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DEVELOPMENT OF SAMPLE PREPARATION TECHNIQUES FOR THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BIOGENIC AMINES IN FOODS

by

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Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

July 2010

ACKNOWLEDMENTS

It is difficult to excel or achieve success without hard work and effort.

Throughout my PhD studies, many individuals helped to ease the difficulties that I faced.

First and foremost, my deepest gratitude goes to my supervisor Professor Bahruddin Saad for his guidance and encouragement throughout these years. He has been a wonderful mentor regardless of various constraints and hurdles. His sincerity in passing down his knowledge through his wisdom is much appreciated. I would also like to thank my co-supervisors Dato' Professor Muhammad Idiris Saleh and Dr Abdussalam Salhin for their help throughout the research. Not to be forgotten, Prof Ismail Ab Rahman for supervising some of my work. Special thanks also to Dr Chanbasha Basheer and Professor Hian Kee Lee for the attachment to the laboratory of the Department of Chemistry, National University of Singapore.

Futhermore, I would like to thank the staff of the School of Chemical Sciences, USM especially to Mr Ariffin Majid for his great help and assistance in my research. I wish to thank Universiti Teknologi MARA for financial support throughout my studies under the Young Lecturer Scheme program.

Last but not least, I am indebted to my fellow labmates and friends who helped me during the difficult times through their support, encouragement, sharing of ideas and

the good times in the last four years. I wish all of them successfully completion of their studies.

Notwithstanding involvement of others, nobody can deny that the closest party who have supported me is my family. Their existence provide an abundance of inspiration and their sacrifice has lead me to be a better person. To them, I dedicate this thesis.

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LIST OF ABBREVIATIONS

A18C6	1-aza-18-crown-6
AB18C6	4'-aminobenzo-18-crown-6
AccQ	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
AGM	Agmatine
ASE	Accelerated solvent extraction
BA	Biogenic amines
BET	Brunauer-Emmett-Teller
BJH	Barrett-Joyner-Halenda
CAD	Cadaverine
C18	Octadecylsilane groups
C^4D	Capacitively coupled contactless conductivity detector
CE	Capillary electrophoresis
DA18C6	1,4,10,13-tetraoxa-7,16-diazacyclo octadecane
DAO	Diamine oxidase
Db-Cl	Dabsyl chloride
DLLME	Dispersive liquid-liquid microextraction
Dn-Cl	Dansyl chloride
EDX	Energy dispersive x-ray microanalysis
EF	Enrichment factor
ELISA	Enzyme-linked immunosorbent assays
EME	Electro membrane extraction
FITC	Fluoresceine isothiocyanate
FMOC	9-fluorenylmethyl chloroformate
FQ	3-(2-furoyl)-quinoline-2-carboxaldehyde
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry

HIS	Histamine
HPLC	High performance liquid chromatography
ISs	Immunosorbents
k	Selectivity coefficient
K_d	Distribution coefficient
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase microextraction
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MIPs	Molecularly imprinted polymers
MSBC	Mesoporous silica immobilized with chloropropyltrimethoxysilane
MSPD	Matrix solid phase dispersion
NDA	Naphthalene-2,3-dicarboxaldehyde
OPA	*o*-phthalaldehyde
PAHs	Polycyclic aromatic hydrocarbons
PDMS	Polydimethysiloxane
PEA	β-phenylethylamine
PFE	Pressurized fluid extraction
PLE	Pressurized liquid extraction
PUT	Putrescine
RAMs	Restricted access materials
R^2	Square of regression coefficient
RSD	Relative standard deviation
SAX	Strong anion-exchange

SAE	Sonication accelerated extraction
SBSE	Stir bar sorptive extraction
SCX	Strong cation-exchange
SDME	Single drop microextraction
SEM	Scanning electron microscope
SFE	Supercritical fluid extraction
SLM	Supported liquid membrane
SPD	Spermidine
SPE	Solid phase extraction
SPM	Spermine
SPME	Solid phase microexteaction
St-DVB	Styrene-divinylbenzene
TEOS	Tetraethoxysilane
TGA	Thermogravimetric analysis
TLC	Thin layer chromatography
TRP	Tryptamine
TYR	Tyramine
US FDA	US Food & Drug Administration
US EPA	US Environmental Protection Agency
UV	Ultraviolet
WCX	Weak cation-exchange

PEMBANGUNAN KAEDAH PENYEDIAAN SAMPEL UNTUK PENENTUAN AMINA BIOGENIK DI DALAM MAKANAN MENGGUNAKAN KROMATOGRAFI CECAIR PRESTASI TINGGI

ABSTRAK

Amina biogenik (BA) dikaji dengan meluas disebabkan oleh ketoksikan dan kemungkinan dapat digunakan sebagai penanda kimia dalam penentuan kualiti makanan dan kajian kanser. Tesis ini difokuskan kepada pembangunan kaedah penyediaan sampel untuk penentuan BA di dalam makanan menggunakan kromatografi cecair prestasi tinggi dan pengesan ultralembayung. BA triptamina (TRP), putrescina (PUT), histamina (HIS), tiramina (TYR) dan spermidina (SPD) telah diekstrak menggunakan asid hidroklorik dan asid trikloroasetik, dan ditentukan di dalam enam puluh dua sampel makanan biasanya dimakan di Malaysia. Sampel makanan ini adalah budu, cincalok, ikan ditinkan, ikan kering masin, hasilan daging, jus buahan, sayuran/buahan ditinkan dan hasilan kacang soya. Selepas pengekstrakan, sampel ini telah diterbit dengan menggunakan dansil klorida. Kandungan purata TRP, PUT, HIS, TYR dan SPD di dalam lapan sampel budu masing-masing adalah 82.7, 38.1, 187.7, 174.7 dan 5.1 mg kg^{-1}. BA yang didapati di dalam cincalok adalah PUT, HIS dan TYR, masing-masing dengan kandungan purata 330.7, 126.1 dan 448.8 mg kg^{-1}. Dengan pengecualian pekasam dan belacan, kandungan BA di dalam ikan kering masin adalah rendah, manakala kandungan BA di dalam hasilan daging, jus buahan dan sayuran/buahan ditinkan tidak dikesan atau rendah.

Bahan penjerap (A18C6-MS, DA18C6-MS dan AB18C6-MS) berasaskan kepada ligan eter mahkota 1-aza-18-mahkota-6, 1,4,10,13-tetraoksa-7,16-diazasiklo oktadekana dan 4-aminobenzo-18-mahkota-6 telah disediakan melalui pemegunan kimia ligan-ligan ini kepada bahan penyokong silika liang meso. Bahan penjerap dicirikan dengan menggunakan FTIR, analisis mikro mikroskopi pengimbasan elektron-serakan tenaga sinar-X, analisis unsur, ujian penjerapan/penyahserapan nitrogen dan analisis termogravimetri. Keupayaan bahan penjerap ini untuk mengekstrak BA dikaji melalui kaedah serapan kelompok dan turus. Pengekstrakan ini dinilai melalui fungsi pH, masa sentuh atau kadar alir, kepekatan BA dan guna semula. Dalam keadaan yang optimum, semua penjerap menunjukkan kepilihan yang paling tinggi terhadap SPD berbanding BA lain (HIS, PUT, TRP, TYR). Walaubagaimanapun, keupayaan muatan dan kepilihan AB18C6-MS adalah tertinggi dalam kehadiran BA lain. Kesemua kaedah yang dicadangkan telah digunakan untuk mengekstrak SPD dan ditunjukkan melalui perolehan semula yang baik (71.2-99.8 %) dari kedua-dua kaedah apabila menggunakan matriks makanan yang berbeza. Kaedah turus telah dipilih disebabkan mudah untuk dilaksanakan.

