.



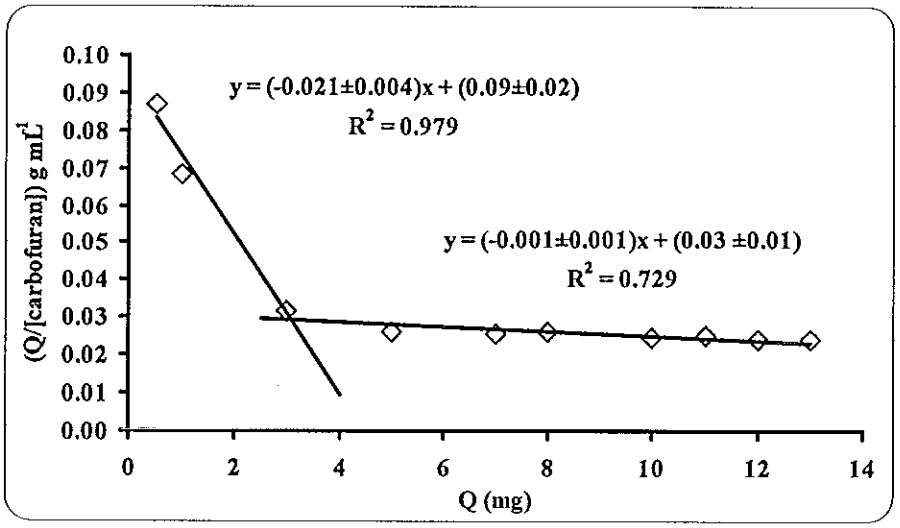


Figure 3.27 Scatchard plot of the binding of carbofuran. Q: bound carbofuran; [carbofuran]: concentration of free carbofuran (n=5) (a) MIP 2 (b) non-MIP 2.

Table 3.19 Equilibrium dissociation constant and maximum number of binding sites value of two classes of binding sites in MIP and non-MIP.

Constant	MIP 1		non-MIP 1		MIP 2		non-MIP 2	
	High affinity	**Low affinity**	**High affinity**	**Low affinity**	**High affinity**	**Low affinity**	**High affinity**	**Low affinity**
K_D (mol L^{-1})	15 ± 2	145 ± 29	53 ± 19	187 ± 88	43 ± 13	666 ± 71	49 ± 13	1010 ± 142
Q_{max} (mg g^{-1})	4 ± 3	11 ± 2	9 ± 2	5 ± 2	5 ± 1	22 ± 5	3 ± 1	11 ± 2

3.4.6 Imprinted factor

The amount of carbofuran bound to the polymers was determined by equilibrium binding experiments. The specificity of the polymers was estimated by the distribution coefficients of carbofuran between polymer and solution. The distribution coefficient (K_d) is defined as Equation 3.8 (Zhu *et al.*, 2002):

$$K_d = \frac{C_p}{C_l} \tag{3.8}$$

Where C_p: the concentration of the carbofuran in the polymer (mmol/g polymer)

C_l: carbofuran concentration in solution (mmol/mL)

C_p was calculated according to Equation 3.9

$$C_p = \frac{Q}{\text{mass of polymer in grams}} \tag{3.9}$$

Where Q: the amount of carbofuran bound to MIPs

The molecular imprinting factor (IF) was used to evaluate the imprinting effect. Then, the IF was calculated according to Equation 3.10.

$$IF = \frac{K_d\,(MIP)}{K_d\,(non-MIP)} \qquad (3.10)$$

The obtained IF values of MIP 1 and MIP 2 were 2.0 ± 0.2 and 2.4 ± 0.2. Between the two, MIP 2 shows higher IF value. The results indicated that the hydrogen bonding from hydroxyl group of methacrylic acid (MIP 2) is stronger than amine group of acrylamide (MIP 1) and this is essential for the efficient improvement in the affinity and specific binding of the imprinted polymer (Ikegami *et al.*, 2004; Jun *et al.*, 1998).

3.4.7 Reproducibility of synthesized MIP

To ensure batch-to-batch reproducibility, results from three different batches of synthesized MIP were compared. The percentage of relative standard deviation of capacity property was considered. The results obtained from MIPs of different batch gave % RSD lower than 10 % (Table 3.20 and Figure 3.28) provided *i.e.* relatively high reproducibility of synthesized MIP was obtained.

Table 3.20 Binding capacity of MIPs and non-MIPs at different times of synthesized.

Times	MIP 1	non-MIP1	MIP2	non-MIP2
1	39 ± 4	18 ± 1	48 ± 2	19 ± 1
2	39 ± 2	20 ± 1	43 ± 2	18 ± 2
3	38 ± 2	20 ± 2	50 ± 4	17 ± 4
Average	39	19	47	18
SD	0.6	1.3	3.3	1.0
% RSD	**1.4**	**6.9**	**7.1**	**5.8**

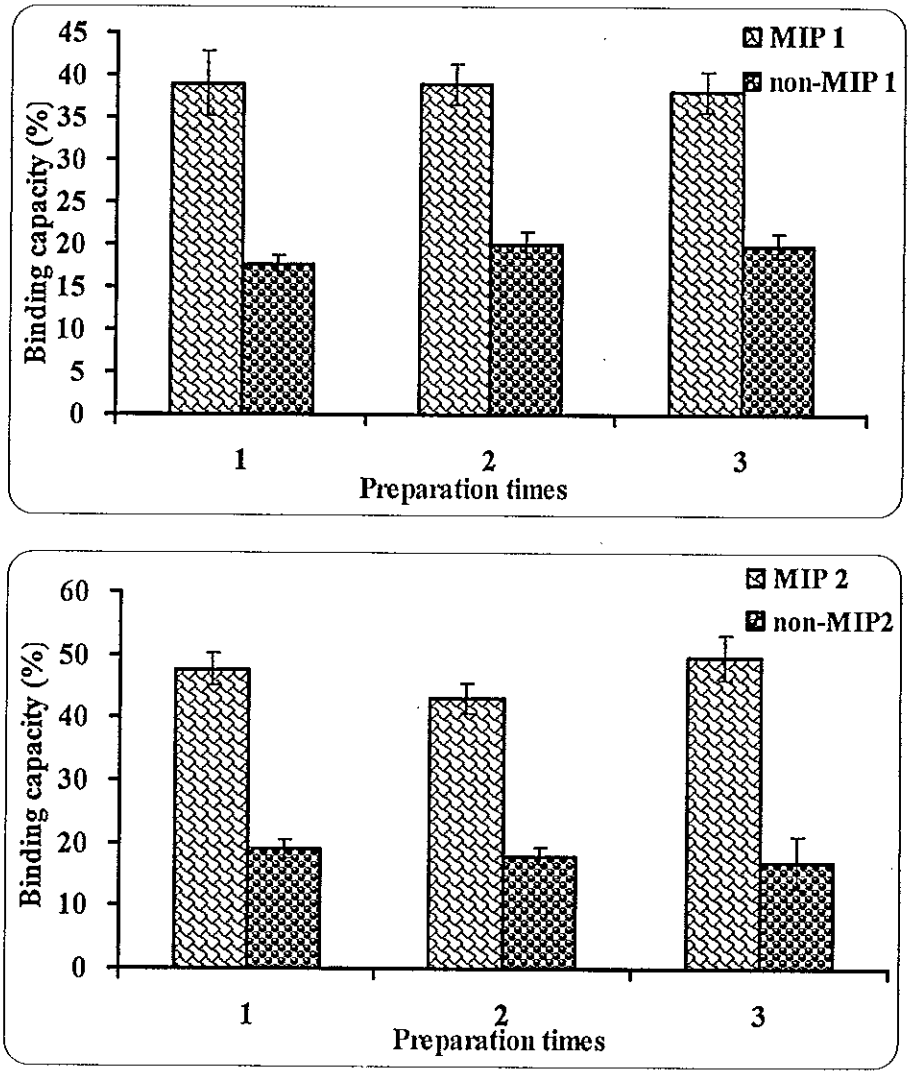


Figure 3.28 Reproducibility of synthesized (a) MIP 1 (b) MIP 2.

3.5 Sample preparation

The synthesized MIPs were used as the sorbent in solid phase extraction and solid phase dispersion techniques, so called molecular imprinted solid phase extraction (MISPE) and molecular imprinted solid phase dispersion (MISPD), respectively to extract carbofuran from water in the sample preparation step. The parameters affecting the extraction efficiency of both techniques were studied including method validation.

3.5.1 Molecular imprinted solid phase extraction (MISPE)

Solid phase extraction (SPE) is a sample preparation technique based on the use of a solid sorbent material to adsorb the specific compounds for rapid sample clean-up or preconcentration. After conditioning of the packing material, the interested analyte are adsorbed on the solid sorbent phase. A washing step allows selective removal of interfering compounds, followed by an eluting step where the analyte is eluted with a solvent and collected fraction. The eluting solvent should facilitated fast dissociation of the analyte from the solid phase. The SPE process has three steps *i.e* complete retention followed by washing and elution from the sorbent. These were optimized for the extraction of carbofuran.

3.5.1.1 Flow rate of loading sample

One of the most important considerations in the practice of SPE is the control and optimization of flow rate. The flow rate of the sample solution through the sorbent is important because mass transfer resistance affects the chromatographic performance of the cartridge (Meloan, 1990). The flow rate is independent of retention and much more to do with the kinetic sorptive properties of MISPE. *i.e* when the flow rate is too high for a given sorptive properties of MISPE. In other words, when the flow rate is too high for a given MISPE step, it will allow minimal time for analyte - sorbent interaction so analyte molecules pass through the MISPE cartridge without sufficient residence time to adsorb or desorbs from the MISPE sorbent.

In the conditioning step, the cavities (binding sites) of the MIP for carbofuran are activated in order to maximize the interactions with the target analyte present in the sample. After that the sample was loaded, the sample medium has a direct influence on the recognition properties of the imprinted polymer. Thus, if the sample is percolated through the MIP, a selective loading step can be achieved, in which only the target analyte is selectively retained on the MIP while the sample matrix is non-retained. The results of this study (2.8.1.1) are shown in Figure 3.29 which found that when sample flow rate increased, responses decreased. This is due

to the shorter time available for interaction between analyte and polymer in the MIP cartridge. However, when considered the response obtained from 0.2 and 0.5 mL min^{-1}, they differ < 10 %. Therefore, to reduce the time, 0.5 mL min^{-1} was selected as the optimum loading sample flow rate.

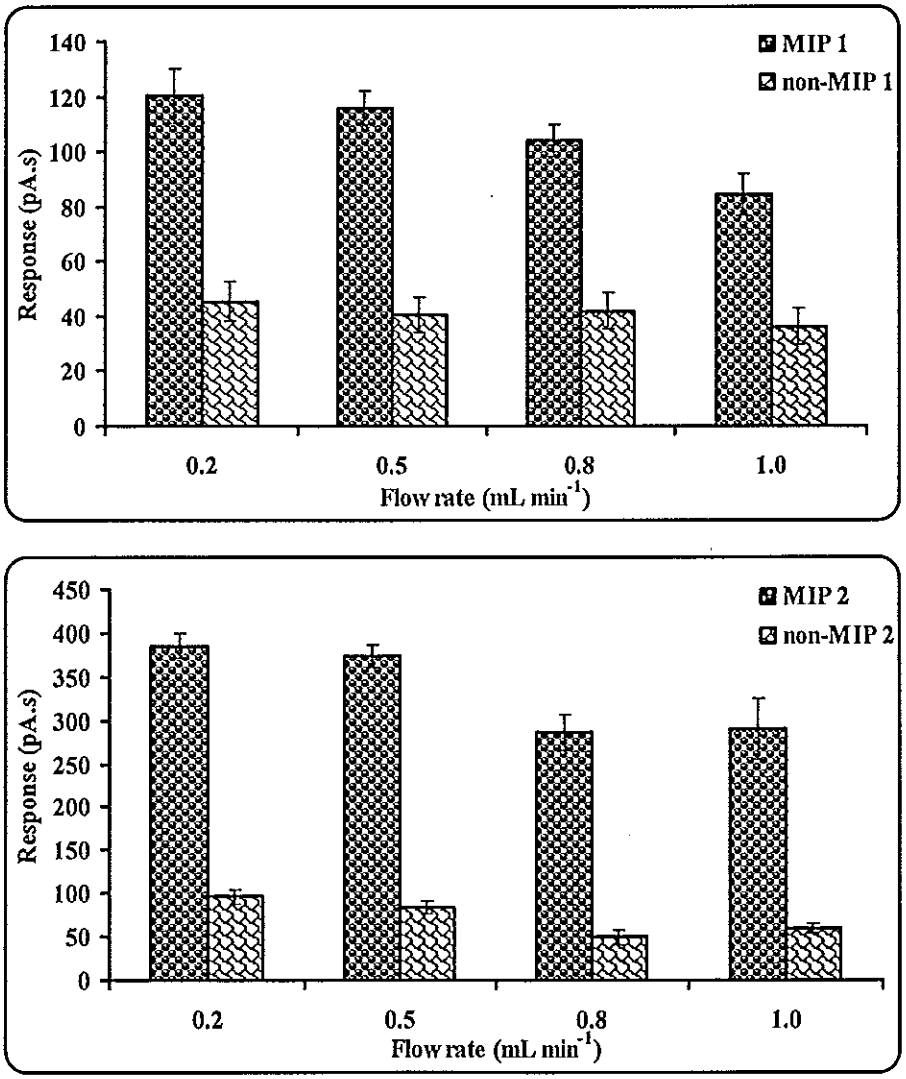


Figure 3.29 The response of carbofuran various loading sample flow rate (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.2 Drying time

When water samples are percolated through MIPs, the clean-up step can be a problem because the washing solvent used normally is non-polar, and that may give rise to miscibility problems. To avoid this drawback, the MIP should be dried (*e.g.*, by drawing air through the polymer). If small amounts of water remained on the cartridge after applying the sample, the binding ability of the MIP may be influenced in the subsequent selective clean-up step that led the decreasing in binding properties and selectivity became from the swelling of MIP (Caro *et al.*, 2006). Therefore the optimization of MISPE cartridge drying time after sample loading is also an important aspect (section 2.8.1.2). Figure 3.30 shows the responses at different drying times. The results found that drying time has no effect on MISPE 1. However the drying time of 10 minutes gave the highest response for MISPE 2. The difference can refer to the swelling of MIP 2 which is more than MIP 1. Therefore, MISPE 1 cartridge can be washed immediately after sample loading but MISPE 2 cartridge needed time to dry for 10 minute after sample loading.

