OPTIMISATION AND VALIDATION OF A NEW MICROEXTRACTION METHOD EMPLOYING SURFACTANT FOR THE DETERMINATION OF SELECTED POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN SUGARCANE SAMPLES

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by

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CERTIFICATE

This is to certify that the dissertation entitled *"Optimisation and validation of a new microextraction method employing surfactant for the determination of selected Polycyclic Aromatic Hydrocarons (PAHs) in sugarcane samples"* is the bonafide record of research work done by Ms Siti Farahiyah Binti Ahmad Shater during the period February 2015 to January 2016 under my supervision.

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LIST OF ABBREVIATIONS

AF-LPME	Agarose-film liquid phase microextraction
AMDI	Advanced Medical and Dental Institute
CE	Capillary electrophoresis
CMC	Critical micelle concentration
CPE	Cloud point extraction
DLLME	Dispersive liquid-liquid microextraction
DLLME-SFO	Dispersive liquid-liquid micreoextraction-solidification of floating organic solvent
D-LPME	Dynamic-liquid phase microextraction
FA-HLLME	Floatation-assisted homogenous liquid-liquid microextraction
FLU	Fluoranthene
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
HLB	Hydrophilic- lipophilic balance
LLE	Liquid-liquid extraction
LODs	Limit of detection
LOQs	Limit of quantification
LPME	Liquid-phase microextraction
MIPs	Molecular imprinted polymer
NaCl	Sodium chloride
PAHs	Polycyclic aromatic hydrocarbon
PHE	Phenanthrene
RSDs	Relative standard deviation

S-LPME	Static-liquid phase microextraction
SPE	Solid phase extraction
UAME	Ultrasound assisted microextraction
UASEME	Ultrasound assisted surfactant enhanced emulsification microextraction
USM	Universiti Sains Malaysia

LIST OF SYMBOLS

%	Percent
˚C	Degree celcius
µgmL-1	Microgram per mililiter
µg L-1	Microgram per liter
µL	Microliter
Mg	Milligram
Min	Minute
mL	Mililiter
Mm	Milimeter
N	Number of replication
Rpm	Rotation per minute
S	Second
v/v	Volume per volume
w/v	Weight per volume
ppm	Part per million
ppb	Part per billion
R	Coefficient of determination

ABSTRACT

In the present study, a microextraction method termed ultrasound-assisted surfactant enhanced emulsification microextraction (UASEME) has been developed for the determination of phenanthrene (PHE) and fluoranthene (FLU) in sugarcane samples. Optimised extractions were obtained using the following conditions: extraction solvent, toluene; volume of extraction solvent, 30 µL; surfactant, Tween 20; volume of surfactant, 15 µL; extraction time; 2 min and with no salt addition. Under the optimized conditions, the method demonstrated good linearity ($r^2 \geq 0.9932$) over a concentration range of 1 to 1000 µg L^{-1}. The method showed limit of detections (LODs) and limit of quantification (LOQs) for both analytes were 0.30µg L^{-1} and 1.0 µg L^{-1}, respectively. Good reproducibility with relative standard deviations (RSDs) in the range of 1.62 – 10.32 % (n = 3) and satisfactory recoveries (91.75 - 104.1%) was obtained for spiked polycyclic aromatic hydrocarbons compounds in sugarcane juice samples. The proposed method has been successfully applied to nine sugarcane samples and the blank extraction samples indicated that all samples were free from FLU but the samples were contaminated with PHE at below the LOQ concentration. The proposed UASEME method proved to be simple, rapid, environmentally friendly and suitable for the determination of PHE and FLU in sugarcane samples.