Pengekstrakan mikro fasa cecair gentian berongga melalui kaedah terbitan *in situ* menggunakan dansil klorida juga telah berjaya dibangunkan untuk penentuan BA. Dalam keadaan yang optimum (pelarut pengekstrakan, diheksil eter; fasa penerima, 0.1 M HCl; masa pengekstrakan, 30 minit; suhu pengekstrakan, 26 °C; tanpa penambahan garam), faktor perkayaan 47-456 telah diperolehi. Had pengesan dan pengkuantitian masing-masing adalah di dalam julat 0.01-0.03 dan 0.03-0.10 μg mL^{-1}. Kaedah ini telah berjaya digunakan dalam penentuan sampel cincalok dan sos tomato.

DEVELOPMENT OF SAMPLE PREPARATION TECHNIQUES FOR THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BIOGENIC AMINES IN FOOD

ABSTRACT

Biogenic amines (BA) have been extensively studied in recent years due to their potential toxicity and possible use as chemical markers for assessing food quality and in cancer research. This thesis is focused on the development of sample preparation techniques for the high performance liquid chromatographic-ultraviolet determination of BA in food. The BA tryptamine (TRP), putrescine (PUT), histamine (HIS), tyramine (TYR) and spermidine (SPD) were extracted using hydrochloric acid and trichloroacetic acid and determined in sixty-two food items commonly consumed in Malaysia. This includes the local appetizers budu and cincalok, canned fish, salt-cured fish, meat products, fruit juice, canned vegetables/fruits and soy bean products. After the extraction, the samples were derivatized with dansyl chloride. Mean levels of TRP, PUT, HIS, TYR and SPD in eight budu samples were 82.7, 38.1, 187.7, 174.7 and 5.1 mg kg^{-1}, respectively. The main BA found in cincalok were PUT, HIS and TYR where the mean values were 330.7, 126.1 and 448.8 mg kg^{-1}, respectively. With the exception of pekasam and belacan, significantly lower levels of BA were found in salt-cured fish samples. Non detectable or low levels of BA were found in meat products, fruit juice and canned vegetables/fruit samples.

Sorbent materials (A18C6-MS, DA18C6-MS and AB18C6-MS) based on the crown ether ligands, 1-aza-18-crown-6, 1,4,10,13-tetraoxa-7,16-diazacyclo octadecane and 4'-aminobenzo-18-crown-6, respectively, were prepared by the chemical

immobilization of the ligand onto mesoporous silica support. The sorbents were characterized by FTIR, scanning electron microscopy-energy dispersive X-ray microanalysis, elemental analysis, nitrogen adsorption-desorption test and thermogravimetric analysis techniques. The applicability of the sorbents for the extraction of BA by the batch sorption and column method were extensively studied and evaluated as a function of pH, contact time or flow rate, BA concentration and reusability. Under the optimized conditions, all the sorbents exhibited highest selectivity toward SPD compared to other BA (HIS, PUT, TRP and TYR). Among the sorbents, AB18C6-MS offer the highest capacity and best selectivity towards SPD in the presence of other BA. The applicability of the proposed method for the selective extraction of SPD was demonstrated by the reasonable recoveries obtained from both methods (71.2-99.8 %) when different food matrices were used. Between the two methods, the column technique is prefered as it is easier to be implemented.

A hollow fiber liquid phase microextraction with *in situ* derivatization using dansyl chloride was also successfully developed for the determination of BA (TRP, PEA, PUT, CAD, HIS, TYR, SPD) in food samples. Under the optimized conditions (extraction solvent, dihexyl ether; acceptor phase, 0.1 M HCl; extraction time, 30 min; extraction temperature, 26 °C; without addition of salt), enrichment factors varying from 47 to 456 was achieved. The limits of detection and quantification ranged from 0.01 to 0.03 μg mL^{-1} and 0.03 to 0.10 μg mL^{-1}, respectively. The method was successfully applied to cincalok and tomato ketchup samples.

CHAPTER ONE

INTRODUCTION

1.1. Sample preparation in chemical analysis

Despite the great technological advances in the analytical field, most sophisticated instruments cannot handle complex sample matrices directly and, as a result, a sample preparation step is commonly involved in an analytical procedure. The main objective of the sample preparation step is to isolate and concentrate the analytes of interest from interfering sample components, and to convert the analytes to a form that is compatible with the instrument for the final analysis. Sample preparation has long been recognized as the main bottleneck in the analytical process, it is the most time consuming, error prone and labour-intensive. Efficient sample preparation is therefore important for a successful analysis. Generally, a clean sample helps to improve the separation and detection, while poorly treated sample may invalidate the whole assay.

Liquid-liquid extraction (LLE) remains the most common method in sample preparation. Most of the official methods still use LLE techniques, such as those published by the US Environmental Protection Agency (US EPA). The technique provides large potentials for tuning the extraction by chemical means (e.g., by pH adjustments, selecting solvents with specific properties or incorporating different specific reagents) (Jönsson and Mathiasson, 2000).

However, LLE is time consuming, tedious and uses large amounts of potentially toxic organic solvents. As LLE often involves multi-step operation, it often results in the loss of analytes, frequently contributing as a major source of error in analysis (Psillakis, 2002). Due to the limited selectivity, particularly for trace level analysis, there is a need to clean-up or enrich the analyte prior to the instrumental analysis (Ridgway *et al*., 2007). Furthermore, the LLE technique is plaqued by the formation of emulsion and is difficult to automate (Psillakis, 2002).

Another popular sample preparation technique for the extraction of analytes in solid samples is soxhlet extraction, invented in 1879 by Franz von Soxhlet. Main applications of Soxhlet extraction are for environmental samples, such as soil (Hwang and Cutright, 2004). It has also been used for the analysis of food, such as for the extraction of lipids from wheat grains (Zarnowskia and Suzuki, 2004) and pharmaceutical samples (Devine *et al*,, 2006). This technique, although exhaustive, is not selective and further clean-up such as solid phase extraction is necessary. Due to the elevated temperatures involved, Soxhlet extraction can degrade thermally labile compounds. The time required is often long (typically 1-6 h) and a significant volume of organic solvent (50-200 mL for a 10 g sample) is required (Ridgway *et al*., 2007). Nevertheless, soxhlet extraction is still widely found in laboratories and form a standard procedure for many solid-liquid extractions (Virot *et al*., 2007).