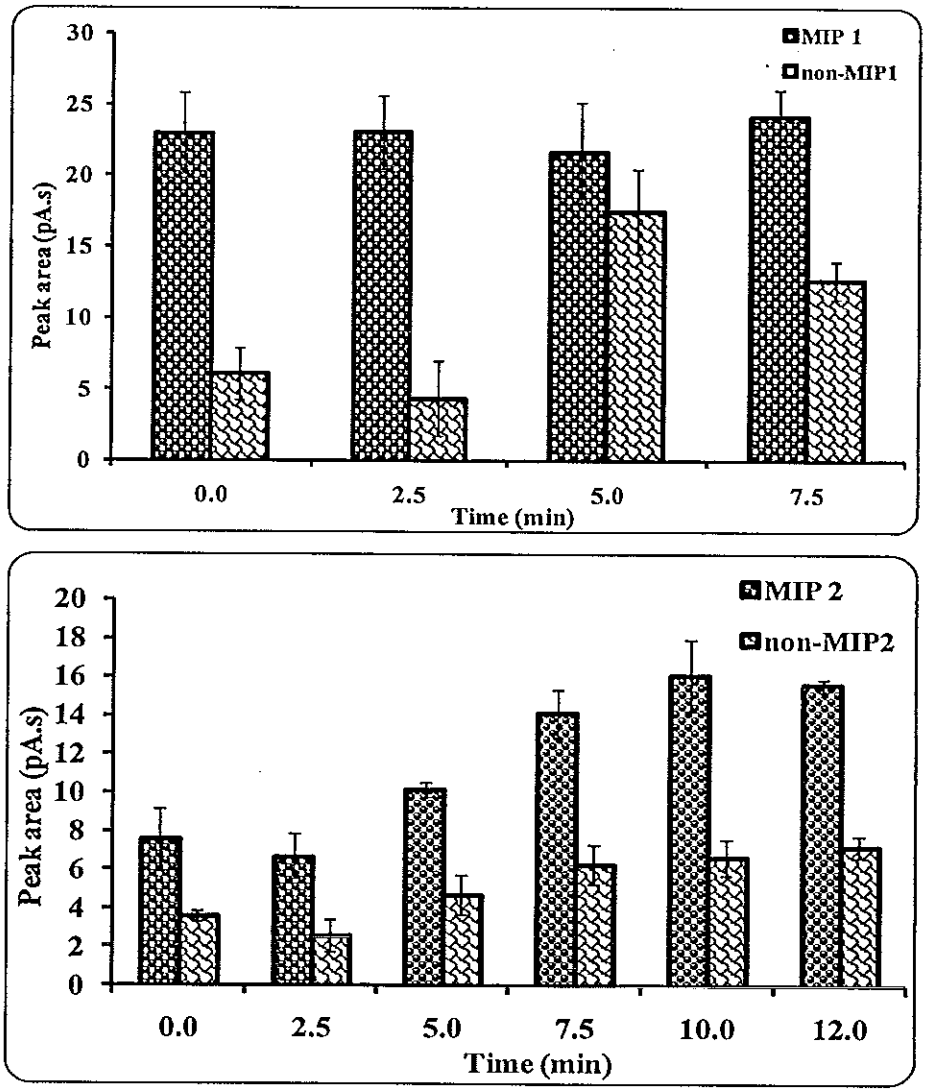


Figure 3.30 The response of carbofuran various drying time (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.3 Type of eluting solvent

Although a clean-up step seems to be essential to achieve a selective extraction, it can be avoided if the elution step is selective enough. In this case, although the analyte and the other components in the sample matrix are retained on the MIP by non-selective interactions during the loading step, a selective elution of the target analyte can be performed using an appropriate solvent. To attain high enrichment factors, it is necessary to use small volumes of solvent, but the

interactions between the MIP and the analyte are sometimes so strong that the volume of eluting solvent has to be increased. To avoid this, mixtures of organic solvents or an organic solvent with water or with a modifier, such as acetic acid or pyridine, can be used. On the one hand, water disrupts the specific non-covalent interactions and on the other, the modifier competes with the binding sites to interact with the target molecule. Both represent effective ways of rapidly eluting the analyte. The results from the affect of various type of eluting solvent (2.8.1.3) are shown in Figure 3.31 where methanol provides the highest response for both MISPE 1 and MISPE 2. This is due to hydrogen bonding interactions lost its molecular recognition ability in an aqueous media or organic solvents with high polarity (Xu *et al.*, 2007). Therefore methanol was selected as the eluting solvent.

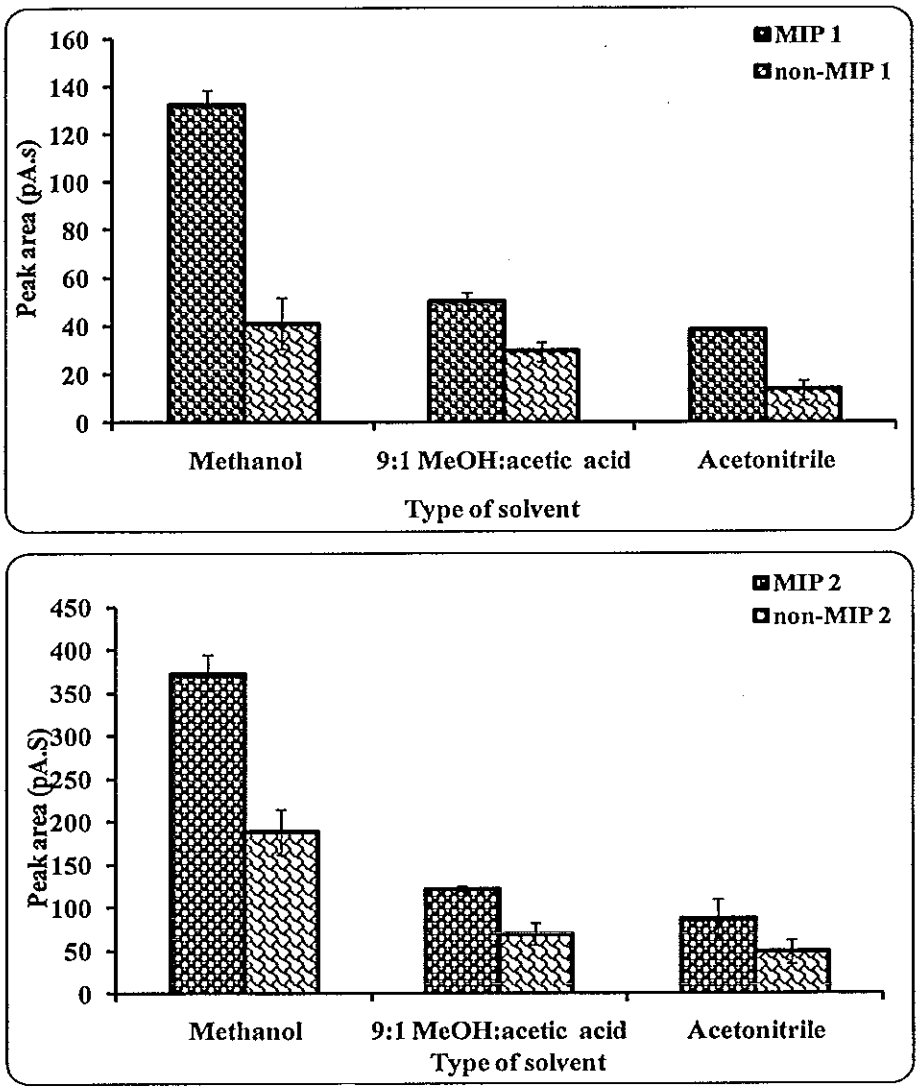


Figure 3.31 The response of carbofuran various type of eluting solvent (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.4 Volume of eluting solvent

The minimum volume of eluting solvent requires to complete the elution of analyte was investigated (2.8.1.4). The use of small volume can reduce the experiment time and solvent consumption. After loading sample on MISPE cartridge, the adsorbed carbofuran was eluted by methanol. The results (Figure 3.32) found that there is no change after 2.5 mL, therefore, 2.5 mL of methanol was chosen as the optimum volume for both MIPs.

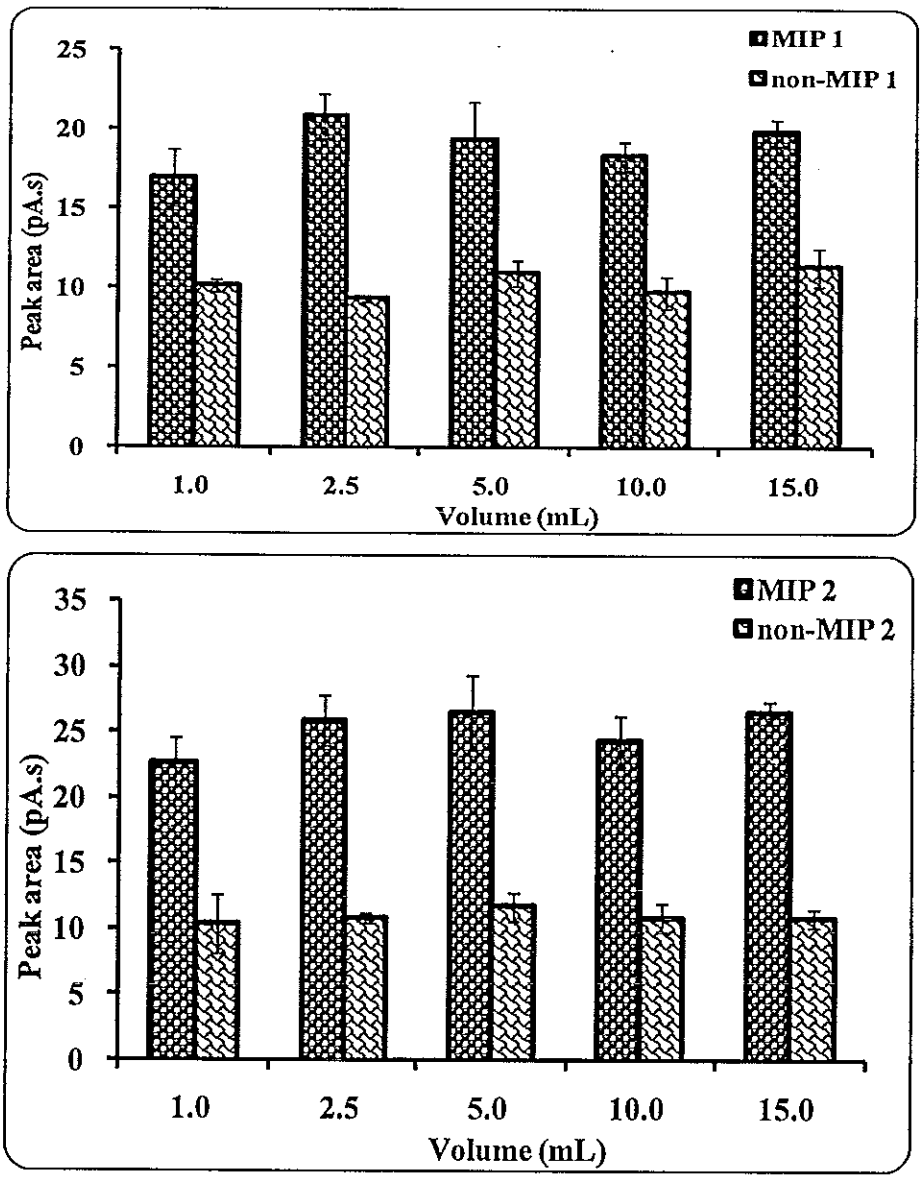


Figure 3.32 The response of carbofuran various volume of methanol (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.5 Flow rate of eluting solvent

When the flow rate is either too high or low, it will give low response and lead to poor recovery. Most developed methods aim to determine the fastest flow rate that still yields good recovery. This typically ensure enough residence time for the eluting solvent to sufficiently interact with the sorbent. By properly controlling and optimizing flow rate parameters for an MISPE procedure, one can increase the

ruggedness of an MISPE method eliminating one potential cause often attributed to low and variable recoveries (Sigma-Aldrich., 2005).

In this study the eluting solvent flow rate was optimized as described in 2.8.1.5 (0.2 to 1.0 mL min^{-1}) and the results are shown in Figure 3.33. The response decreased when increase flow rate for MISPE 1. Lower flow rate than 0.2 mL min^{-1} was not studied due to limit of SPE control unit. The flow rate of 0.2 mL min^{-1} was selected for this step for MISPE 1. For MISPE 2, the response at 0.2 and 0.5 ml min^{-1} are not different therefore, to obtain fast sample preparation time 0.5 mL min^{-1} was chosen.

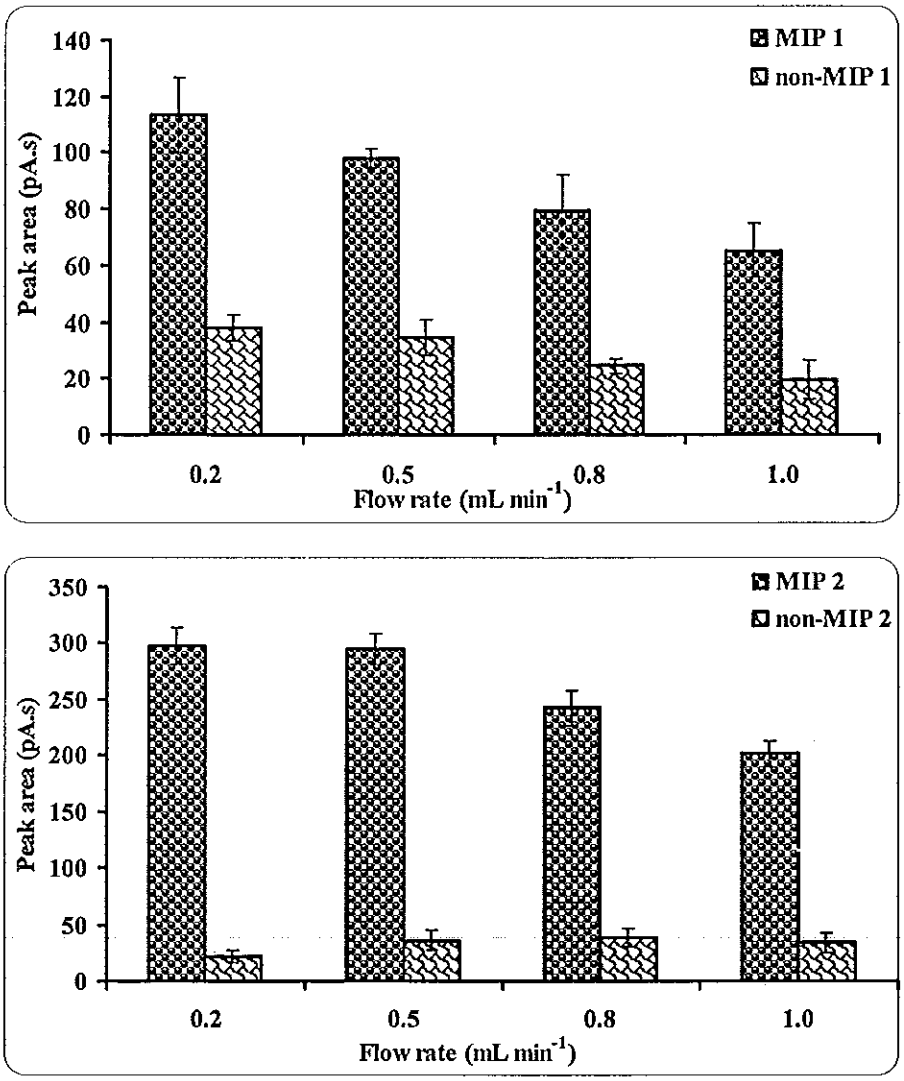


Figure 3.33 Response of carbofuran at various eluting flow rate (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.6 Type of washing solvent

When applying real water samples to the MISPE, other hydrophobic molecules from the matrix would also bind with the same mechanism. To remove these, a selective washing solvent must be used which disrupts nonselective hydrophobic bonds, but allows selective binding of carbofuran to the polymer. Consequently, to achieve a selective extraction, a clean-up step with an organic solvent is introduced prior to the elution step. This clean-up is more critical in MISPE procedures than in conventional SPE (Caro *et al.*, 2006). For this purpose, low-polarity organic solvents have been used. Figure 3.34 shows the results from this study 2.8.1.6 *i.e.* the effect of various organic solvent on response of carbofuran and carbaryl that adsorbed on MISPE 1 and 2, respectively. The results showed that, hexane gave the lowest binding capacity of carbaryl. Since hexane is the least polar solvent; the elution is the reverse order of their hydrophobicity *i.e.* the most hydrophobic carbaryl elutes first (Pap *et al.*, 2002).