ABSTRAK

Dalam kajian ini, kaedah pengekstrakan yang diistilahkan sebagai pengekstrakan mikro ultrabunyi dibantu pengemulsi (UASEME) telah dibangunkan bagi penentuan fenantherena (PHE) dan fluoranthena (FLU) di dalam sampel air tebu. Pengekstrakan optimum diperoleh menggunakan keadaan seperti berikut: pelarut pengekstrakan, toluene; isipadu pelarut pengekstrakan, 30 µL; pengemulsi, Tween 20; isipadu pengemulsi, 15 µL; masa pengekstrakan, 2 min dan tiada penambahan garam. Dalam keadaan optimum, kaedah ini menunjukkan kelinearan yang baik (r^2> 0.9932) dalam julat kepekatan 1 hingga 1000 µg L^{-1}. Kaedah ini menunjukkan had pengesanan (LODs) dan had kuantifikasi (LOQs) bagi kedua-dua analit ialah 0.3µg L^{-1} dan 1.0 µg L^{-1}, masing-masing. Kebolehulangan yang baik dengan nilai sisihan piawai relatif dalam julat 1.62 – 10.32 % (n = 3) dan pengembalian (91.75 - 104.1%) yang memuaskan diperoleh bagi sebatian hidrokarbon aromatik polisiklik yang ditambah di dalam sampel air tebu. Kaedah UASEME yang dicadangkan ini telah berjaya diaplikasikan kepada sembilan sampel air tebu dan pengekstrakan sampel kosong menunjukkan semua sampel tidak mengandungi FLU, tetapi semua sampel mengandungi PHE pada kepekatan di bawah nilai LOQ. Kaedeh UASEME yang dicadangkan ini terbukti sebagai kaedah yang ringkas, cepat, mesra alam dan sesuai digunakan bagi penentuan PHE dan FLU di dalam sampel air tebu.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Incomplete combustion of organic materials is the primary source of polycyclic aromatic hydrocarbons (PAHs).Concentration of this particular compound may increase tremendously due to unvented heating sources, while tobacco smoking also be one of the contributors to ambient air level arise. However, the main sources of PAHs exposure to human are from food and drinking water. This is because, this compounds were formed during cooking or atmospheric deposition on vegetables, grains and also fruits (WHO, 2000). The presence of these compounds in food has been proved to trigger the growth of cancer cell in human. Therefore, US Environmental Protection Agency (EPA) had included these compounds in carcinogenic and mutagenic group. Several studies conducted using animal as a subject indicated that, some of the compounds in PAHs group are able to trigger a number of adverse effect such as toxicity of reproductive system, toxicity to immune system and induce cancer cells (WHO, 2000).

Air, water, soil and food sources are the contributors to the exposure of PAHs to human living. The exposure of this compound could be through ingestion, inhalation and skin contact either during occupational setting or non-occupational setting. Although human can expose to PAHs through inhalation, the volatility characteristic may influence the mobility of PAHs in environment (European Commission, 2002). If the source of PAHs for a smoker is from ciggarate smoke, then the non-smoking person are exposed through diet. Many studies have shown that carcinogenic PAHs are present in food (Farhadian *et al.*, 2010; Chen & Chen, 2005; Mottier *et al.*, 2000). Aaslyng *et al.* (2013) also stated that food is the main source for human exposure to PAHs compound. However, the concentrations of PAHs vary according to types of foodstuffs. Usually, contamination of food by

PAHs is from processing procedure and cooking method such as grilling, and barbequing. Therefore, it is vital to develop a new method that is easy, precise, efficient and reliable for detection of PAHs compounds in food and beverages such as sugarcane products. This method can be applied in detecting that particular compound in order to avoid negative health effect to the consumer.

Numerous established analytical methods have been reported for the detection of PAHs including the application of fluorescence spectrophotometry (Krupadam *et al.,* 2009), capillary electrophoretic method (Terabe *et al.,* 1984), gas chromatography (Zhang *et al.,* 2014) and high performance liquid chromatography (Mao *et al.,* 2012). Gas chromatography and high performance liquid chromatography combined with spectrofluorometric detector are the most widespread techniques that were used for PAHs analysis (Purcaro *et al.,* 2007). Although the chromatography method is the most popular technique, the analysis of trace compound like PAHs need extensive sample pretreatment due to very low concentration in sample and also the matrix effect of the sample. With the main objectives to preconcentrate the target anaytes and to reduce the interference caused from the complicated matrix sample, a new sample pretreatment techniques are required before proceeding to final analysis (Mao *et la.*, 2012).

Liquid-liquid extraction (LLE) (Caruso and Alaburda, 2009), and solid phase extraction (SPE) (Caruso and Alaburda, 2009; Da Porto and Moret, 2007) are example of conventional sample preparations that have been carried out for determination of PAHs. However, these classical pre-concentration method involved several phase operations with consumption of large volume of toxic organic solvent that lead to environment-unfriendly (Hosseini e*t al.,* 2012). Researchers have been devoted to the development of microextraction method to solve these problems as it is environmental friendly and reduced usage of organic solvent. Several microextraction mehods have been developed for the determination of PAHs compounds include liquid phase microextraction (LPME) (Zanjani *et al.,* 2007), static- liquid phase microextraction (S-LPME) (He and Lee, 1997), dispersive liquid-liquid microextraction (DLLME) (Rezaee *et al.,* 2006), and ultrasound assisted emulsification

microextraction (UAME) (Cacho *et al.,* 2016). Although there are various established analytical methods nowadays, there is still limited access of microextraction method for the analysis PAHs from sugarcane samples.