Automated Soxhlet extraction systems are available and these have been claimed to greatly reduce the extraction times and perform boiling, rinsing and solvent recovery automatically. Up to six samples can be extracted simultaneously and lower volumes of solvent can be anticipated (Ridgway *et al*., 2007). This technique, included its automation and a comparison to other techniques has been reviewed by Luque de

Castro and García-Ayuso (1998). Other development include the use of focused microwave-assisted soxhlet extraction (Luque-García and Luque de Castro, 2004) and microwave-integrated soxhlet (Virot *et al*., 2007) to improve the extraction efficiencies.

1.2. Modern sample preparation techniques

Although conventional sample preparation techniques are still in use, the trend in recent years has been towards (Smith, 2003):

- The ability to use smaller initial sample sizes.
- Greater selectivity in extraction.
- Potential for automation or for on-line methods, reducing manual operations, errors and time required.
- More environmentally friendly approach (green chemistry) with less waste and the use of significantly small volumes or no organic solvents.

Driven by these purposes, advances in sample preparation have resulted in a number of techniques such as sonication accelerated extraction (SAE), microwave accelerated extraction (MAE), pressurized liquid extraction (PLE) (also known as pressurized fluid extraction (PFE) and by the Dionex tradename 'accelerated solvent extraction' (ASE)), supercritical fluid extraction (SFE), solid phase extraction (SPE) and matrix solid phase dispersion (MSPD). More recently, microextraction techniques such as solid phase microextraction (SPME), stir bar sorptive extraction (SBSE), single drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME) and liquid phase microextraction (LPME) approaches have been used. Microextraction techniques have been regarded as the most

attractive sample preparation technique as it enables rapid analysis at low operating cost, minimization of organic solvents and high enrichments can be achieved. These techniques, in combination either with gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) or capillary electrophoresis (CE) can be used for the analysis of analytes in complex matrices.

1.2.1. Sonication accelerated extraction (SAE), microwave accelerated extraction (MAE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE)

The similarity between these techniques is the possibility of working at elevated temperatures and pressures, which drastically improve the speed of the extraction process. Moreover, the manipulation of physical properties can result in lower surface tension, increased analyte solubilities, higher diffusion and even alteration of solvent polarity. A review of these techniques, including the instrumentation involved and several applications were given in the literature (Camel, 2001; Zougagh *etal*., 2004; Björklund *et al*., 2006; Schantz, 2006). Table 1.1 summarizes the SAE, MAE, PLE and SFE techniques and presents their advantages and drawbacks.

SAE is inexpensive and easy to use. In this method, acoustic vibrations are applied to the sample. The method relies on the particles being broken down mechanically, which improve solvent access to the interior components. However, repeated extractions may be required for effective extraction and thus still consume large volumes of solvent (Eskilsson and Björklund, 2000). MAE is an interesting

alternative due to its medium investment costs and the possibility of performing multiple extractions (up to 50 cells at a time) with low solvent consumption and fast extraction. MAE uses microwave energy to heat sample-solvent mixtures in sealed or open vessels. The main disadvantage is the long cool down times required for the extraction cells. The extraction solvents available for MAE are somewhat limited to those solvents that can absorb microwaves, although the use of solvent mixtures with or without dipoles opens up a variety of potential solvent mixtures (Camel, 2001; Björklund *et al*., 2002; Schantz, 2006).

In the PLE technique, the solid or semisolid sample is placed in a close container (cell), and sand, sodium sulphate or hydromatrix is often used as a dispersant in the cell. Solvent is then added to the cell at the start of the heating cycle. During the heating cycle, the solvent is pumped in and out of the cell to maintain the pressure and to perform a number of static cycles as required (Schantz, 2006). The main advantage of PLE is that existing soxhlet methods can to a great extend be converted to PLE methods with small changes (Zougagh *et al*., 2004). In particular, PLE has been recognized as an official method by the US EPA (Camel, 2001).

Of much interest is the recent use of PLE with subcritical water as the extraction solvent. This fluid has been successfully used for several applications and is a promising alternative to the supercritical carbon dioxide (CO_2). However, even though subcritical water shows great potential as a ‘clean’ solvent, it yields dilute liquid extracts which require concentration steps and more matrix is extracted as compared to CO_2 extraction (Camel, 2001). The main shortcoming of the method is very high investment costs. Moreover, the method suffers from disadvantage of low extraction efficiency (Chen *et al*., 2008).

The SFE technique, introduced in the 1980s, is based mainly on supercritical CO_2 for the extraction. As CO_2 is a non-polar substance, it is able to dissolve non-polar to moderately polar compounds, such as polycyclic aromatic hydrocarbons (PAHs) and halogenated pesticides, or lipids and fats, but is generally unsuitable for many pharmaceuticals and drug samples. The addition of polar modifiers (e.g., methanol, 1-20 %) to supercritical CO_2 expands its extraction range to include more polar analytes and increases the scope of the method (Smith, 2003; Zougagh *et al*., 2004). Of much interest with SFE is the ability to extract labile or thermally sensitive analytes (Buldini *et al*., 2002). Another advantage of the technique is the possibility of performing rather selective extractions with no external clean-up or filtration. However, the main drawback is the very high investment and maintenance costs. Moreover, the method involves time consuming method development and requires skilled personnel (Camel, 2001; Zougagh *et al*., 2004).

Table 1.1 Comparison between the sonication accelerated extraction, microwave accelerated extraction, pressurized liquid extraction and supercritical fluid extraction techniques (Eskilsson and Björklund, 2000; Björklund *et al*., 2002)

	Extraction technique			
	SAE	MAE	PLE	SFE
Brief description	Sample is immersed in solvent in a vessel and placed in an ultrasonication bath	Sample is immersed in a microwave-absorbing solvent in a closed vessel and irradiated with microwave energy	Sample and solvent are heated and pressurized in an extraction vessels. When the extraction is finished, the extract is automatically transferred into a vial	Sample is loaded in a high pressure vessel and extracted with supercritical fluid (e.g., CO_2). The analytes are collected in a small volume of solvent or onto a solid phase trap, which is rinse with solvent in a subsequent step
Extraction time	10-60 min	3-30 min	5-30 min	10-60 min
Sample size	1-30 g	1-10 g	1-30 g	1-5 g
Solvent usage	30-200 mL	10-40 mL	10-100 mL	2-5 mL (solid trap), 5-20 mL (liquid trap)
Investment	Low	Moderate	High	High
Advantages	Multiple extractions	Fast and multiple extraction Low solvent volume Elevated temperatures	Fast extraction Low solvent volume Elevated temperatures No filtration required Automated system	Fast extraction Minimal solvent volume Elevated temperatures Relatively selective towards matrix interferences No clean-up or filtration required Concentrated extract Automated systems
Drawbacks	Large solvent volumes Repeated extractions may be required Clean-up step needed	Extraction solvent must be able to absorb microwaves Clean-up step needed Waiting time for the vessel to cool down	Clean-up step needed	Many parameters to optimize, especially analyte collection
Application	Environmental	Environmental Polymer Pharmaceutical Natural product	Environmental Food Pharmaceutical Biological samples	Environmental Food Pharmaceutical Biological samples

1.2.2. Solid phase extraction (SPE)

Sample preparation using SPE was first introduced in the 1970s (Poole *et al*., 2000). The development of SPE was driven by the need of an alternative method to replace LLE because many polar analytes are often partially soluble in water and cannot be extracted with good recoveries using organic solvents (Hennion, 1999). SPE is now regarded as a mature technology and has a strong foothold in the marketplace and has found applications in many areas including environmental, pharmaceutical, clinical, food and industrial (Poole, 2003). SPE has been accepted as an alternative sample preparation method to the LLE technique in many US EPA standard method for the analysis of organic compounds in drinking water and wastewater (Hennion, 1999).