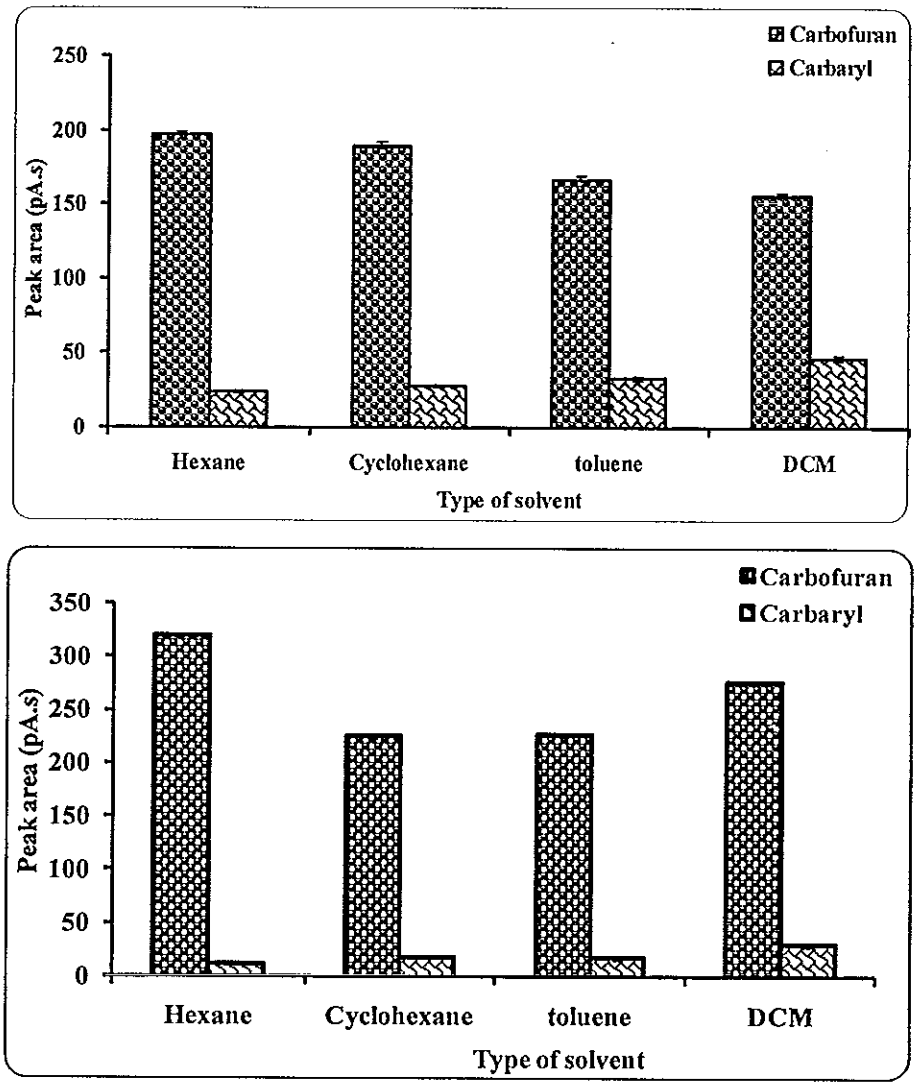


Figure 3.34 The response of carbofuran and carbaryl various type of washing solvent (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.7 Volume of washing solvent

Appropriate volume of hexane used in the washing step was determined (2.8.1.7). The results are shown in Figure 3.35. The volume of 5.0 mL was enough and effectively to remove non-specific binding for both MISPEs. This was selected as washing volume to remove interference from MISPE cartridge.

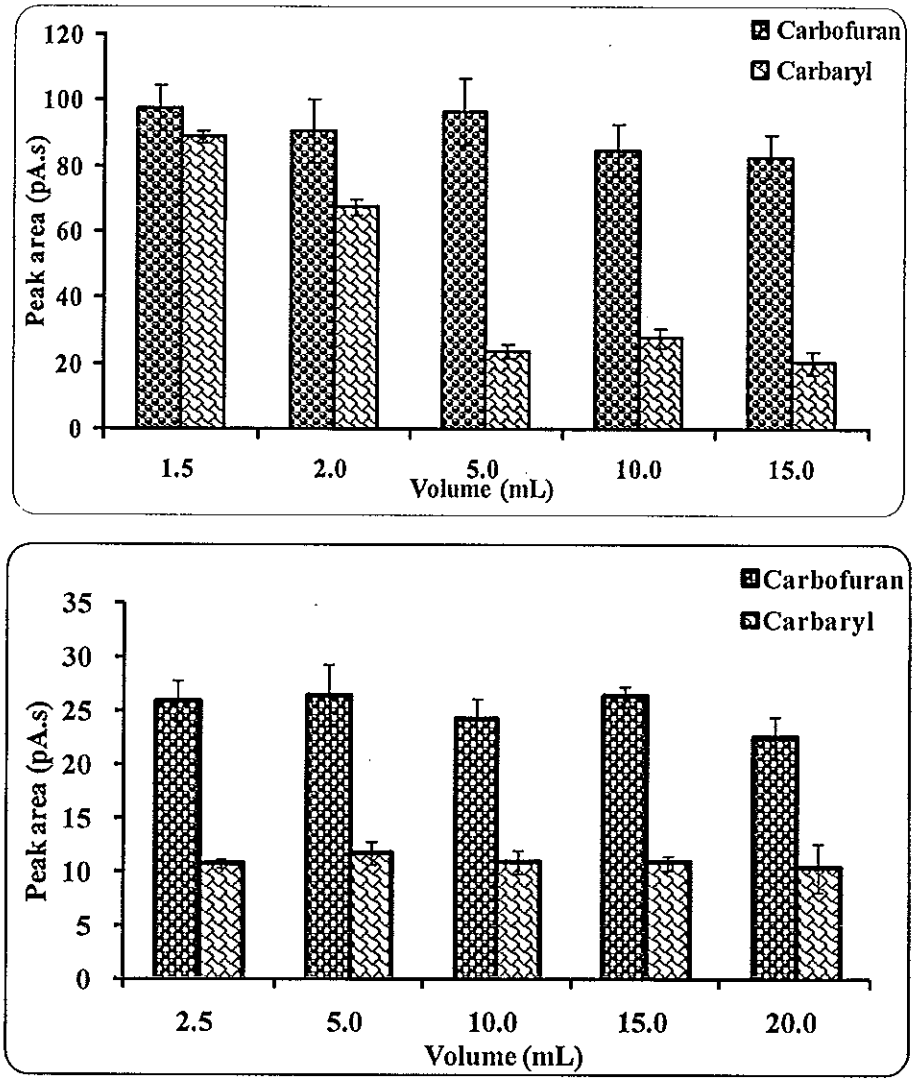


Figure 3.35 The response of carbofuran and carbaryl various volume of hexane (n=5) (a) MISPE 1 (b) MISPE 2.

The optimum conditions of MISPEs for the analysis of carbofuran in real water sample with GC-FID are summarized in Table 3.21.

Table 3.21 The optimum conditions of MISPEs for the analysis of carbofuran in real water sample with GC-FID.

Parameters	Studied range	Optimum condition	
		MISPE 1	MISPE 2
Type of eluting solvent	methanol, 9:1 mathanol:acetic acid, acetonitrile	methanol	methanol
Eluting flow rate (mL min^{-1})	0.2 - 1.0	0.2	0.5
Volume of eluting solvent (mL)	2.5 - 15.0	2.5	2.5
Type of washing solvent	hexane, cyclohexane, toluene and dichloromethane	hexane	hexane
Volume of washing solvent (mL)	1.5-15.0	5.0	5.0
Sample flow rate (mL min^{-1})	0.2 - 1.0	0.5	0.5
Drying time (min)	0 - 12.5	0	10

3.5.1.8 Breakthrough volume

It is well known that the concentration of analytes in environmental samples is normally very low so a large volume of sample has to be percolated through the MISPE in order to preconcentrate. The ability to improve sensitivity by extracting larger sample volume has been mentioned (Andersson, 2000). The greater the breakthrough volume and the capacity the better the limit of detection (Delaunay *et al.*, 2000). The breakthrough volume for carbofuran on each MIP sorbent was determined by passing increasing volume (25–200 mL) of water sample spiked with 100, 200 and 300 μg L^{-1} standard carbofuran solution (2.8.1.8). The results for MISPE 1 are shown in Figure 3.36 (a). In case of MISPE 2 only high concentration was tested (Figure 3.36 (b)) because from MISPE 1 showed it was shown that low concentration

would not reach the breakthrough. Measurement of the peak areas of the eluant from the sorbent revealed that sample volumes up to 175 mL and 125 mL for concentration at 300 μg L^{-1} could be used under the proposed experimental conditions for MISPE 1 and 2, respectively. For high sample volumes the extraction performance was irreproducible possibly due to the uncertainty encountered during the handling of large sample volumes and as the procedure continues, clogging of the cartridge is observed. However, in this work only 100 mL was used to pass to the MISPEs cartridge since this volume is enough for the preconcentration to achieve LOD low enough to meet the regulation of EPA for drinking water.

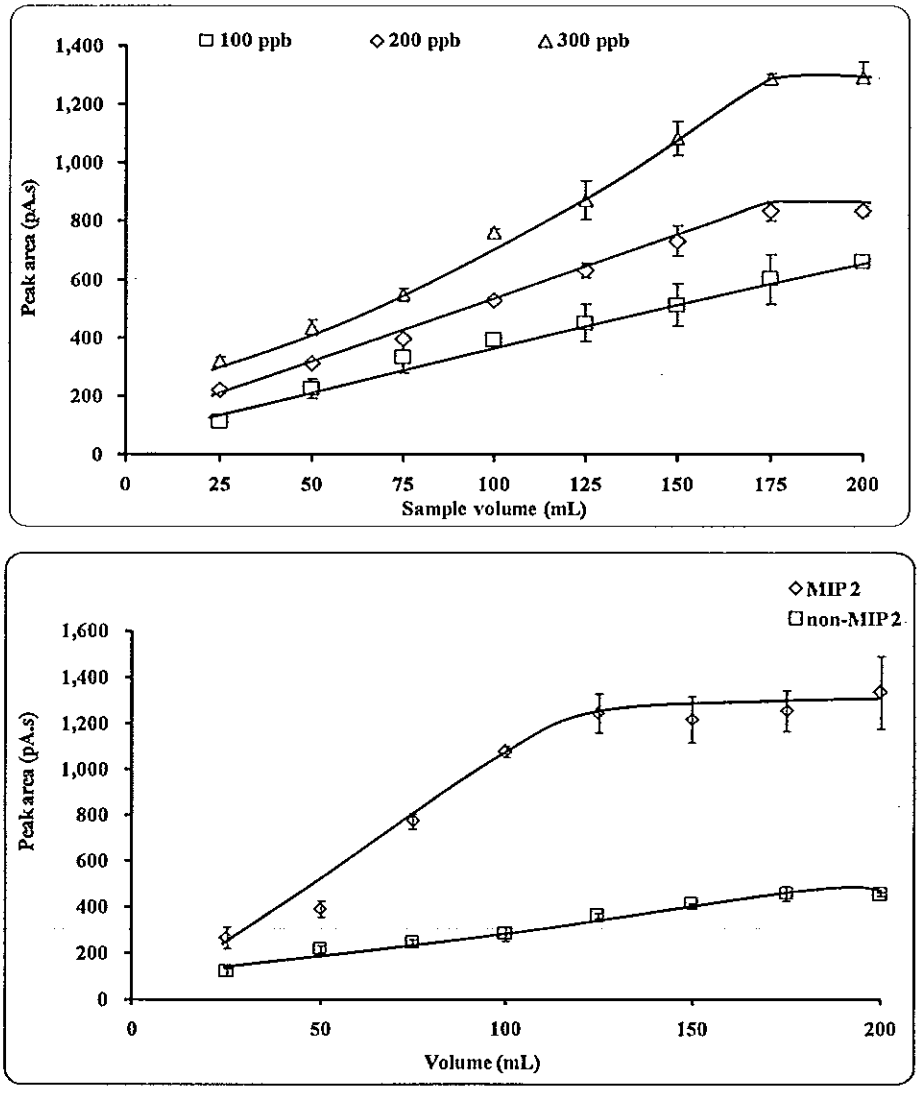


Figure 3.36 Breakthrough volumes at various concentrations of carbofuran (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.9 Selectivity

In this study we investigated a MIP imprinted for carbofuran for its separation capability against other carbamate pesticides that have similar structure with carbofuran: carbaryl and carbosulfan (Figure 3.37).

For carbosulfan, the chromatogram can not be separated from carbofuran. Therefore to test the selectivity of synthesized MIPs, only carbosulfan spiked DI water 100 mL was passed through the cartridge at various concentrations. For carbaryl, it was mixed with carbofuran at the same concentration and spiked into 100 mL DI water. The sample was passed through the cartridge and extracted under SPE procedure at the optimum conditions. The results (Figure 3.38) found that when the concentration of carbaryl and carbosulfan increased, the response did not increase while for carbofuran the response increased with concentration. Therefore it can be concluded that MIP for carbofuran is selective.

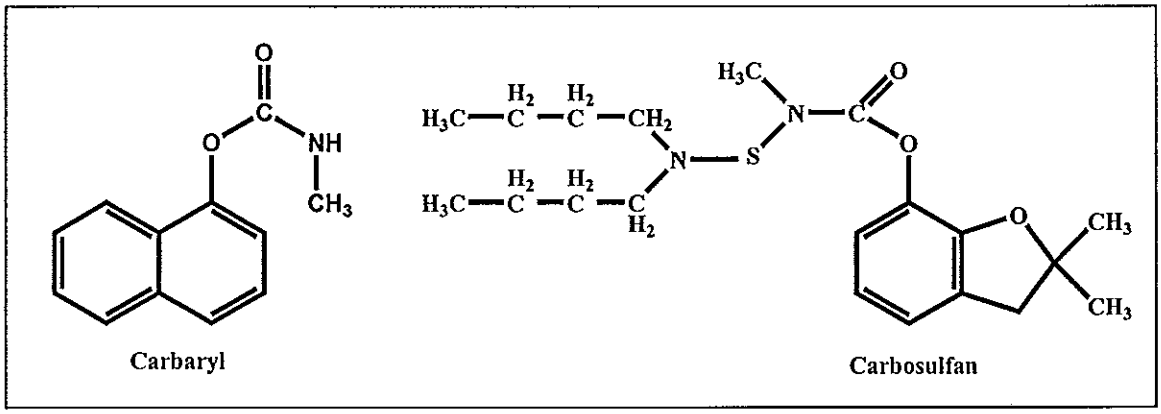


Figure 3.37 Structure of carbaryl and carbosulfan.

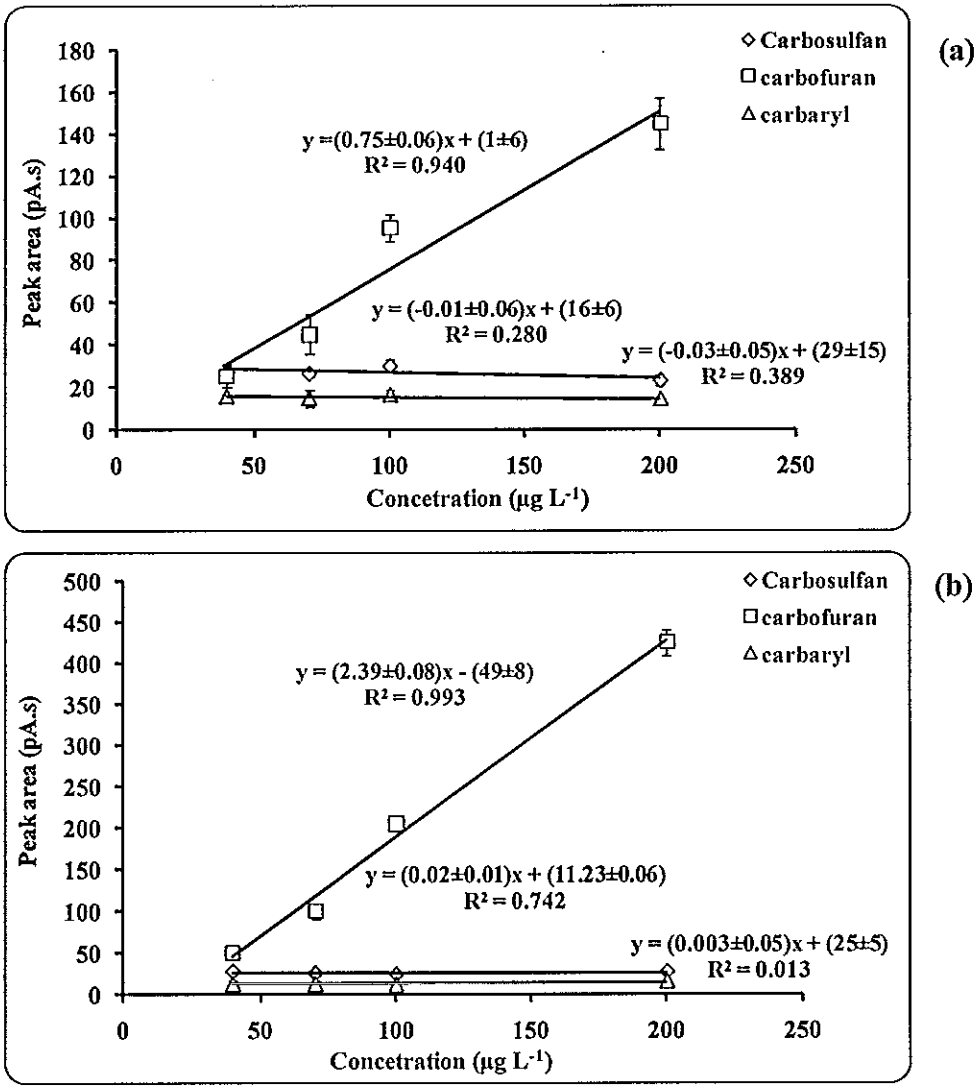


Figure 3.38 The response of carbamate pesticides at various concentrations (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.1.10 Reusability

The reusability of each MISPE cartridge was tested by monitoring its extraction efficiency for carbofuran with repeating SPE procedure many times (2.8.1.10). Figure 3.38 shows that between 1-5 times the responses reduced only slightly after each use (3 % of MISPE 1 and 2% of MISPE 2). For the sixth time the response showed significant reduction (16% of both MISPEs). The extraction

efficiency of MISPE cartridge reduced with repeated use because of the swelling of the polymer. Therefore one cartridge can be used up to 5 times.