Development of Ultrasonic-Assisted Emulsification Microextraction (UAEM) was done by Rugeiro and his group in 2008 (Rugueiro *et al.,* 2008). In this method, extraction solvent will disperse into an aqueous solution with the aid of ultrasound –assisted emulsification without the use of dispersive solvent (Cheng *et al.,* 2011). Ultrasound radiation was proved to be an efficient tool in the formation of emulsification phase and accelerate the process of mass transfer between two immiscible (Fontana *et al.,* 2009). The improvement of the above method was successfully done by Wu et al. (2010). They introduce surfactants as emulsifier in the UAEM method for detection of selected carbamate compounds in water samples. This new method was greatly shortened the time of extraction needed due to the effective combination of surfactant and ultrasound radiation (Cheng *et al.,* 2011). Surfactants also known as surface active agent that having both hydrophobic head and hydrophilic tail. This special characteristic give extra advantages to react both in water and immiscible phase. Other than that, it also can act as emulsifier which is having the ability to catalyse the dispersion of water-immiscible phase into the aqueous phase. Therefore, this Ultrasound-Assisted Surfactant Enhanced Microextraction (UASEME) methods was successfully integrate the advantages of DLLME and UAME methods.

In this study, a new microextraction method termed UASEME incorporated with Tween 20 has been carried out prior to GC-MS determination of selected PAHs from sugarcane samples. Six parameters have been optimised include types of extraction solvents, types of surfactants, volume of extraction solvents, volume of surfactants, salt addition and extraction time. Based on the result, the purposed method is highly recommended the analysis of selected PAHs in sugarcane matrices.

1.2 Background of the Problem

In Malaysia, agriculture sector covers up to 11.1% of the work force (World Fact Book, 2014). It was estimated that the gross domestic product from agriculture sector is 11.4% (World Fact Book, 2014). Although sugarcane was not the largest agriculture industry in Malaysia, but it has been listed in top ten of crop production in Malaysia by Agriculture and Agri-food Canada (2014).

Sugarcane is a giant grass (*Poacae*) from species *Saccharum officinarum L*. Most of the sugarcane plantation owners prefer to burn their plantation area during harvesting season. Protection of workers from sharp leaves, insect bites, poisonous snakes, and also facilitate manual harvest are among the reason behind this action (Godoi *et al.,* 2004; Zamperlini *et al.,* 2000). Other than that, burning their sugarcane plantation is one of the strategies to rise up the sugar content by weight due to evaporation of water (Zamperlini *et al.,* 2000). However, there is an adverse side effect from the burning process. The burned sugarcane contained PAHs compound and the presence of this compound fromed the burning process (Azevedo *et al.,* 2002; Godoi *et al.,* 2004; Serra *et al.,* 1995). Studies conducted by Bosso (2004) shows that excretion of PAHs through urine during harvesting season are nine times higher compared to the rural workers and the control group (Bosso, 2004).

Due to the major drawbacks of conventional extraction methods for detection of the PAHs in very low concentration levels, new development of sample preparation methods were focusing on simplicity, time saving, environmental friendly and efficient. In this study, new approach of the sample preparation method known as UASEME incorporated with Tween 20 as surfactant was established for the analysis of selected PAHs compounds in sugarcane drink samples combined with GC-MS determination. The proposed UASEME method can be used as an alternative approach for environmental- friendly microextraction of PAHs in sugarcane samples as it requires small amounts of organic solvent and surfactant in each analysis.

1.3 Research Objectives

The general objective of this research is to develop a simple, efficient, fast and environmental friendly ultrasound assisted surfactant enhance emulsification microextraction (UASEME) combined with gas chromatography-mass spectrometry (GC-MS) for detection of selected polycyclic aromatic hydrocarbon (PAHs) in sugarcane samples.

Specific objectives:

- To optimize GC-MS conditions for separation of selected PAHs compounds, namely, phenanthrene and fluoranthene.
- To develop and validate UASEME method using Tween 20 as the selected surfactant.
- To explore the application of the developed UASEME method to the analysis of selected PAHs in several sugarcane samples collected from local store in Kedah and Penang, Malaysia.

1.4 Scopes of the Study

This study specialise on the application of UASEME method using Tween 20 as surfactant. The determination of selected PAHs compounds in sugarcane samples were done by using GC-MS. Optimisation of six parameters were conducted and these optimisation conditions were applied during the extraction testing. HP-5MS capillary column GC-MS had been used throughout this experiment for separation and quantification of selected PAHs compounds.