SPE benefits from shorter processing times, low solvent consumption, simpler processing procedures and makes on-line determination possible by hyphenation with chromatographic techniques (Poole, 2003). Many reviews (Hennion, 1999; Huck and Bonn, 2000; León-González and Pérez-Arribas, 2000; Liŝka, 2000; Poole *et al*., 2000; Poole, 2003) and books (Thurman and Mills, 1998; Fritz, 1999) provided in-depth and comprehensive coverage of the SPE technique.

The recent innovation in SPE is the introduction of a disk format which is less subject to chanelling problems, offering large cross-sectional area and lower bed mass (Hennion, 1999). Disks differ from cartridges or syringes in that the disk is a membrane loaded with a solid sorbent, whereas the cartridge or syringe contains the sorbent (Thurman and Snavely, 2000). Disk technology has contributed directly to the automation of SPE through development of the 96-well SPE plates, mainly for sample clean-up in the pharmaceutical and biotechnological industries. Micropipette

tips are also available and uses a conventional pipette tip that is fitted with a SPE disk (Poole, 2003).

A wide range of sorbents ranging from the chemically bonded silica to the carbon or ion-exchange materials to the polymeric materials based on styrene-divinylbenzene (St-DVB) are available. Recently, selective sorbents such as immunosorbents (ISs), molecularly imprinted polymers (MIPs), restricted access materials (RAMs) and hydrophilic polymeric sorbents for the polar compound have been introduced (Fontanals *et al*., 2005).

The interest in ISs is due to its high selectivity, which is particularly suited to complex biological and environmental samples. ISs with covalently immobilized antibodies or antigens have high affinity to the corresponding antigens, or antibodies, allowing the extraction, concentration and clean-up of target analytes from complex matrices in a single step. Methods for the analysis of mycotoxins, phenylurea, herbicides and polycyclic aromatic hydrocarbons, are now commercially available (Delaunay *et al*., 2000; Poole, 2003). The on-line combinations of ISs and liquid chromatography have also been documented (Rodriguez-Mozaz *et al*., 2007). However, one major disadvantage of this technique is the need to initially develop the antibody, which make it impractical for one off analyses. The analyte-antibody interaction can also be affected by the sample matrix, leading to low extraction recoveries (Ridgway, 2007). Although the ISs have high selectivity, they are unstable in most cases and are expensive (Delaunay *et al*., 2000; Chen *et al*., 2008).

Rather than being dependent on antibody production, attempts have been made to mimic the specificity of immunosorbents with synthetic MIPs or plastic antibody in

the analysis of drugs and other compounds in environmental and biological samples (Andersson, 2000). A key feature in this technique is the polymerization of functional and cross-linking monomers in the presence of a template molecule (the analytes). MIP with specific cavities formed from a template molecule possesses specific molecular recognition sites adapted to the three-dimensional shape and functionalities of the analyte of interest. General advantages of MIPs, as compared to ISs is the selectively of analyes towards the template molecule, offering the advantages of an easy, low cost, rapid preparation, high thermal and chemical stability, and extremely long shelf-life without any need for special storage conditions (Andersson, 2000; Rodriguez-Mozaz *et al*., 2007; Chen *et al*., 2008).

In addition to MIPs, various RAMs have been used as extraction sorbents. These sorbents are developed particularly for the analysis of biological samples, such as plasma and serum. It is designed to prevent the macromolecules (e.g., proteins) from accessing the retention regions of target analytes either by a physical diffusion barrier such as a pore diameter of the internal surface of reversed phase sorbents or by a chemical diffusion barrier created by a macromolecular network at the outer surface of particle (Figure 1.1) (Ridgway, 2007; Chen *et al*., 2008). It can serve as a pre-column to preliminarily clean-up the biological fluids and to pre-separate and preconcentrate the target analytes. The interaction sites within the pores are accessible to small molecules only and the analytes are retained by hydrophobic or electrostatic interactions. Various RAMs sorbents are available with different surface chemistries. With RAM automated on-line SPE, direct injection of complex biological fluids into liquid chromatography is possible (Souverain *et al*., 2004).

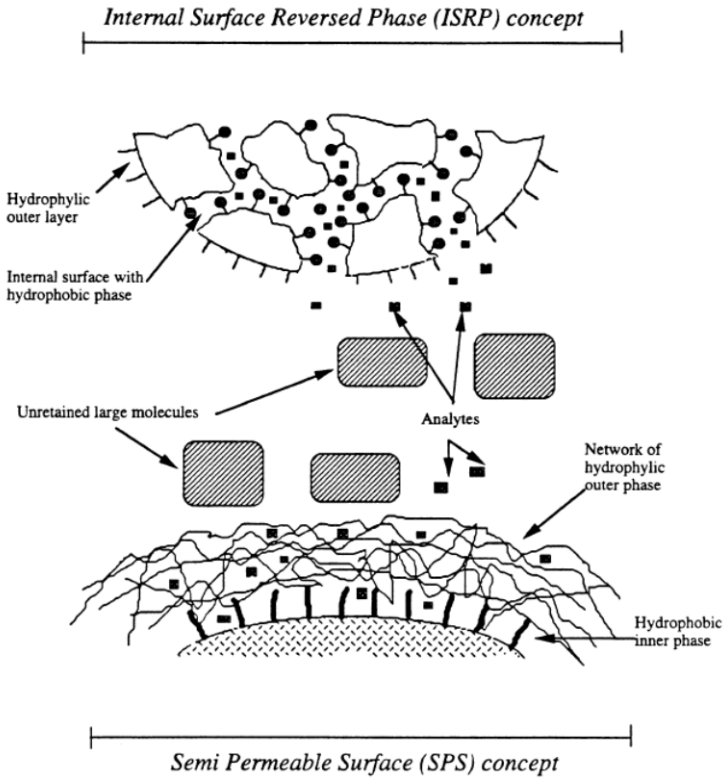

Figure 1.1 Schematic representation of the separation mechanism of RAMs (Poole, 2003).