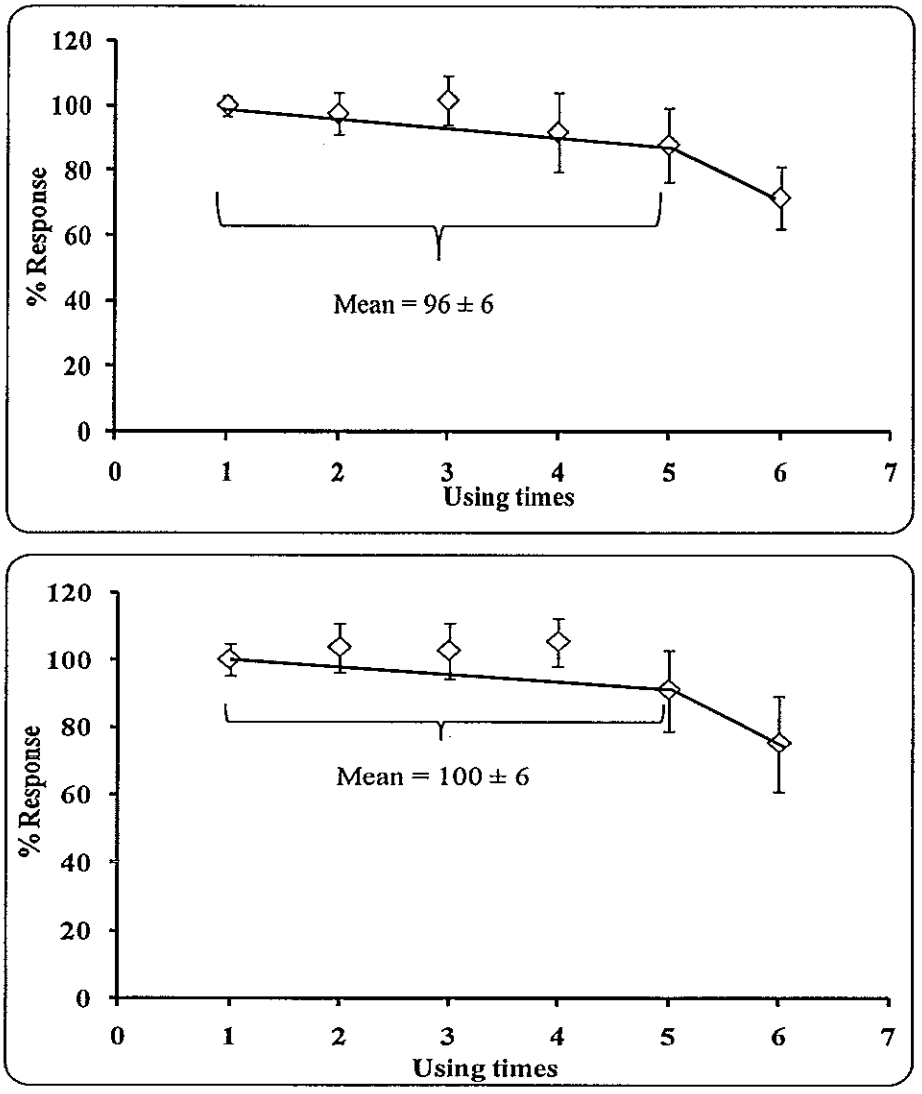


Figure 3.39 The reusability (n=5) (a) MISPE 1 (b) MISPE (2).

3.5.1.11 Repeatability

Five MISPE cartridges from the same synthesis were used to investigate the repeatability as in 2.8.1.11. The results Figure 3.40 shows very good repeatability with low % RSD. This indicated that a monolithic network polymer particles was formed by the bulk polymerization, but the polymeric block needs to be

crushed and to obtained particles of irregular shape. However, for MISPE applications, the irregularity in particle size is not a particular problem (Caro *et al.*, 2006).

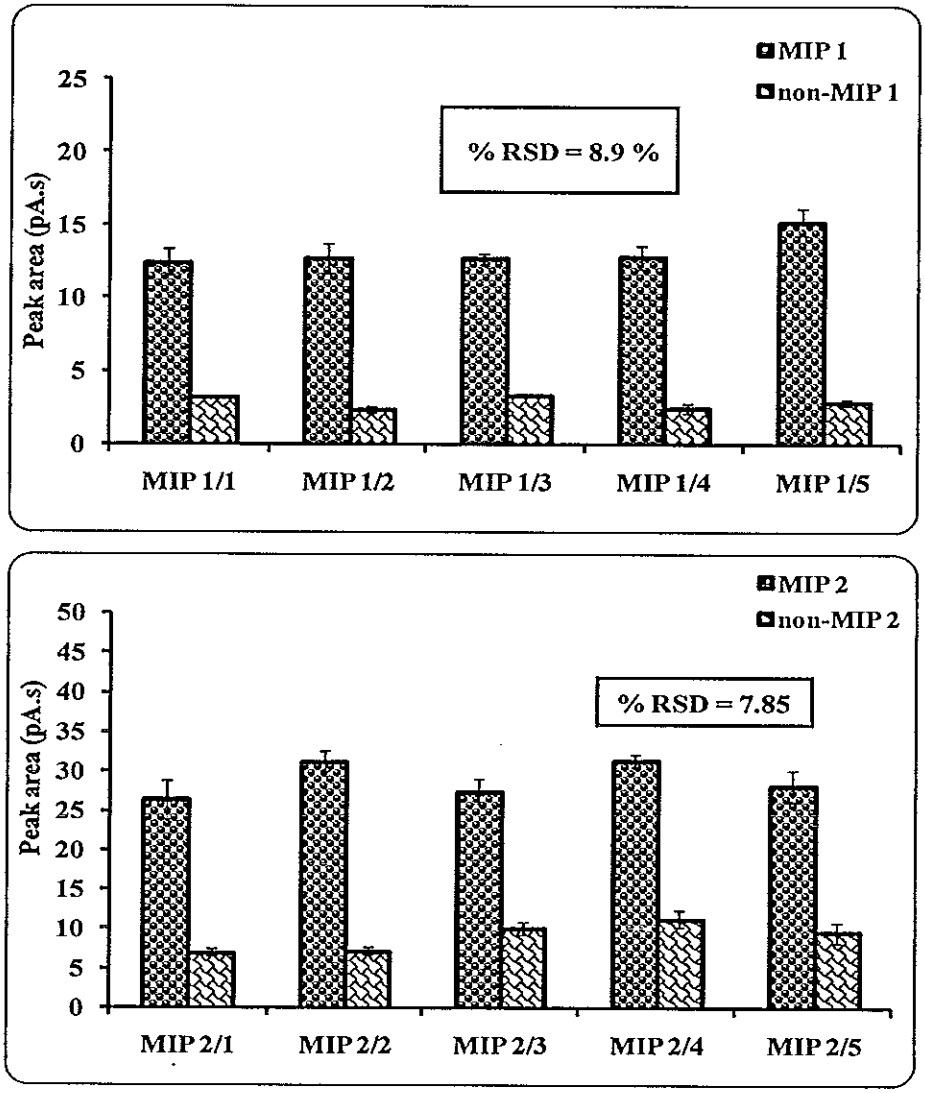


Figure 3.40 The repeatability (n=5) (a) MISPE 1 (b) MISPE 2.

3.5.2 Molecular imprinted solid phase dispersion (MISPD)

Solid phase dispersion was also studied since it was physically and functionally different from classical SPE in several ways: 1) it accomplishes complete sample disruption and dispersal onto particles, providing an enhanced surface area for subsequent extraction of the sample. In SPE sample disruption must be conducted as a

separate step in preparing samples for SPE and many of the sample components must be discarded in the process of making the sample suitable for addition to an SPE column. 2) In SPE the sample is usually absorbed onto the top of the column packing material, not throughout the column as in SPD. 3) The physical and chemical interactions of the components of the system are greater in SPD and different, in many respects, from those seen in classical SPE or other forms of liquid chromatography (Barker, 2007). Several factors were investigated for their effect in conducting MISPD extractions include shaker strength and time, washing and eluting solvent volume.

3.5.2.1 Shaker strength and time

To enhance contact between template and binding site of MIP, the shaker machine was used. However, shaker in excess of the optimal rate may also cause lower extraction efficiency because it reduces the opportunity of template to bind with the binding site. The results from this study (2.8.2.1) Figure 3.41 found that when increased the shaker strength; the shaking time was shorter. The shortest time for MISPD 1 was at shaker strength 270 oscillation min^{-1} and 308 oscillation min^{-1} used for MISPD 2. Consequently, the use of MISPD 1 as sorbent was extracted for 10 minutes at shaker strength 270 oscillation min^{-1} and MISPD 2 was extracted for 10 minutes at shaker strength 308 oscillation min^{-1}.

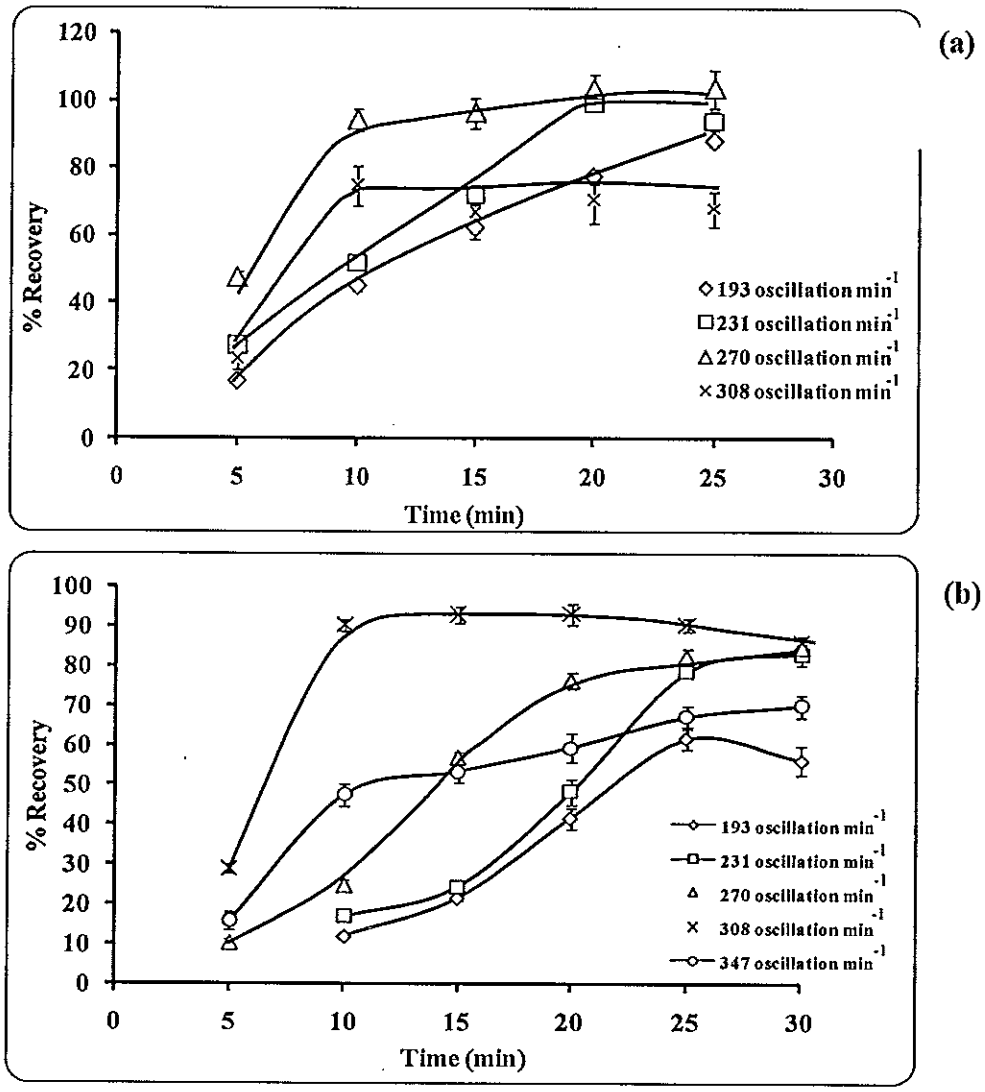


Figure 3.41 The effect of shaker strength and extraction time (n=5) (a) MISPD 1 (b) MISPD 2.

3.5.2.2 Washing solvent volume

Hexane was chosen as the washing solvent, the same as MISPE technique. The appropriate volume of washing solvent was studied (2.8.2.2). The results Figure 3.42 show that 1.0 mL of hexane can effectively remove the non-specific binding for MIP 1 and MIP 2. Therefore, this volume was selected as washing volume.