1.5 Significance of the Study

The main advantage of UASEME is the extraction time is shorten as compare to other methods such as USAEME and DLLME (Cheng *et al.*, 2011). This method also offers high efficiency when comparing with USAEME and DLLME for detection of fungicides in water samples (Cheng *et al.*, 2011). Aside from the stated advantages, this method applied the usage of surfactant that is more

environmental friendly as compare to disperser solvent that widely used in conventional DLLME. Surfactant has a few similarities with the disperser solvent such as, it has ability to dissolve in both aqueous and organic phases, and reducing the interfacial tension between two immiscible phases. This surfactant may help in term of decreasing the surface tension of water by adsorbing at the liquid-gas interface (Moradi *et al.*, 2010). Other than that, enrichment factor may increase the yield of analyte in low volume of solvent.

CHAPTER 2

LITERATURE REVIEW

2.1 Polycyclic Aromatic Hydrocarbons

One of the large groups of organic compounds that made up of one to six benzene rings fused together is polycyclic aromatic hydrocarbon. Basically, PAHs are divided into two groups which is small and large. Small PAHs composed up to six fused aromatic rings and large PAHs composed of more than six aromatic rings. They are highly lipophilic and relatively low solubility in water (hydrophobic). Most of these compounds are absorbable by environmental particles aided by their low vapour characteristic can be adsorbed on particles in environment due to their low vapour pressure characteristic in the air. Pyrolytic process including incomplete combustion of organic substances and human activities such as smoking, and cooking are a few generator for the formation of PAHs (Lee *et al.*, 1981; WHO, 2000).

2.1.1 Routes of Exposure

Human can be exposed to PAHs through air, water, soil and food sources. There are two ways of human exposure to PAHs which are during occupational setting or non-occupational setting. Ingestion, inhalation and skin contact are three routes that exist in both setting.

2.1.1.1 Air

The concentrations of PAHs in air varies from 5 to 200,000 nanograms/ cubic meter (ng/m^3) (Georgiadis and Kyrtopoulos, 1999). The exposure of human through environmental air level are lower as compared to those involved with specific occupational exposures. However, when it spreads over large urban population, it is considered as public health concern (Zmirou *et al.*, 2000). Smoking

and environmental smoke are considered as the common source of human exposure of PAHs through air. A previous study revealed that one cigarette can produce almost 20-40 ng of benzo (a) pyrene (ATSDR, 2000). Other study also compared unfiltered cigarettes and filtered cigarettes. A pack of unfiltered cigarettes yield 0.7 µg/ day of benzo (a) pyrene which is higher than filtered cigarettes; 0.4 µg/ day (Sullivan and Krieger, 2001).

2.1.1.2 Water and Soil

PAHs can enter the aquatic environment through leaching from soil into water. Sewage discharge from industrial and accidental spills during transportation of oil at sea can lead to contamination of aquatic ecosystem. The concentration of one of the PAHs compound in drinking water is extremely lower when comparing with those in untreated water (U.S Environmental Protection Agency's (EPA)). However the levels of PAHs will tremendously increase when asphalt or coal tar was applied in storage tank coating and water distribution pipes (European Commission, 2002).

The presence of PAHs in groundwater due to leaching process is limited although this compound is adsorbed strongly to the organic fraction of soils. This is because, the compound cannot penetrate deeply into most of the soils. Many plants would not have easy access of PAHs from soil. However, measurable amounts of PAHs were found in soil, primarily from airborne fallout. As stated by IARC, the level of PAHs compound in samples taken from cities and congested areas were commonly below than 2,000 µg/ kg (IARC, 1973).

2.1.1.3 Foodstuffs

Exposure of non-smoking individuals to PAHs is through the diet. A study carried out in 2004 showed that up to 70% of PAHs exposure for non-smokers were from diet (Skupinska *et al.*, 2004). Aaslyng *et al.* (2013) also stated that food is one of the main sources of exposure to PAHs. The concentration of PAHs is dependent on foodstuffs. Processing procedures and cooking are the primary sources of

contamination by PAH. Several parameters such as duration of cooking, uses of fuel, heat source distance, drainage of fat, and cooking type may give various concentration levels of PAHs (European Commission, 2002).