In recent years, research on new SPE materials has focused on the development of new hydrophilic polymeric materials for polar analytes. The hydrophilic sorbents can be prepared by co-polymerizing monomers that contain suitable functional groups or by chemically modifying the styrene-divinylbenzene (St-DVB) hydrophobic polymers with a polar moiety. The polarity of the sorbent surface and a large surface area allow a greater number of interactions (π-π and polar) with the analytes, thus leading to higher recoveries. They have been used in the field of environmental and biological fluid (Fontanals *et al*., 2005; Fontanals *et al*., 2007).

Other interesting sorbents are the mixed-mode ion-exchange sorbents. The sorbents have a combination of the ion-exchange and reversed-phase functional moieties on one resin, thus are able to produce a mixed mechanism of interaction, i.e., by hydrophobic and ionic interactions. The strong retention of analytes by

ion-exchanger and the use of efficient rinse solvents will naturally result in cleaner extracts compared with the single-mode sorbents. These type of sorbents can thus be applied mainly to the extraction of acidic, neutral and basic pharmaceuticals, pollutants and many other types of analytes such as from food (Rosales-Conrado *et al*., 2005), biological fluids (Huq *et al*., 2005) and wastewater (Benito-Peña *et al*., 2006). Alternative sorbents for the extraction of polar compounds such as multi-walled carbon nanotubes (Fang *et al*., 2006) and mesoporous silica based modified with β-cyclodextrin were also reported (Liu *et al*., 2004).

1.2.3. Matrix solid phase dispersion (MSPD)

MSPD was introduced in 1989 and was later patented in 1993 (Barker, 2007). MSPD is primarily used because of its flexibility, short extraction times, requiring small amounts of sorbent and solvent, and consequently low costs. It offers the possibility of simultaneously performing extraction and clean-up in one step. MSPD is capable of preparing, extracting and fractionating solid, semi-solid and viscous samples (Chen *et al*., 2008). MSPD has been frequently applied to the isolation of drugs, herbicides, pesticides and other pollutants from animal tissues, fruits and vegetables (Barker, 2000).

It operates by blending a sample with a solid support to simultaneously disrupt and disperse the desired components on a solid support which is commonly a silica-based material (e.g., derivatized silica, silica gel, sand and florisil). The blended mixture is then packed into a column and a sequential elution is conducted with solvents to collect the analytes by fractionation. This process is showed in Figure 1.2. (Barker, 2007). Hot water was proven to be a fast and efficient eluting solvent for various biological matrices (Bogialli *et al*., 2005). The MSPD may be directly used for

further instrumental analysis, but additional (co-column or external column) SPE is suggested to remove the co-eluted interferences or to clean-up the analytes by further fractionation (Ramos *et al*., 2004). The principles and basic procedure of MSPD have been described in several reviews (Barker, 2000; Kristenson *et al*., 2006; Barker, 2007).

This method is however fairly labour intensive and time consuming for a large number of samples. So far no papers reported on the on-line coupling of MSPD to LC or GC units (Kristenson, 2006). The continuing improvement and development of new supports and bonded-phases and the potential of miniaturization and direct coupling or automation with other techniques will make MSPD more useful in the near future.

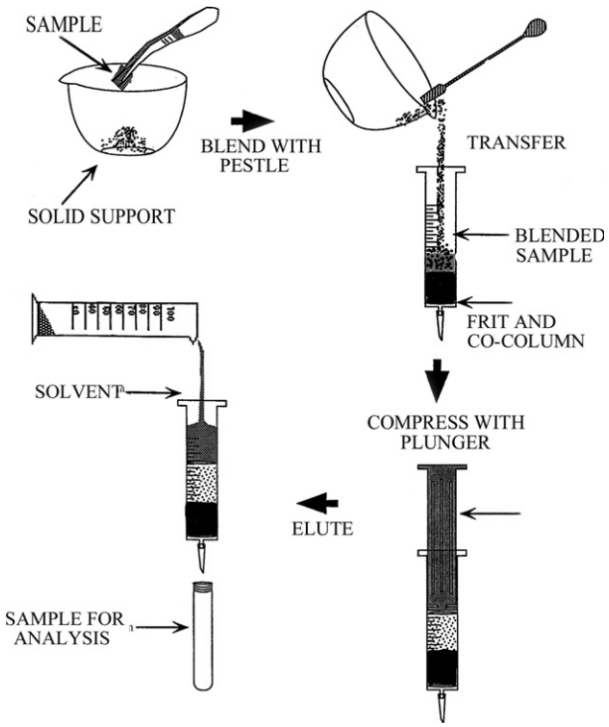

Figure 1.2 Step in a typical MSPD extraction process (Barker, 2007).

1.2.4. Solid phase microextraction (SPME)

SPME is a miniaturized version of sample preparation introduced by Pawliszyn and co-workers in 1990. SPME is easy to operate and automate, and use minimum amounts of solvent. A polymer coated on a fused silica fiber (which fits inside the needle of a syringe-like SPME holder) is used as an extraction device, and the extracted analytes can be directly analyzed by GC, GC-MS, HPLC, LC-MS and CE (Figure 1.3) (Theodoridis *et al*., 2000; Kataoka, 2002). The SPME technique enables the simultaneous extraction and pre-concentration of analytes from gaseous, liquid and solid samples. It is highly sensitive and can be used for polar and non-polar analytes with different types of matrices. SPME has been widely used for the determination of analytes in clinical, pharmaceutical, biological, environmental and food samples (Kataoka, 2005; Nerín *et al*., 2009). SPME can also be applied to the determination of inorganic analytes (Malik *et al*., 2006; Díez and Bayona, 2008). The details of SPME developments and applications have been summarized in several recent reviews (Theodoridis *et al*., 2000; Kataoka, 2000; Kataoka, 2002; Zambonin, 2003; Kataoka, 2005; Lambropoulou *et al*., 2007) and book (Pawliszyn, 1999).

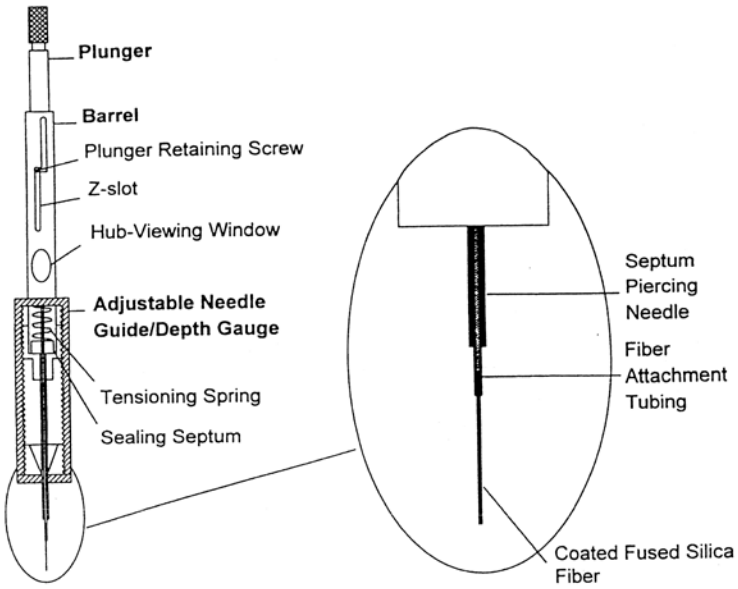

Figure 1.3 Design of the SPME device (Theodoridis *et al*., 2000).