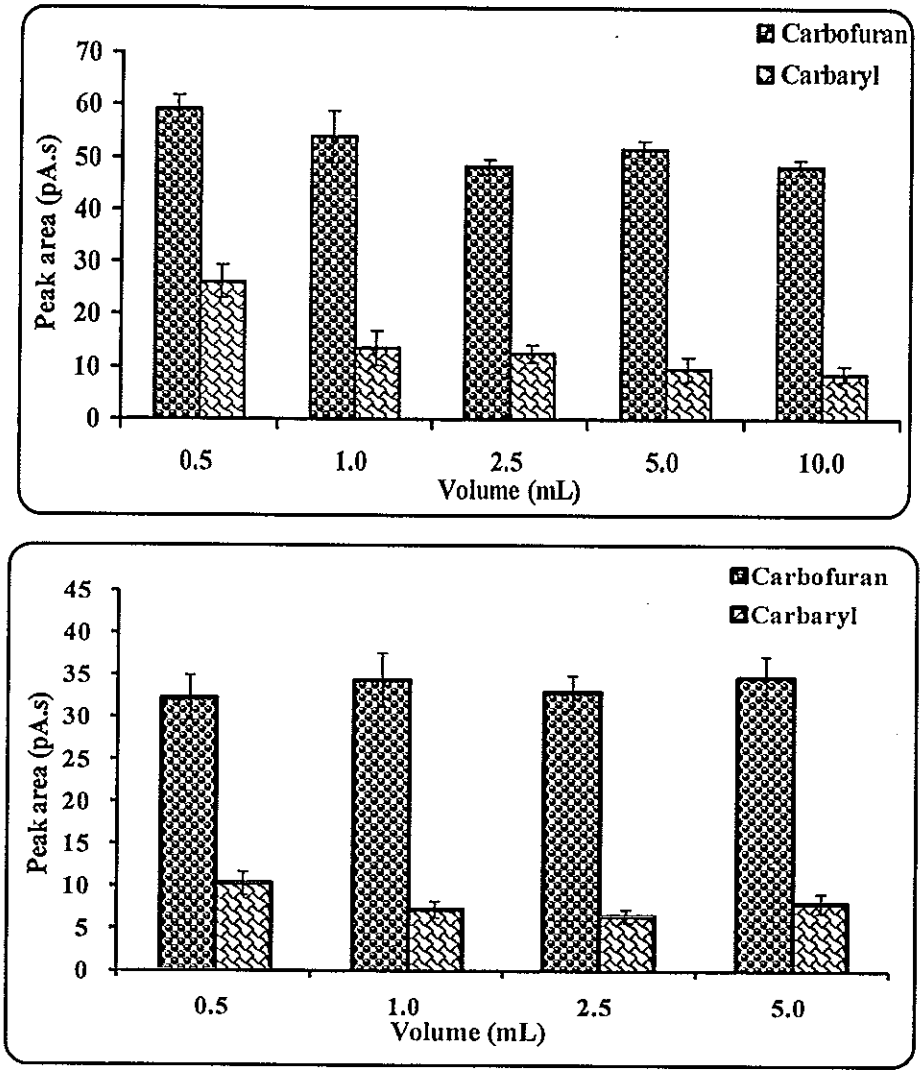


Figure 3.42 Effect of hexane volume on the response carbofuran and carbaryl (n=5) (a) MISPD 1 (b) MISPD 2.

3.5.2.3 Eluting solvent volume

The volume of solvent used in the eluting process was also an important parameter because it would affect the extraction efficiency. If the volume is too small it may not be sufficient to elute all the analyte on MIP sorbent. Methanol was chosen as the eluting solvent, the same as MISPE technique. The volume of methanol was studied (2.8.2.3) and the results in Figure 3.43 found that 5.0 mL provided the high response and consumed small amount of methanol.

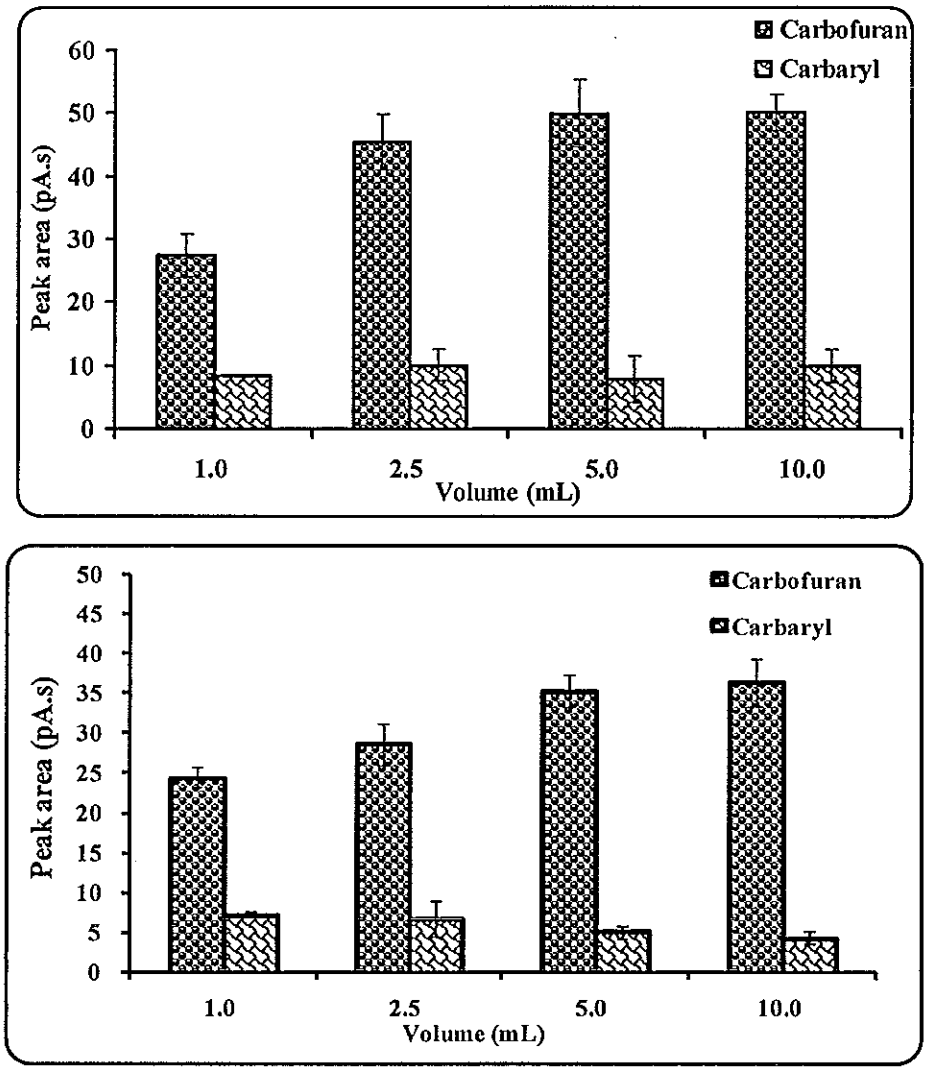


Figure 3.43 Effect of hexane volume on the response carbofuran and carbaryl (n=5) (a) MISPD 1 (b) MISPD 2.

The optimum conditions of MISPD for the analysis of carbofuran in real water sample with GC-FID are summarized in Table 3.22.

Table 3.22 The optimum conditions of MISPD for analyze of carbofuran in real water sample with GC-FID.

Parameters	Optimum condition	
	MISPD 1	MISPD 2
Type of eluting solvent	methanol	methanol
Eluting flow rate (mL min^{-1})	0.2	0.5
Volume of eluting solvent (mL)	5.0	5.0
Type of washing solvent	hexane	hexane
Volume of washing solvent (mL)	1.0	1.0
Sample flow rate (mL min^{-1})	0.5	0.5

3.5.2.4 Repeatability

The repeatability of the over-all procedure has been assessed by extraction 10 times (2.8.2.4). Each batch was analyzed with GC-FID (5 injections) and the RSD values were lower than 10% for all cases (Table 3.23 and Figure 3.44).

Table 3.23 The % recovery of 40 μg L^{-1} carbofuran spiked water from 10 extraction tubes.

Extraction vial	% Response			
	MISPD 1	**% RSD**	**MISPD 2**	**% RSD**
1	99 ± 8	8.1	91 ± 3	3.0
2	105 ± 4	4.1	84 ± 6	7.2
3	102 ± 6	6.6	97 ± 10	10.3
4	86 ± 5	5.8	100 ± 4	3.8
5	99 ± 2	1.7	97 ± 7	7.0
6	101 ± 8	8.8	90 ± 5	5.5
7	89 ± 10	12.6	88 ± 4	4.9
8	90 ± 9	10.1	83 ± 5	5.7
9	80 ± 3	4.3	76 ± 8	10.0
10	102 ± 10	10.1	92 ± 6	6.0
Average	**95 ± 8**	**7 ± 3**	**90 ± 7**	**6 ± 2**

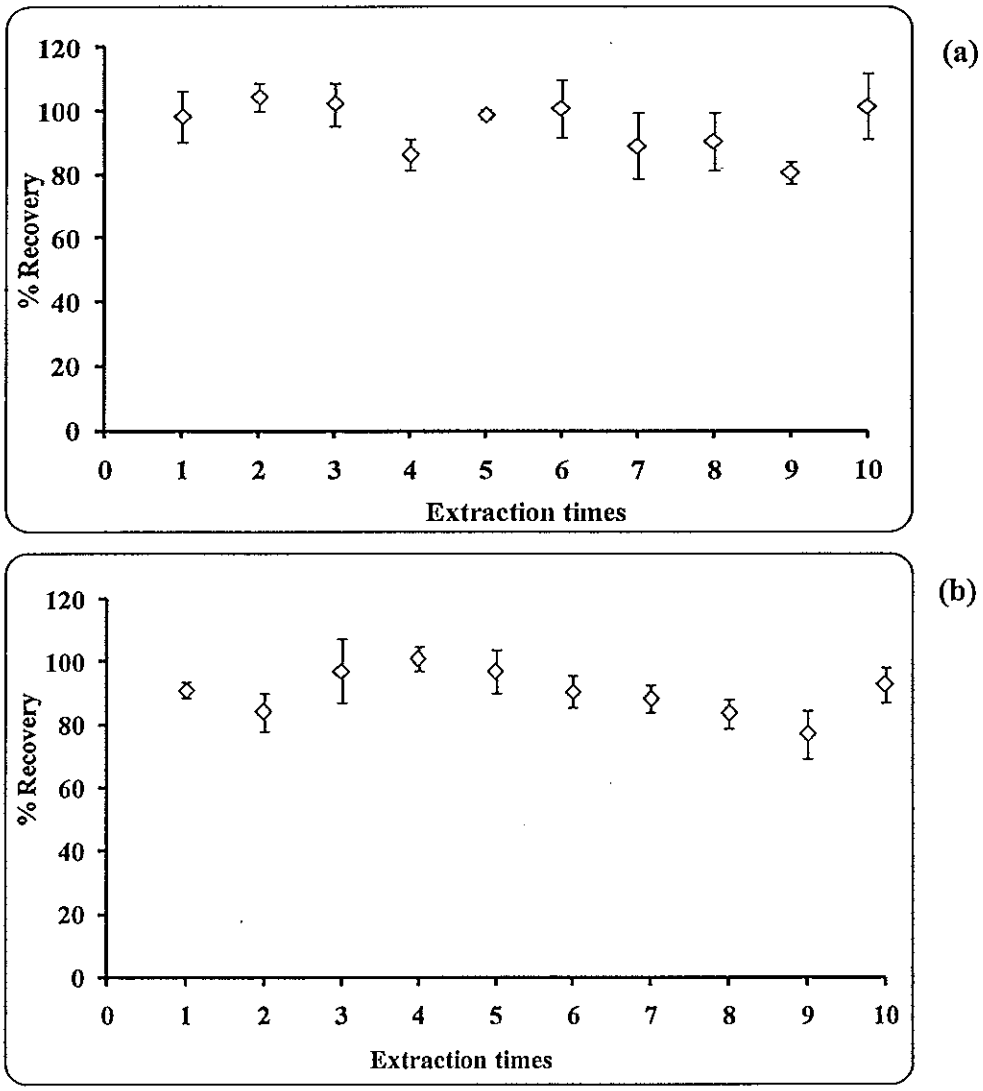


Figure 3.44 The repeatability (a) MISPD 1 (b) MISPD 2.

3.6 Method validation

3.6.1 Linear dynamic range and calibration curve

The linear dynamic range of MISPE and MISPD technique were investigated by serial dilution of stock standard solution as describe in experiment 2.9. Linearity is achieved when the coefficient of determination is equal or greater than 0.99 (FDA, 2000). Figure 3.45 shows the response of carbofuran obtained from MISPE method. Figure 3.46 shows the response of carbofuran obtained from MISPD method.

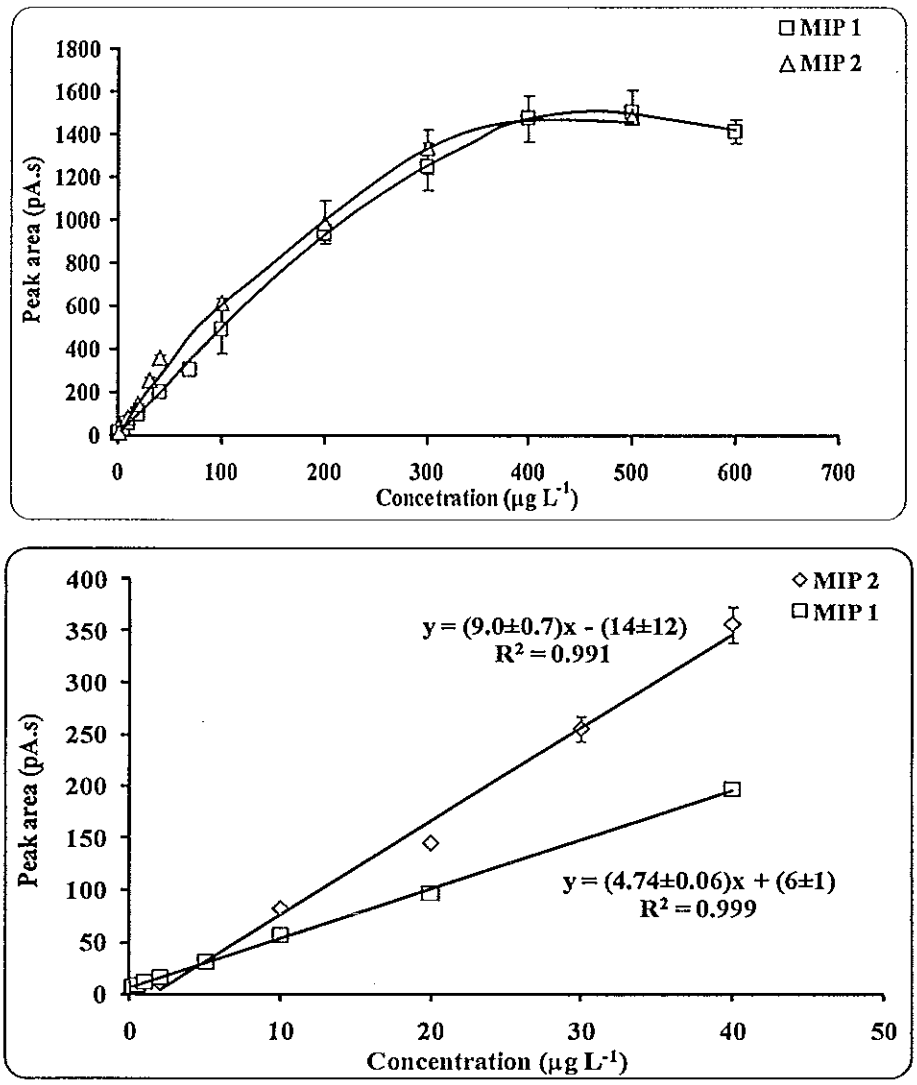


Figure 3.45 (a) Studied concentration range (b) linear dynamic rang of carbofuran in MISPE method (n=5).

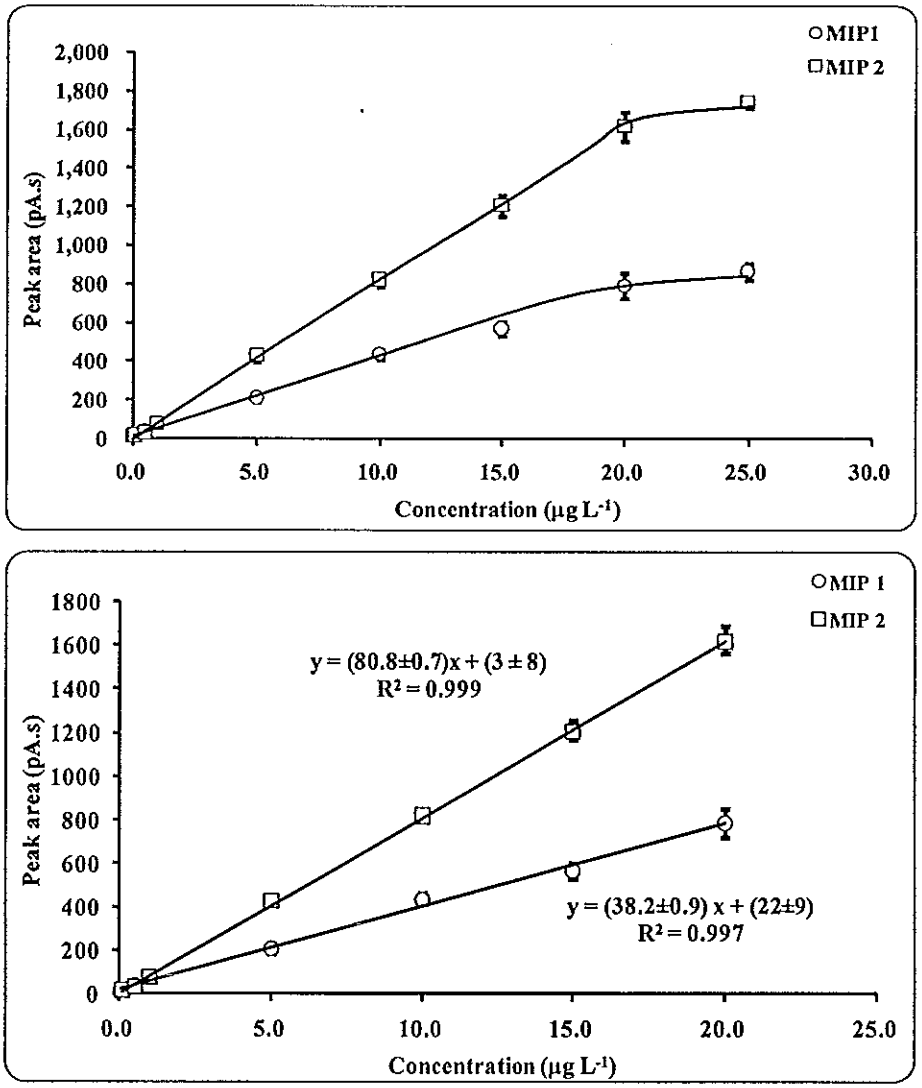


Figure 3.46 (a) Studied concentration range (b) linear dynamic range of carbofuran in MISPD method (n=5).

When comparing between MISPE and MISPD, the MISPE provided wider linear dynamic range than the MISPD but MISPD has higher sensitivity (Table 3.24).

Table 3.24 Comparison between linear dynamic range of MISPE and MISPD.

Technique	Linear dynamic range (μg L^{-1})	
	MIP 1	**MIP 2**
MISPE	0.2 - 200.0	2.0 - 40.0
MISPD	0.1 – 20.0	0.1 – 20.0

3.6.2 Limit of detection and limit of quantitative (LOD and LOQ)

The limit of detection and limit of quantitative of MISPE and MISPD technique were investigated by using the IUPAC method (Long and Wineforder, 1983). From Equation 1.9 and the data in Table 3.25, LOD and LOQ were calculated and are shown in Table 3.26. When comparing LOD, LOQ and sensitivity between MISPE and MISPD, MISPD provided better performances than MISPE. This behavior is attributed to two reasons; the sample could contact on the sorbent though the column without disrupting their structure. According to the latter, the adsorbed analyte should create a three-dimensional layer in the solid-liquid interface which significantly enhances contact with the components of the bulk solvent (Parisis *et al.*, 2005).

Table 3.25 The maximum response of 20 blank injections in MISPE and MISPD.

Injection times	Maximum response (pA.s)	
	MISPE	**MISPD**
1	2.70	0.21
2	3.90	0.22
3	1.80	0.31
4	2.50	0.21
5	2.50	0.16
6	1.20	0.21
7	2.40	0.16
8	2.70	0.21
9	2.00	0.16
10	4.50	0.24
11	2.90	0.26
12	3.90	0.21
13	3.80	0.39
14	2.60	0.35
15	2.10	0.29
16	5.20	0.16
17	3.50	0.35
18	1.30	0.16
19	1.70	0.21
20	2.00	2.00
Average	2.76	0.32
sd	1.07	0.40

Table 3.26 Comparison between LOD and LOQ of MISPE and MISPD technique.

Technique	LOD ($\mu g\ L^{-1}$)		LOQ ($\mu g\ L^{-1}$)	
	MIP 1	**MIP 2**	**MIP 1**	**MIP 2**
MISPE	0.677 ± 0.009	0.36 ± 0.03	2.26 ± 0.02	1.19 ± 0.09
MISPD	0.031 ± 0.001	0.015 ± 0.002	0.105 ± 0.002	0.050 ± 0.005

3.6.3 Precision

Precision is the closeness of agreement between independent test results obtained under optimum conditions. It is measuring of how close results are to one another and is usually expressed as a relative standard deviation (RSD) which describe the spread of results (Bruce *et al.*, 1998; EURACHEM., 1998). It is generally dependent on analyte concentration. The precision is calculated on the basis of five replications of real water samples. In this study, each water samples were evaluated at two spiking level of 5.0 $\mu g\ L^{-1}$ and 20.0 $\mu g\ L^{-1}$ of carbofuran followed by both sample preparation procedures (MISPE and MISPD) before analysis with GC-FID system at the optimum conditions. Five replication analyzes were performed at each concentration. The results showed good precision with relative standard deviations (RSD) lower than 19 % at spiked concentration 5.0 $\mu g\ L^{-1}$ and 20.0 $\mu g\ L^{-1}$ for both techniques (Table 3.27). These values were better than the recommendation by EPA method 531.2 that accepted RSD less than or equal to 20 % (EPA, 2001).

Table 3.27 Precision of carbofuran of various water samples at spiked concentrations of 5.0 μg L^{-1} and 20.0 μg L^{-1} for MISPE and MISPD techniques.

Sample no.	% RSD of MISPE		% RSD of MISPD	
	5.0 μg L^{-1}	20.0 μg L^{-1}	5.0 μg L^{-1}	20.0 μg L^{-1}
1	5.6	7.1	3.9	6.6
2	13.9	4.1	8.4	7.5
3	18.9	4.6	6.7	10.0
4	15.7	2.1	7.4	7.1

* 5 replications (% RSD < 19%)

3.6.4 Accuracy

Accuracy, in terms of mean recovery was assessed by replicated analytes of water samples in order to evaluate these sample preparations technique gave the true value. The recovery of carbofuran in water samples were tested by spiking known amounts of carbofuran standard solution into water sample at concentration levels 2.0, 20.0 and 40.0 μg L^{-1}, as descried in 2.9.4. The response obtained from standard solution and water sample were compared. Percentage recovery (%R) is calculated followed Equation 2.5. The results are shown in Table 3.28, acceptable recoveries (70-120 % by EPA method 351.2) were obtained, range from 80 – 101 % and 71-110 % for SPE and SPD, respectively.

Table 3.28 Recovery of carbofuran of water samples spiked at various concentrations.

Concentration ($\mu g\ L^{-1}$)	Recovery (%) MISPE		Recovery (%) MISPD	
	MIP 1	**MIP 2**	**MIP 1**	**MIP 2**
2	80 ± 4	86 ± 2	71 ± 3	80 ± 5
20	101 ± 1	88± 1	109 ± 2	110 ± 3
40	84 ± 1	98 ±1	108 ± 3	102 ± 5

3.6.5 Matrix effect

The matrix had a significant effect on the sample preparation process since real water sample consists of various components that could interfere with the interest analytes. If the analytes are not aware of these interferences, it can lead to either a suppression or enhancement of the sample signal compared to the calibration signal for the same analytes. Interference would usually affect the slope of the calibration curve, so that it will be different from the slope of the analyte (EU, 2007), therefore the slope of the calibration curve in the methods of addition may affect the linearity of the curve. This effect has the potential to indicate the possible present of hidden interference (EURACHEM., 1998). The investigation was carried out in 2.9.5. The optimum conditions were set for determining the spiked sample and standard. The results show in Figures 3.47 and 3.48 for MISPE and MISPD, respectively.

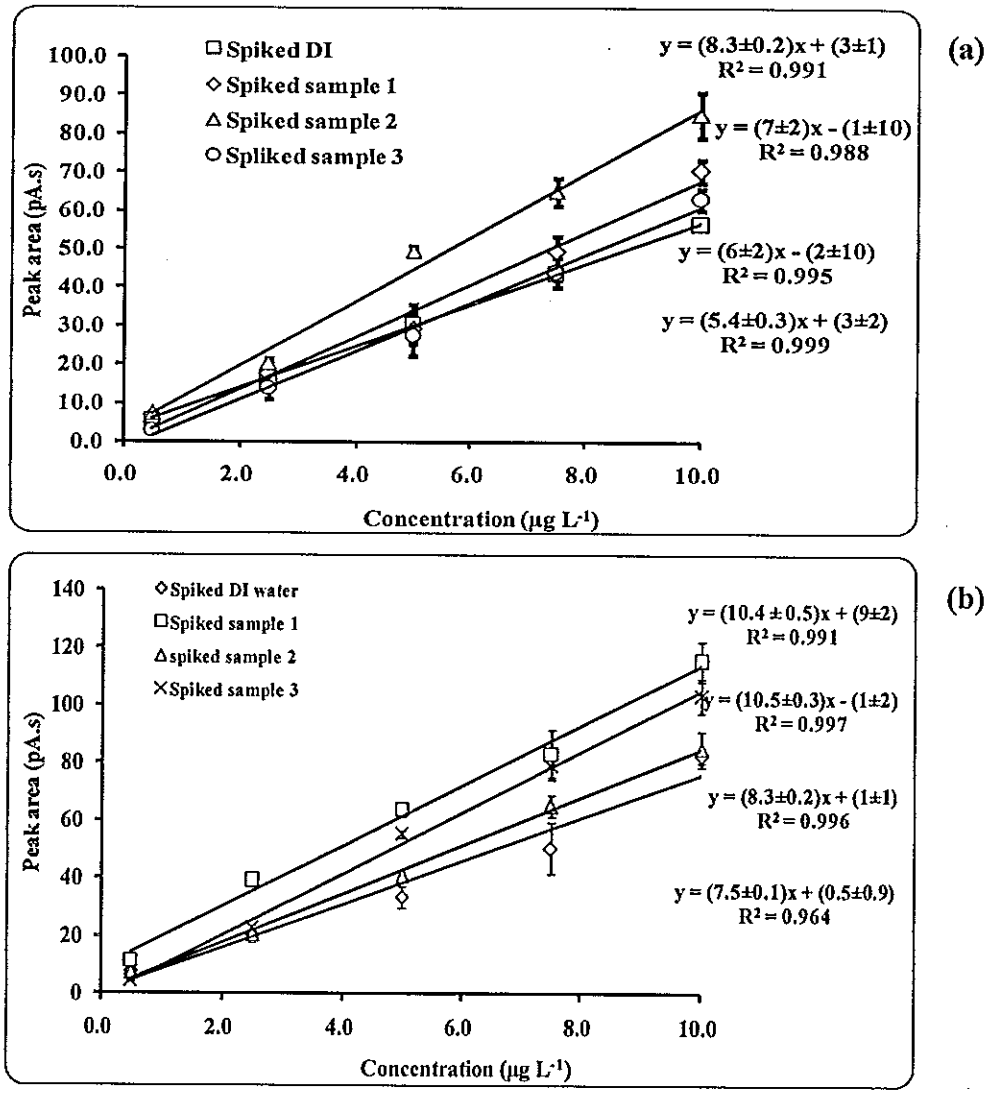


Figure 3.47 Matrix – matched calibration curve of carbofuran in water samples (n=5) (a) MISPE 1 (b) MISPE 2.

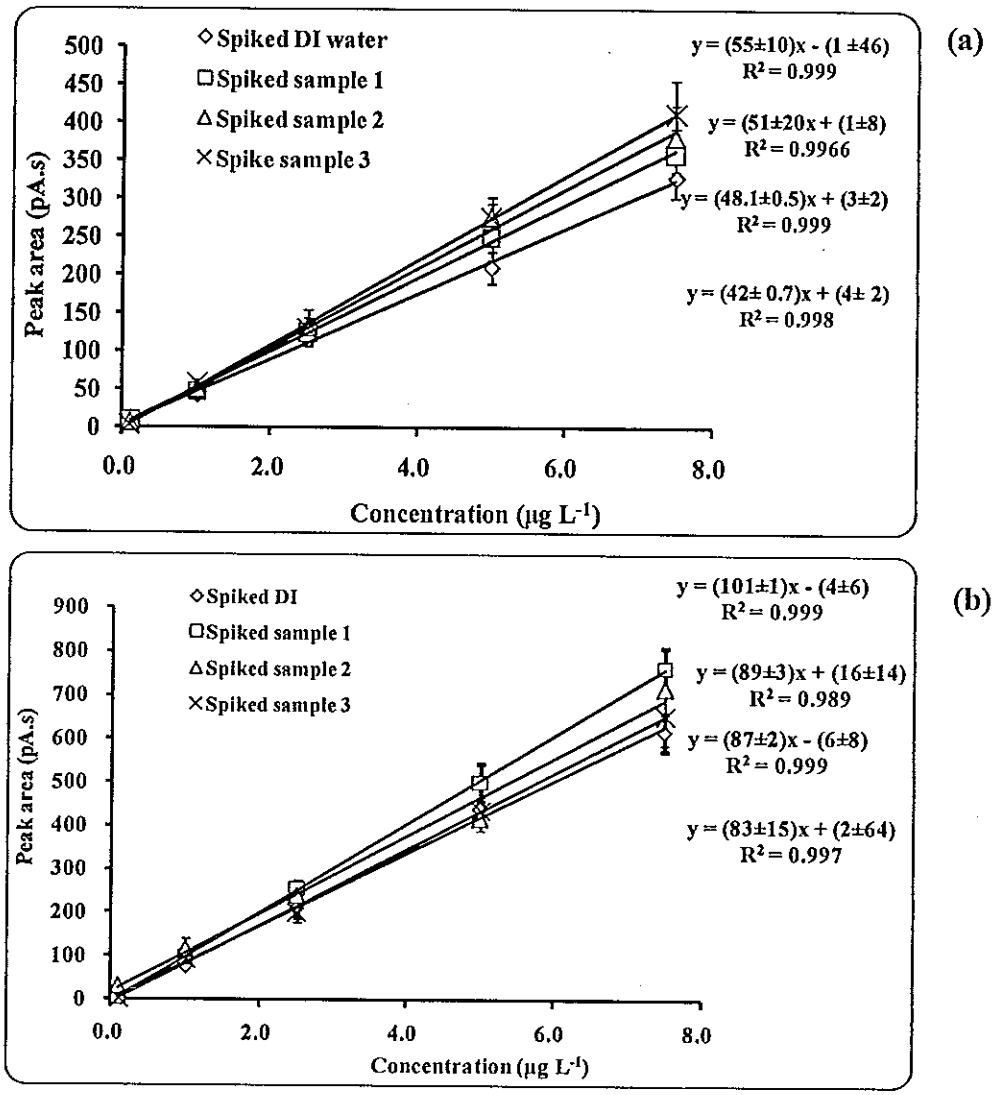


Figure 3.48 Matrix – matched calibration curve of carbofuran in water samples (n=5) (a) MISPD 1 (b) MISPD 2.

To confirm the matrix interference a statistical test known as a significance test was employed to test whether the difference between the two results is significant, or whether it can be accounted for merely by random variations. Significance tests are widely used in the evaluation of experiment results. In this experiment the use of different concentrations is a controlled factor since the concentrations are chosen by the experimenter. The curve on which the experiment is performed introduces uncontrolled variation. The slope of standard curve and matrix match calibration curve were tested using two-way ANOVA (analysis variance).

In making a significance test, the truth of a hypothesis which is known as a null hypothesis (H_0), that interaction of both slopes is not significant and alternative hypothesis (H_1), the interaction of slope significant. If *P* value is less than α (level of significance), then null hypothesis was rejected at that significant level (Miller and Miller, 2000). The *P* value was calculated by R software (R Development Core Team., 2006).

The results from significant test for the comparison of standard curve and matrix matched calibration curve are shown in Table 3.29 for MISPE 1.

Table 3.29 Statistical value for the comparison between the slopes of carbofuran spiked DI water calibration curve and matrix-match calibration curve using two-way ANOVA by R software.