SPME fibers are available in different film thicknesses with single or combined coatings or co-polymers (Theodoridis *et al*., 2000). Recent advances in new coatings are focused on sol-gel technology. Kumar *et al*. (2008) reviewed the development on SPME fibers by sol-gel methods and its applications to different analytes (PAHs, aromatic amines, phenols and pesticides). Additionally, monolithic sorbents with different functional groups (Zhang *et al*., 2006), MIPs (Hu *et al*., 2008), polypyrrole polymer (Wu and Pawliszyn, 2001) and immunoaffinity-based coating (Eugênia *et al*., 2007) have shown promising results.

Despite the advancements in the development of new sorbent coatings, derivatization of polar analytes is still important, because it enables their hydrophobicity and thermal stability to be increased. Recently, Stalikas and Fiamegos (2008) has elaborated derivatization strategies in SPME that can be carried out in three different modes; direct derivatization in the sample matrix (before SPME), derivatization in the injection port and on-fiber derivatization after and/or during SPME.

The small dimension and almost solvent-free feature of SPME enables *in vivo* sampling without severe damage to live organisms. The reported *in vivo* methods include monitoring the biogenic volatile organic compounds emitted from plants, isolating the insect semiochemicals and other microbiological inspections (Augusto and Valente, 2002). Direct extraction from flowing blood (Lord *et al*., 2003) and sampling of volatiles emitted by humans (Zhang *et al*., 2005) and insects (Djozan *et al*., 2005) have also been achieved.

Miniaturized cool coated fiber SPME device with carbon dioxide cooling has been developed (Figure 1.4) (Chen and Pawliszyn, 2006). The technology enables the

heating of a sample while maintaining the fiber coating at relatively low temperatures. The application of this system was particularly useful for the extraction of solid samples and volatiles components, such as fragrance in foodstuffs (Chen *et al*., 2007; Carasek and Pawliszyn, 2006) and environmental samples such as PAHs (Chen and Pawliszyn, 2006). In both cases the use of a cold SPME device resulted in much higher sensitivities compared to ordinary fibers.

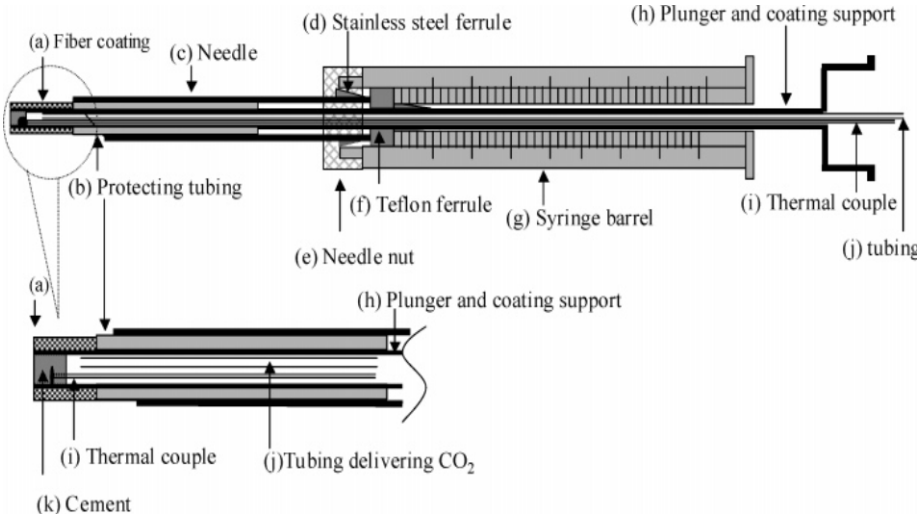

Figure 1.4 The internally cooled SPME device (Chen and Pawliszyn, 2006).

1.2.5. Stir bar sorptive extraction (SBSE)

SBSE is an extraction technique that was developed by Baltussen *et al*. in 1999 to overcome the limited extraction capacity of SPME fibers (Baltussen *et al*., 1999). The technique utilizes stir bars (0.3-1.0 mm) that had been coated with polydimethylsiloxane (PDMS). The amount of PDMS coated is 25-125 µL, which is substantially higher than that on a SPME fiber with a maximum volume of 0.5 µL. The phase ratio of SBSE is about 50-250 times larger than SPME, resulting in lower limit of detection (LOD) of 0.1 ng L^{-1} and much higher recoveries and preconcentration capacities (Kawaguchi *et al*., 2006a). Analytes are sampled by

introducing the PDMS stir bar directly into the liquid sample and rotated or suspended in the gaseous (headspace) matrices for a fixed time to perform the extraction. After sampling, the isolated molecules on the stir bar are desorbed either thermally for GC or into an aqueous solution for LC analysis (Bicchi *et al*., 2005). Instrumentation capable of automating the thermal desorption of the stir bars into a GC has become commercially available (De Jager *et al*., 2009). SBSE has been successfully applied for the enrichment of organic compounds in environmental (Sanchez-Ortega *et al*., 2009), foods (De Jager *et al*., 2009) and biological fluids (Melo *et al*., 2009).

The disadvantages of SBSE is that the operation is in most cases manual and coating of the stir bar can be damaged during the high-speed stirring process (Chen *et al*., 2008). A low stirring speed could protect the coating, but the equilibrium time and extraction efficiency would be affected. Due to the non-polar character of PDMS, therefore, SBSE has been mainly applied to extract non-polar and weakly polar compounds. To overcome this limitation, several authors have proposed new strategies, such as *in situ* derivatization (Kawaguchi *et al*., 2008a), the dual-phase stir bar involving PDMS combined with specific adsorbents (e.g. activated carbons) (Bicchi *et al*., 2005), PDMS/β-cyclodextrin based on sol-gel technique (Hu *et al*., 2007), as well as a glass fiber strip coated with polyacrylate (Rodil, 2007) to recover compounds with higher polarity. A novel polymeric phase based on polyurethane foams proved to be useful for the enrichment of the more polar analytes in aqueous media has also been introduced (Portugal *et al*., 2008).

Recently, a new coating based on vinylpyrrolidone and divinylbenzene monolithic materials for SBSE was prepared. The coating not only could directly concentrate

non-polar and polar organic compounds in environmental samples effectively without derivatization, but can also extract heavy metal ions (e.g., Cu^{2+}, Pb^{2+}, Cr^{3+} and Cd^{2+}) through coordination reaction between nitrogen and oxygen donor atoms in the coating and metal ions (Huang *et al*., 2009). The application of SBSE to metal ions and the further development of new coating and design for other trace analysis in different matrices open interesting possibilities that will extend the applicability of SBSE in the future.