Matrix	D_f	*Sun Sq*	*Mean Sq*	*F*	*P*
Sample 1	4	2657	664	3.54	0.015*
sample 2	4	36709	9177	20.79	2.57×10^{-9}***
sample 3	4	41270	2541	13.02	5.05×10^{-8}***

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Where D_f : the degree of freedom, it refers to the number of independent deviation,

$D_f = n$-1 (n is the number of concentration = 5-1=4)

Sum S_q: the sum of squares, it refers to an interim quantity used in the calculation of an estimate of the population variance

Mean S_q: the mean square, it refers to a sum of squared terms divided by the number of degrees of freedom

F: the ration of two sample variances, i.e. the ratio of the squares of the standard deviations, $\frac{s_1^2}{s_2^2}$

P: Probability

From the results of the interaction between response and concentration in standard and matrix groups, it can be concluded that the slope of regression line in each group of standard curve and matrix matched calibration curve were significantly different at various levels. Thus the interference is present. If interference is present, the matrix matched calibration curve used for an accurate determination of analyte in real water sample (EURACHEM, 1998). Similar results were obtained for MISPE 2 and MISPD 1 and 2 *i.e.* there were matrix effect for all cases.

3.7 Qualitative and quantitative analysis of carbofuran in water sample

3.7.1 Qualitative analysis

The optimum conditions of GC-FID were used to analyze carbofuran in water samples. For qualitative analysis, the most frequently used technique for tentative identification of an eluted component is based on the retention time (t_R) of the eluted component and those of known standard solution (Gudzinowicz, 1967). The average t_R of carbofuran was 3.65 ± 0.02 minutes (Figure 3.49).

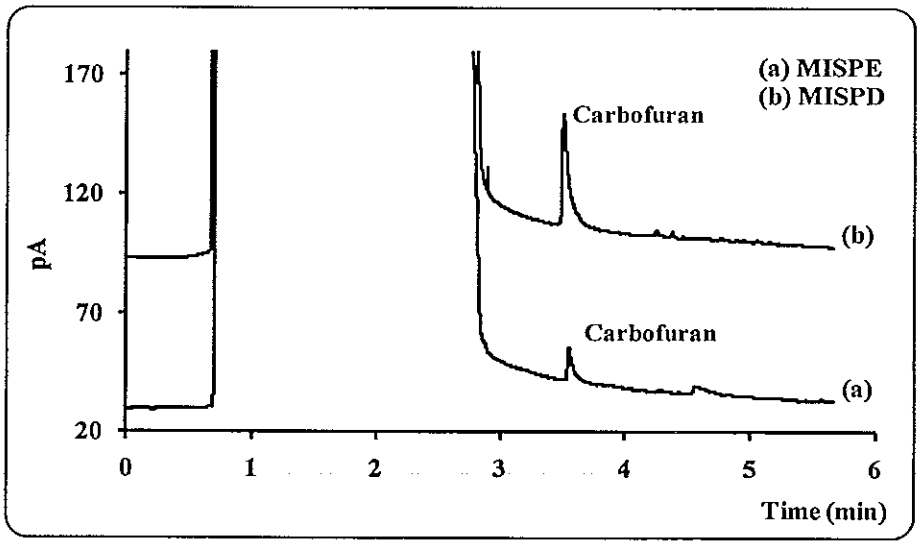


Figure 3.49 The chromatograms of MISPE and MISPD technique at the optimum conditions.

3.7.2 Quantitative analysis

The quantitative analysis of carbofuran was done by considering peak area that was related to the concentration of the analyte. The 11 sampling sites (Table 3.30) of real water were collected from PSU water reservoir, Well water from Thambon Bang Riang, Kuan Nieng district Songkhla which have the reported that contaminated by carbamate pesticide (Suwansa-ard *et al.*, 2005) (Figure 3.50), and Phom Ki Ri district, Nakorn Si Thammarat. Samples were filtered twice through GF/F microfiber in order to remove particulates that can plug in the binding site of MIP, then, extracted by MISPE or/and MISPD using both MIPs. The extractants were analyzed by GC-FID using optimum conditions. Carbofuran concentrations in the samples were evaluated by using matrix matched calibration curve. Concentration of carbofuran in the sample was found to be in the range of non detectable to 80 $\mu g\ L^{-1}$ (Table 3.31). From the results, only sampling site 7 was found the contaminated of carbofuran, it may be they just plants the vesgetables therefore carbofuran used as the seedling dip, releaded and contaminated in water sample. For the other sampling sites non detectable, there have two assumptions; the first, carbofuran was not used or the second is carbofuran was degraded or dilute to very low concentration. When carbofuran could not be detected, the confirmation was carried out by standard addition method as described in 2.10.2.2. An example of standard addition (site 1, PSU water reservoir) is shown in Figures 3.51 and 3.52 for MISPE and MISPD, respectively. Only sampling site two (Phom KI RI district) contaminated carbofuran at 0.08 ± 0.03 and $0.09 \pm 0.02\ \mu g\ L^{-1}$ for MISPE 1 and MISPE 2, respectively. The other sampling sites were not detectable.

The results obtained from MISPE and MISPD methods for both MIP 1 and 2 were also compared by spiked 10 concentrations of standard carbofuran (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 $\mu g\ L^{-1}$) then tested by the Wilcoxon singed rank test (Miller and Miller, 2000). In this test, the null hypothesis (there is no difference between the two methods) is rejected at a significance level ($P < 0.05$) if the experimental value is less than or equal to the critical value (Table 3.32) (Miller and Miller, 2000). The results in Tables 3.33-3.36 showed that the null hypothesis is retained, there is no evidence for systematic difference between the results obtained

from MISPE and MISPD method for both MIP 1 and 2 ($P < 0.05$). From all these results it can be concluded that MISPD can be used in of place of MISPE method and can provide the same results. MISPD has more advantages than MISPE, *i.e.* it provides higher sensitivity, lower LOD and LOQ, consume small amount of sample and short extraction time.

Table 3.30 The details of 11 sampling sites.

Site	Place	Cultivated plant	Type of water	Technique for sample preparation
1	PSU water reservoir	-	surface	MISPE and MISPD
2	Phom KI RI district	cucumber	pond	MISPE
3	Moo 3, Bang riang	cucumber, lamon basil	well	MISPE
4	Moo 2, Bang riang	cucumber	pond	MISPE
5	Moo 4, Bang riang	Chinese cabbage, winged bean	pond	MISPE
6	Moo 6, Bang riang	-	pond	MISPE
7	Moo 8, Bang riang	cabsicum frutescence	pond	MISPE and MISPD
8	Moo 3, Bang riang	cucumber	pond	MISPE and MISPD
9	Moo 3, Bang riang	sweet basil	tap	MISPE and MISPD
10	Moo 2, Bang riang	maize	canal	MISPE and MISPD
11	Moo 2, Bang riang	winged bean	canal	MISPE and MISPD

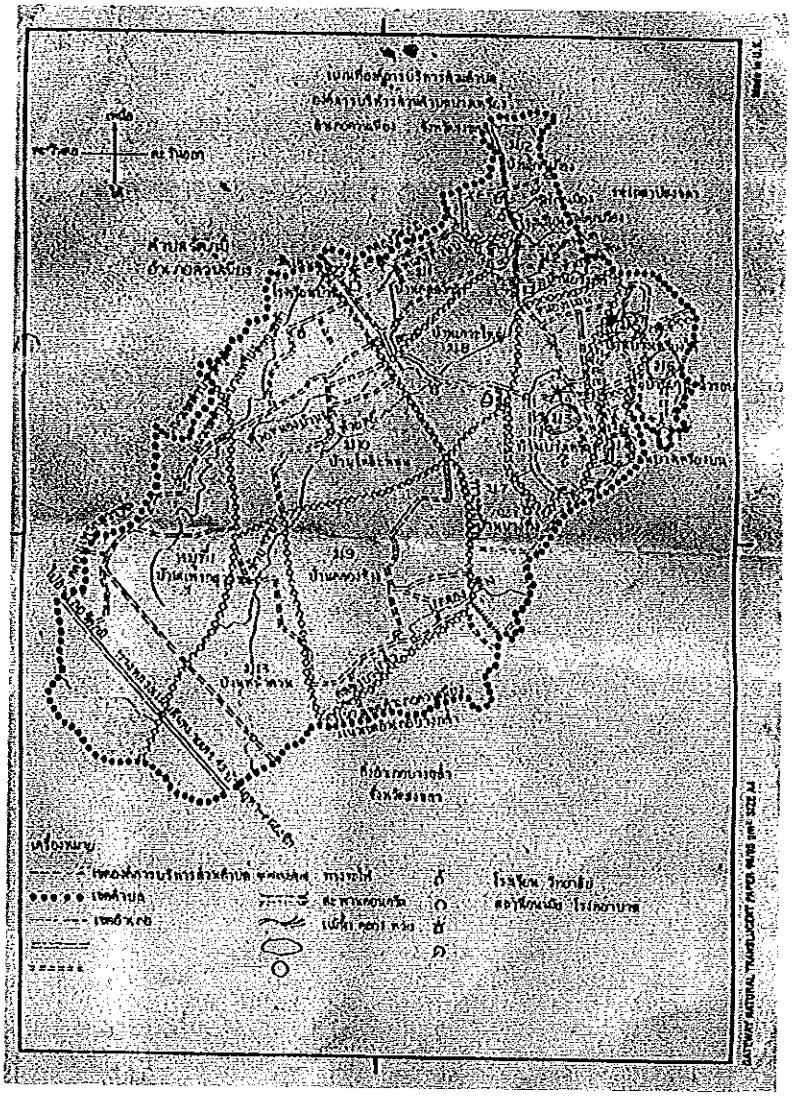


Figure 3.50 The map of Thambon Bang riang, Kuan nieng district, Songkhla.

Table 3.31 Carbofuran concentrations in water samples.

Site	Concentration of carbofuran from each technique ($\mu g\ L^{-1}$)*			
	MISPE		MISPD	
	MIP 1	MIP 2	MIP 1	MIP 2
1	ND	ND	ND	ND
2	ND	ND	-	-
3	ND	ND	-	-
4	ND	ND	-	-
5	ND	ND	-	-
6	ND	ND	-	-
7	76 ± 5	78 ± 6	80 ± 3	79 ± 8
8	ND	ND	ND	ND
9	ND	ND	ND	ND
10	ND	ND	ND	ND
11	ND	ND	ND	ND

* 5 Replications, ND = non detectable

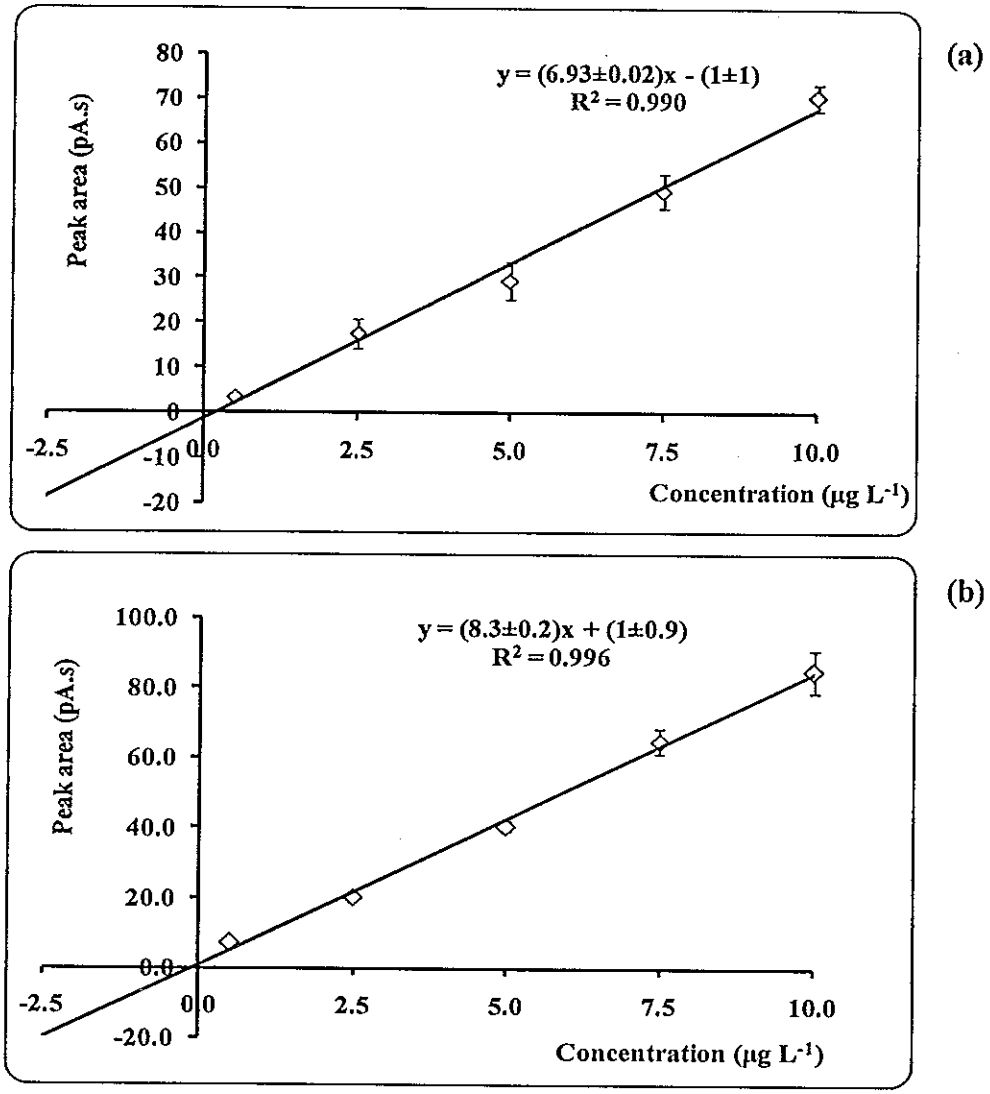


Figure 3.51 The standard addition curve of sample site 1 (n=5) (a) MISPE 1 (b) MISPE 2.

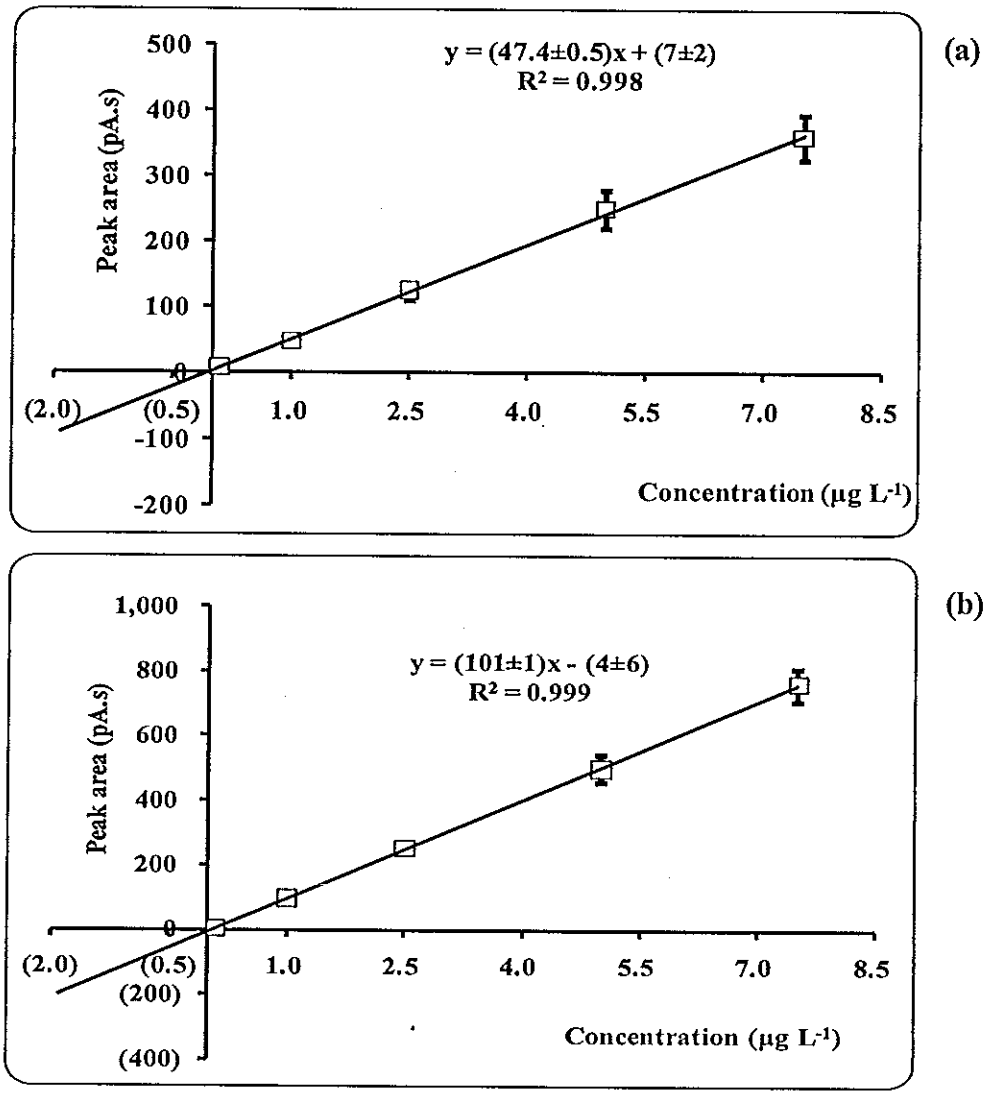


Figure 3.52 The standard addition curve of sample site 1 (n=5) (a) MISPD 1 (b) MISPD 2.

Table 3.32 Wilcoxon signed rank test. Critical values for the test statistic at *P=0.05* (Miller and Miller, 2000).

n	One-tailed test	Two-tailed test
5	0	NA*
6	2	0
7	3	2
8	5	3
9	8	4
10	10	8
11	13	10
12	17	13
13	21	17
14	25	21
15	30	25

* NA indicated that the test cannot be applied

Table 3.33 The Wilcoxon sign rank test for comparison of the concentration of carbofuran in spiked sample from MISPE method that used MIP 1 and 2. The null hypothesis (there is no difference between two methods) is rejected if the test statistic T (the lower of the sum of positive rank or negative rank) is less than or equal to the critical value. The null hypothesis can not be rejected if the test statistic T is greater than the critical value.

No. sample	Concentration of carbofuran ($\mu g\ L^{-1}$)		The difference of two method	The difference of two method	Rank
	MIP 1	MIP 2			
1	2.3	2.1	0.19	0.0	1.5
2	4.3	4.3	0.06	0.0	1.5
3	6.5	6.0	0.58	0.1	3.0
4	8.6	9.0	-0.34	0.2	7.5
5	11.1	11.1	-0.05	-0.2	-7.5
6	12.8	11.7	1.11	-0.3	-9.5
7	15.8	15.4	0.47	-0.3	-9.5
8	17.1	16.9	-0.20	0.5	8.0
9	19.8	19.8	0.03	0.6	9.0
10	20.5	20.8	-0.31	1.1	10.0
n					10
negative sum					**-26.5**
positive sum					50.5
test statistic vale at *P<0.05*					8

Table 3.34 The Wilcoxon sign rank test for comparison of the concentration of carbofuran in spiked sample from MISPD method that used MIP 1 and 2. The null hypothesis (there is no difference between two methods) is rejected if the test statistic T (the lower of the sum of positive rank or negative rank) is less than or equal to the critical value. The null hypothesis can not be rejected if the test statistic T is greater than the critical value.

No. sample	Concentration of carbofuran ($\mu g\ L^{-1}$)		The difference of two method	The difference of two method	Rank
	MIP 1	MIP 2			
1	2.0	2.2	-0.20	0.0	1.5
2	4.3	4.3	0.04	0.0	1.5
3	5.9	6.4	-0.42	-0.2	3
4	7.9	8.3	-0.40	-0.3	4
5	10.8	10.4	0.40	-0.4	-6
6	12.8	11.3	1.55	-0.4	-6
7	13.8	14.4	-0.66	0.4	6
8	15.9	16.9	-1.03	-0.7	-8
9	19.4	19.7	-0.31	-1.0	-9
10	19.9	19.9	0.00	1.5	10
n					10
negative sum					-29
positive sum					**26**
test statistic vale at *P<0.05*					8

Table 3.35 The Wilcoxon sign rank test for comparison of the concentration of carbofuran in spiked sample from MISPE and MISPD method that used MIP 1. The null hypothesis (there is no difference between two methods) is rejected if the test statistic T (the lower of the sum of positive rank or negative rank) is less than or equal to the critical value. The null hypothesis can not be rejected if the test statistic T is greater than the critical value.

No. sample	Concentration of carbofuran ($\mu g\ L^{-1}$)		The difference of two method	The difference of two method	Rank
	SPE	SPD			
1	2.3	2.0	0.29	0.2	1.0
2	3.9	4.3	-0.36	0.3	2.0
3	6.5	5.9	0.59	-0.4	-3.5
4	8.6	7.9	0.70	0.4	3.5
5	11.1	10.8	0.25	0.6	6.5
6	11.3	12.8	-1.48	0.6	6.5
7	15.8	13.8	2.07	0.7	7.0
8	16.9	15.9	1.04	1.0	8.0
9	19.8	19.4	0.45	-1.5	-9.0
10	20.5	19.9	0.58	2.1	10.0
n					10
negative sum					**-12.5**
positive sum					44.5
test statistic vale at *P<0.05*					8

Table 3.36 The Wilcoxon sign rank test for comparison of the concentration of carbofuran in spiked sample from MISPE and MISPD method that used MIP 2. The null hypothesis (there is no difference between two methods) is rejected if the test statistic T (the lower of the sum of positive rank or negative rank) is less than or equal to the critical value. The null hypothesis can not be rejected if the test statistic T is greater than the critical value.

No. sample	Concentration of carbofuran ($\mu g\ L^{-1}$)		The difference of two method	The difference of two method	Rank
	SPE 1	SPD 1			
1	2.1	2.2	-0.10	0.01	1
2	4.3	4.3	0.01	-0.10	-2.5
3	6.0	6.4	-0.42	0.10	2.5
4	9.0	8.3	0.63	0.16	4
5	11.1	10.4	0.70	-0.42	-6.5
6	11.7	11.3	0.42	0.42	6.5
7	15.4	14.4	0.94	0.63	7
8	17.1	16.9	0.16	0.70	8
9	19.8	19.7	0.10	0.88	9
10	20.8	19.9	0.88	0.94	10
n					10
negative sum					-9
positive sum					48
test statistic vale at *P<0.05*					8

CHAPTER 4

Conclusions

Analysis of carbofuran contaminated in water sample was carrier out by gas chromatography coupled with flame ionization detector (GC-FID). Molecular imprinted polymer (MIP) was synthesized and used as selective sorbent for carbofuran in solid phase extraction (SPE) and solid phase dispersion (SPD) in sample preparation step. Two MIPs were synthesized by using two functional monomers, acrylamide and 2-(diethylamino) ethylmethacrylate (DAM) for MIP 1 and methacrylic acid and DAM for MIP 2. Parameters of MIP synthesis affecting the binding properties were investigated and the optimum conditions are as shown in Table 4.1.

Table 4.1 The optimum conditions for MIP synthesis.

Parameters	Optimum value
MIP 1 (acrylamide and DAM)	
Ratio between acrylamide and DAM	1:2
Amount of crosslinker (%)	93
Type of porogen	Toluene
Concentration of porogen (mol)	0.050
Type of initiator	AIBN
Concentration of initiator (mol)	0.019
Polymerization temperature (°C)	60
Polymerization time (hours)	24
MIP 2 (methacrylic acid and DAM)	
Ratio between methacrylic acid and DAM	1:1
Amount of crosslinker (%)	93
Type of porogen	Toluene
Concentration of porogen (mol)	0.010
Type of initiator	AIBN
Concentration of initiator (mol)	0.019
Polymerization temperature (°C)	60
Polymerization time (hours)	24
Template removal (soxhlet extraction time)	6

Characteristic of the synthesized MIPs are shown in Table 4.2.

Table 4.2 Physical characteristic of molecular imprinted polymers.

Properties	MIP 1	non - MIP 1	MIP 2	non - MIP 2
% swelling (mL mg^{-1})	40 ± 5	47 ± 3	43 ± 3	42 ± 10
Particle size (μm)	93 ± 14	81 ± 15	118 ± 9	70 ± 7
Bluk density (g mL^{-1})	0.361 ± 0.008	0.42 ± 0.02	0.36 ± 0.02	0.38 ± 0.01
Capacity (mg L^{-1})	12	-	11	-

Analysis of contaminated carbofuran in water sample was carried out by gas chromatography coupled with flame ionization detector (GC-FID). Chromatographic separation was carried out by a fused silica ULTRA-2 capillary column: 25 m × 0.53 mm I.D. × 0.32 μm film thickness of 5% phenyl 95% dimethylsiloxane. Conditions for GC-FID were optimized and the results are shown in Table 4.3.

Table 4.3 Optimum conditions of GC-FID.

Parameter	Optimum conditions
Carrier gas flow rate (mL min^{-1})	3.0
Oxidant gas flow rate (mL min^{-1})	300
Hydrogen gas flow rate (mL min^{-1})	40
Injector temp (°C)	270
Detector temp (°C)	280
Program temperature	
Initial temp (°C)	100
Initial time (min)	1
Ramp rate (°C min^{-1})	30
Final temp (°C)	210
Final time (min)	3

These GC-FID optimum conditions provided wide linear dynamic range 0.5 mg L^{-1} to 600 mg L^{-1}, with high coefficient of determination ($R^2 > 0.99$). The limit of detection and limit of quantitative were 0.247 ± 0.002 and 0.823 ± 0.006 mg L^{-1}, respectively with % RSD lower than 4 %. However, these LOD values do not cover the value of maximum contaminant levels for carbofuran in drinking water by set EPA, *i.e.* 40 μg L^{-1}, and 0.1 μg L^{-1} for EU (López-Blanco *et al.*, 2002). Therefore, a sample preparation technique was necessary.

Molecular imprinted solid phase extraction (MISPE) and molecular imprinted solid phase dispersion (MISPD) were used as the sample preparation

techniques. The influence of several parameters on the efficiency of these proposed method were investigated and the optimum conditions of MISPE and MISPD are shown in Tables 4.4 and 4.5, respectively.

Table 4.4 The optimum conditions of molecular imprinted solid phase extraction (MISPE).

Parameters	Optimum condition	
	MISPE 1	**MISPE 2**
Type of eluting solvent	methanol	methanol
Eluting flow rate (mL min^{-1})	0.2	0.5
Volume of eluting solvent (mL)	2.5	2.5
Type of washing solvent	hexane	hexane
Volume of washing solvent (mL)	5.0	5.0
Sample flow rate (mL min^{-1})	0.5	0.5
Drying time (min)	0	10
Reusability (Times)	5	5

Table 4.5 The optimum conditions of molecular imprinted solid phase dispersion (MISPD).

Parameters	Optimum condition	
	MISPD 1	MISPD 2
Shaker strength (oscillation min^{-1})	270	308
Shaker time (min)	10	10
Type of eluting solvent	methanol	methanol
Eluting flow rate (mL min^{-1})	0.2	0.5
Volume of eluting solvent (mL)	5.0	5.0
Type of washing solvent	hexane	hexane
Volume of washing solvent (mL)	1.0	1.0
Sample flow rate (mL min^{-1})	0.5	0.5

These techniques were used to extract carbofuran, compared to the other two carbamate pesticides *i.e.* carbaryl and carbosulfan which have quite similar structure to carbofuran. It provided the highest selectivity for carbofuran. Moreover, both techniques provided high repeatability. Validation of linear dynamic range, LOD, LOQ, precision, accuracy and matrix effect were investigated. MISPE provide a wider linear dynamic range than MISPD, however the sensitivity, LOD and LOQ obtained from MISPD were better than MISPE (Table 4.6).

Table 4.6 Linear dynamic range, LOD and LOQ by IUPAC method obtained from MISPE and MISPD at optimum conditions.

Sample preparation method	Linear dynamic range ($\mu g\ L^{-1}$)		LOD ($\mu g\ L^{-1}$)		LOQ ($\mu g\ L^{-1}$)	
	MIP 1	MIP 2	MIP 1	MIP 2	MIP 1	MIP 2
MISPE	0.2 - 200.0	2.0 - 40.0	0.677 ± 0.009	0.36 ± 0.03	2.26 ± 0.02	1.19 ± 0.09
MISPD	0.1 – 20.0	0.1 – 20.0	0.031 ± 0.001	0.015 ± 0.002	0.105 ± 0.002	0.050 ± 0.005

The results indicated that these techniques are appropriate and sensitive to detect trace amount of carbofuran in water. When compared the LODs of the proposed methods with other reports (Table 4.7), the proposed method provided lower or the same LOD with these using chemiluminescence (Rodrigues *et al.*, 2007), amperometric biosensor (Albareda-Sirvent *et al.*, 2001) and GC (Lambropoulou *et al.*, 2002; Maloschik *et al.*, 2007) However, they are higher than the one of using chromatographic detection with mass selective detector. However these values covered the value of maximum contaminant levels for carbofuran in drinking water by EPA and EU.

The matrix effect was studied and the statistically test was used to confirm the difference between the slope of spiked DI water calibration curve and matrix matched calibration curve. It indicated that these was matrix effect, therefore for real sample analysis matrix matched calibration curve was used. High recoveries were obtained in acceptable level (EPA, 2001) ranged from 80 – 101 % and 71-110 % for SPE and SPD, respectively. The RSD was lower than 19% at spiked concentration 5.0 $\mu g\ L^{-1}$ and 20.0 $\mu g\ L^{-1}$ for both techniques. Qualitative and quantitative analysis of carbofuran were performed in water samples from Prince of Songkla University (PSU) water reservoir, well water form Thambon Bang Riang, Kuan Nieng district, Songkhla and Phom Ki Ri district, Nakorn Si Thammarat. All samples were analyzed and evaluated by using matrix matched calibration curve. The concentrations of carbofuran in sample were in the range from non detectable to 80 $\mu g\ L^{-1}$.

In conclusion, the proposed methods can be used for determination of carbofuran in the trace level. The synthesized MIP sorbent for carbofuran provided high selectivivity and can be reused up to five times, making it more cost effective than the commercial solid sorbent (C_{18}). The analysis method is reliable, precise and high sensitive. MISPD method has more advantages than MISPE *i.e.* higher sensitivity and lower LOD, consume smaller amount of sample. It is a simple and fast sample preparation technique.

Table 4.7 Comparison of the proposed method with another method for the analysis of carbofuran in water.

Method	Sample preparation	LOD/LOQ (μg L^{-1})	Recovery (%)	Reference
GC-FID	MISPE	0.68 and 0.36/2.26 and 1.19	80 - 101	This work
	MISPD	0.031 and 0.015/0.105 and 0.050	71 - 110	
LC-ESI/MS	SPE	0.0045/-	67 - 73	Rodrigues *et al.*, 2007
Chemiluminescence	-	20/60	93-137	Huertas-Pérez *et al.*, 2005
LC-ESI/MS	SPE	0.1/-	72 -108	Nogueira *et al.*, 2004
HPLC/DAD	SPE	0.06/0.08	90	López-Blanco *et al.*, 2002
	SPME	8.9/10	100	
Amperometric biosensor (AChE inhibitor)	-	0.2/-	101 -104	Albareda-Sirvent *et al.*, 2001
GC-FTD	SPME	0.3/-	87	Lambropoulou *et al.*, 2002
GC-MS	SPME	0.5	84-109	Maloschik *et al.*, 2007
LC–ESI/MS	SPE	0.003/-	85	Nogueira *et al.*, 2003

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List of Publications and Proceedings

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1. Poorahong, S., Thavarungkul, P. and Kanatharana, P. "Development of Supported Liquid Membrane Extraction (SLME) for Polycyclic Aromatic Hydrocarbons in Water" The 5th PERCH-CIC Annual Scientific Congress (PERCH-CIC Congress V), Pattaya, Thailand, 2007.