1.2.6. Single drop microextraction (SDME)

SDME technique was first introduced by Liu and Dasgupta in 1996 (Liu and Dasgupta, 1996). It involves the use of a single liquid drop (typically 1-3 µL) suspended from the tip of a microsyringe needle. The drop is exposed to the sample for a given time, then retracted into the syringe and transferred to the analytical instrument (Xu *et al*., 2007). Figure 1.5 shows the basic configurations of the SDME sampling. It is an elegant method to overcome the limited availability of fiber coatings, as a wide variety of organic solvents and trapping agents can be used and do not suffer from carryover between extractions and fiber degradation that may be experienced using SPME. SDME uses simple and inexpensive apparatus, minimizes solvent consuming and it combines extraction, pre-concentration and sample introduction in one step (Xu *et al*., 2007). SDME has been used for the determination of organophosphorous pesticides in water (Ahmadi *et al*., 2006) and food (Zhao *et al*., 2006), and carbonyl compounds in biological samples (Li *et al*., 2005). Fundamental information and details on SDME configurations have been reviewed (Psillakis and Kalogerakis, 2002; Xu *et al*., 2007).

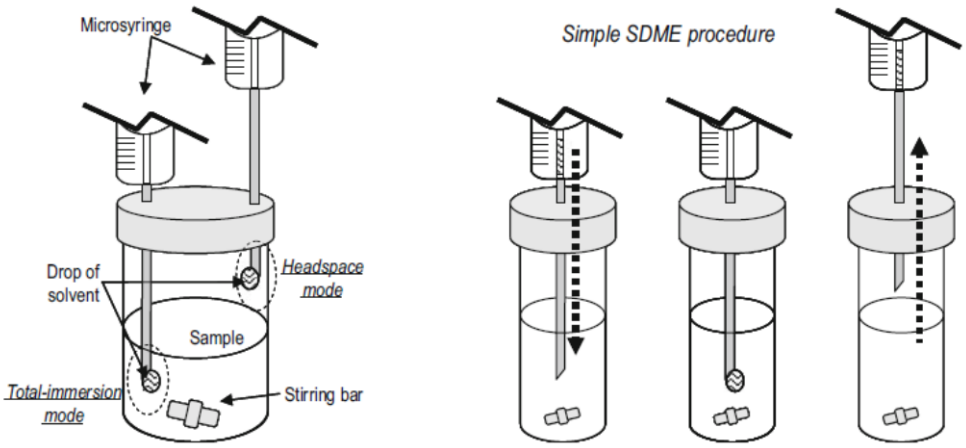

Figure 1.5 Basic configurations for SDME sampling and stages of the extraction (Nerín *et al*., 2009).

The main problem of SDME lies with the adverse consequences of prolonged extraction time and high stirring rate, since they may result in dislodgment of the drop. Formation of air bubbles prevent it to be applied for on-line pre-concentration procedures. Although some progress has been made to automate SDME, cost considerations prevents the approach from being widely accepted (Xu *et al*., 2007). In addition, an extra filtration step is usually needed for the sample solutions with complex matrices, and its sensitivity and the precision still need further improvements (Psillakis and Kalogerakis, 2002; Xu *et al*., 2007).

1.2.7. Dispersive liquid-liquid microextraction (DLLME)

DLLME was first introduced by Assadi *et al*. in 2006 (Rezaee *et al*., 2006). This method is based on a ternary component solvent system in which the extraction solvent and disperser solvent are rapidly injected into the aqueous sample by a syringe. The mixture is then gently shaken and a cloudy solution (water/disperser solvent/extraction solvent) was formed. After centrifugation, the extractive solvent which accumulates at the bottom of the extraction vessel is sampled by a microsyringe and injected into the chromatographic or spectrometric systems

(Xiau-Huan *et al*., 2009). The extraction steps of DLLME are illustrated in Figure 1.6. The advantages of DLLME are the relative simplicity of operation, rapid, high recoveries and enrichment factors.

This technique has been used for trace analysis of organic (e.g., anilines and chlorobenzenes) (Kozani *et al*., 2007; Chiang and Huang, 2008) and inorganic analytes (e.g., palladium and cobalt) (Shokoufi *et al*., 2007) in water samples. Zhao *et al*. (2007) developed a new method for the determination of organophosphorus pesticides in watermelon and cucumber by using DLLME. Another interesting development of DLLME was introduced by Sobhi *et al*. (2008). In this contribution, a small volume (μL) of a suitable organic solvent with a melting point near room temperature is placed on the surface of an aqueous solution and is stirred for a selected time at a chosen temperature. The sample vial is then transferred into an ice bath where the organic solvent solidifies; the solvent is then transferred into a suitable vial and injected into a suitable analysis system. This approach has recently been applied to the analysis of organochlorine pesticides in water (Farahani *et al*., 2008) and mercury in aqueous samples (Baghdadi and Shemirani, 2008). So far, most of the reported extraction solvents are halogenated hydrocarbons. A more extensive range of extraction solvents will extend the range of applicable substrates pre-concentrated by the DLLME technique (Xiao-Huan *et al*., 2009).

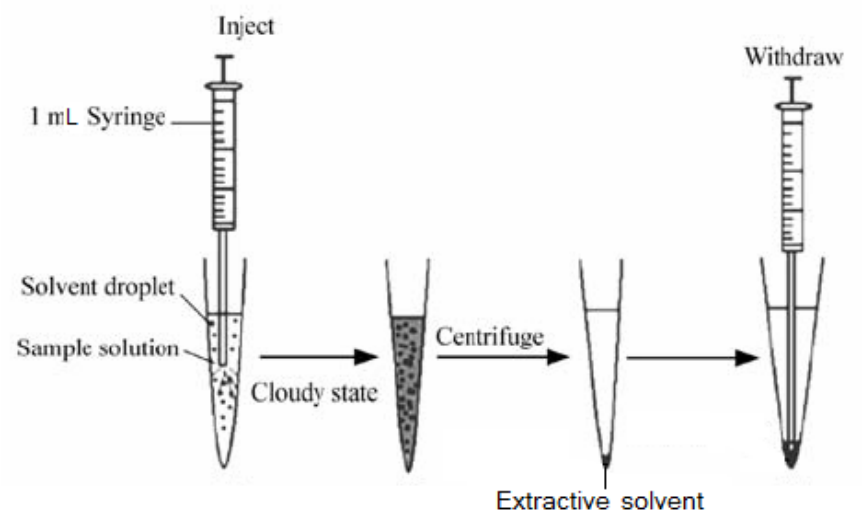

Figure 1.6 DLLME extraction steps (Xiau-Huan *et al*., 2009).

1.2.8. Liquid phase microextraction (LPME)

LPME technique is based on the use of a single, disposable and porous hollow fiber (typically made of polypropylene) and was introduced in 1999 (Pedersen-Bjergaard and Rasmussen, 1999). This technique proved to be extremely simple to use, low-cost and is a virtually solvent-free sample preparation technique. It uses only a minute volume (2-30 µL) of solvent for concentrating analytes from large aqueous samples (ranging between 50 µL and more than 1 L). As a result, the high sample-to-acceptor volume ratio ensures very high analyte enrichment to be obtained (Pedersen-Bjergaard and Rasmussen, 2008).

In a typical LPME set-up, a water immiscible organic solvent is immobilized as a thin supported liquid membrane (SLM) in the pores of the wall of a porous hollow fiber. This is easily accomplished by dipping the hollow fiber for a few seconds in the organic solvent, which immediately flows into the pores by capillary forces. The lumen of the hollow fiber is subsequently filled with a microliter volume of an acceptor solution, and the whole assembly is placed in a sample solution to extract

the target analytes. The analytes are extracted from the sample (aqueous), through the SLM (organic) and into the acceptor solution (aqueous or organic) in the lumen of the hollow fiber. After the extraction, the acceptor solution is directly subjected to a final chemical analysis (e.g., HPLC, GC or CE). The acceptor solution can be an organic solvent providing a two-phase extraction system, which is directly compatible with GC. Alternatively, the acceptor solution can be an aqueous solution providing a three-phase extraction system, which is compatible with HPLC or CE (Figure 1.7) (Pedersen-Bjergaard and Rasmussen, 2008; Rodríguez *et al*., 2008). LPME has been applied successfully for the extraction and clean-up of a wide range of organic and inorganic analytes in environmental, food and biomedical applications (Pedersen-Bjergaard and Rasmussen, 2008).

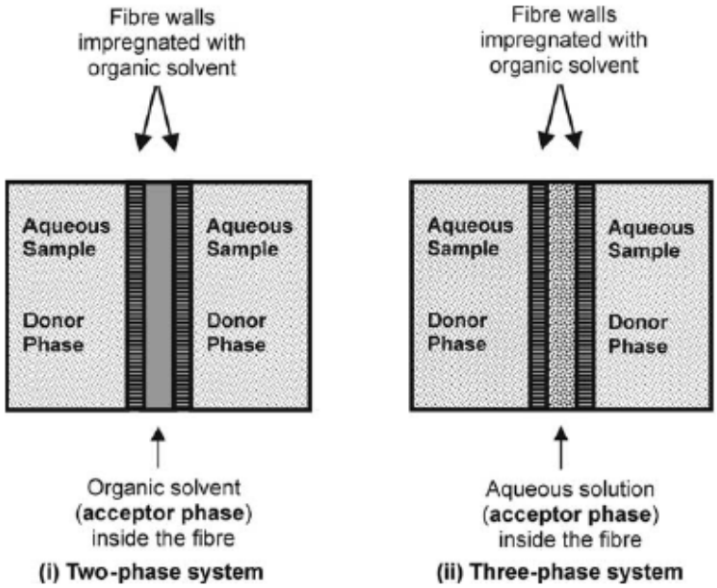

Figure 1.7 Schematic illustration of the two- and three-phase LPME technique (Psillakis and Kalogerakis, 2003).

In the hollow fiber LPME device, the acceptor solution is placed in the lumen of the fiber, was mechanically protected inside the hollow fiber and it was separated from the sample by the SLM (organic solvent) (Figure 1.8). This prevented dissolution of

the extracting phase (acceptor solution) into the sample. Moreover, the hollow fiber enables vigorous stirring and agitation without loss of the extractant phase (as in SDME) and overcomes the many disadvantages of LLE as well as the SPME techniques (e.g., sample carryover between runs) (Psillakis and Kalogerakis, 2003; Rasmussen and Pedersen-Bjergaard, 2004). Another additional advantage is that extraction over a wide range of pH can be carried out when SPE is not suitable (Richoll and Colón, 2006). Moreover, the small pore size of the hollow fiber allows microfiltration of the sample, preventing the extraction of large molecules and particles and thus yielding very clean extracts.

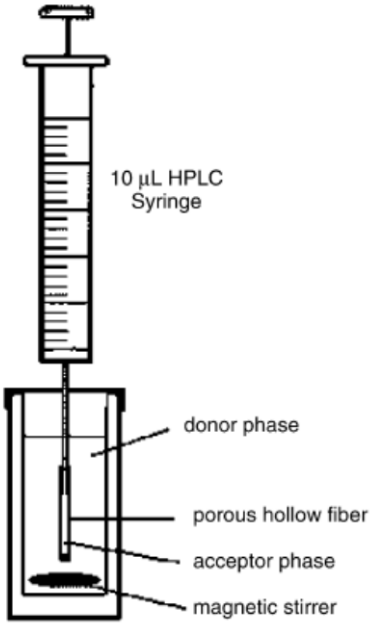

Figure 1.8 Schematic of the LPME device (Psillakis and Kalogerakis, 2003).

A variation of the hollow fiber is the solvent bar microextraction approach (Figure 1.9). In this technique, Jiang and Lee (2004) sealed an organic solvent within a short length of hollow fiber membrane and was used for the extraction of analytes from the stirred aqueous solution. Another variation is dynamic hollow fiber supported headspace extraction. Jiang *et al*. (2005) reported affixing the hollow fiber membrane

to a syringe needle to sample the gaseous headspace for polycyclic aromatic hydrocarbons in soil.

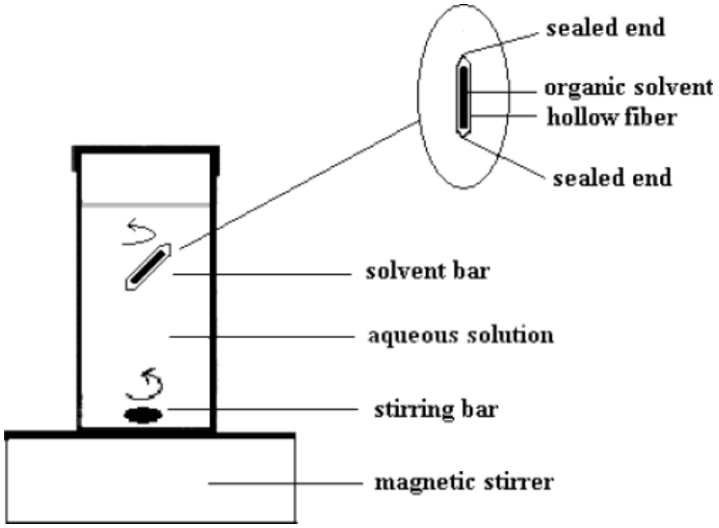

Figure 1.9 Set-up of solvent bar microextraction (Jiang and Lee, 2004).

While enrichment, clean-up and low solvent consumption are the major advantages of the LPME technique, relatively long extraction times is a disadvantage of this technique. Normally, extraction time ranging from 15-60 min may be required to reach equilibrium (Pedersen-Bjergaard and Rasmusssen, 2008). Recently, the kinetics of LPME was improved by application of an electrical potential difference over the SLM. This technique was termed as the electro membrane extraction (EME). The set-up for EME was exactly the same as for the LPME, except for the addition of two electrodes and a power supply (voltage in the range 0-300V) (Figure 1.10). The major benefits of EME were the significant reduction in extraction times (typically 5-10 min per extraction) as compared to the LPME. This technique has been used for the extraction of drugs in biological samples (Gjelstad *et al*., 2006; Kjelsen *et al*., 2008).