A comparison study for the levels of PAHs in duck breast steaks, which go through several type of processing and cooking treatments for selected duration of cooking time (0.5 hour to 1.5 hours) showed that, the highest total amount of PAHs were reported from a samples without skin and undergoing charcoal grilled cooking treatment. The least amount of PAHs were from the liquid smoke flavouring sample (Chen and Lin, 1997). Lorenzo et al., (2010) and Lorenzo (2011) have scientifically proved that many carcinogenic PAHs in food were formed through the smoking of foods. Other than, PAHs also exists in natural food such as vegetables oil that undergo refining treatment, tea, cereals, coffee and roasted peanuts. Some of foodstuffs also have the ability of synthesising PAHs compound or adsorb this compound from water, soil or air (Menzie *et al.,* 1992). As mentioned before, this compound has been shown to be an active contributor to human cancer. Therefore, US EPA had classified them as carcinogenic and mutagenic compounds.

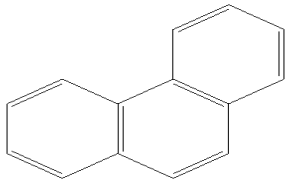
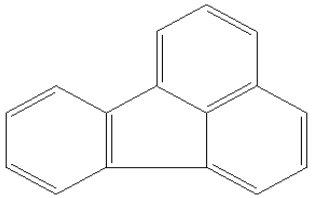
2.1.2 Mechanism of Action

The principal routes of exposure of PAHs to human are through ingestion, inhalation or skin contact (Munoz and Albores, 2011). PAHs can readily invade cellular membrane due to its lipophilic characteristic (Yu, 2005). Once PAHs enter the body, they are metabolised in a number of organs such as liver, kidney and lungs. Afterward, this compound may be excreted through either urine or breast milk. Small amount of PAHs could be stored in adipose tissue. Metabolic action in our body may change the hydrophobic characteristic of PAHs to become more hydrophilic. This hydrophilic characteristic helps to excrete it from our body. PAHs compound capable in triggering the expression of phase 1 and phase 2 metabolising enzymes (Shimada, 2006). These metabolising enzymes include

ado-ketone reductases, cytochrome P-450s, epoxide hydrolase, peroxidases, and sulfotransferase (Williams and Philips, 2000).

PAHs can be activated in three major pathways. The first pathway is the metabolic oxidation process that produced a PAH radical cation. This process connected with the cytochrome P450 peroxidase enzyme activity. The second pathway for PAHs activation is the catalysed oxidation process of dihydrodiol dehydrogenase which can produced PAH-o-quinones. The final pathway is the dihydrodiol epoxides formation, catalysed by cytrochrome P450 enzymes (Guengerich, 2000). Although there are three main pathways, the creation of dihydrodiol epoxides is the most common mechanism, which bind covalently to DNA. Oxidative stress was triggered by PAHs through the process from metabolic activation to diol-epoxides. The inducement of oxidative stress may cause mutagenic reaction in body system. Other than that, the oxidative stress formation rate may give negative impact to the mechanism of DNA repair. The accumulation of DNA mutations may cause carcinogenesis to occur.

Table 2.1: Chemical structure, characteristics, and health hazards of selected polycyclic aromatic hydrocarbon compounds.

PAHs	Chemical structure	Characteristics	Health hazards
Phenanthrene (PHE)		Molecular weight: 178.23 g/mol Log *P*: 4.55 Solubility: Soluble in organic solvent	Skin sensitization, dermatitis, cough, dyspnea, and respiratory neoplasm
Fluoranthene (FLU)		Molecular weight: 202.25 g/mol Log *P*: 5.16 Solubility: Soluble in organic solvent (benzene, ethyl alcohol)	Contact burns to skin and eyes, nausea, cardiac arrhythmias, and liver injury.

Source: 'Open Chemistry Database' http://www.pubchem.ncbi.nlm.nih.gov (Accessed on 24 November 2015)

2.2 Analytical Methods for Analysis of Polycyclic Aromatic Hydrocarbons

2.2.1 Spectrophotometric Method

Spectrophotometric method is an analytical method used to quantify the concentration of solutes in solution. In spectrophotometer, a cuvette read is used to place the solution. Then, the solution will absorb light emitted and the amount of light that is absorbed will be measured. The availability of the instrument, speed, simplicity of procedure, precise and accurate technique are among the reason for selecting spectrophotometric methods (Abdel- Aziz *et al.*, 2014). When compare with other methods such as chromatography and electrophoresis, this method is more economic and simple (Rojas *et al.*, 2009). However, the primary drawback of spectrophotometric multi-component analysis is concurrent determination of two or more active compounds in the same mixture without initial separation (Abdel-Aziz *et al.*, 2014). Analysis of PAHs compound in environmental samples by using fluorescence spectrophotometer has been successfully done by Krupadam *et al.* in 2009. The recoveries of eight PAH compounds between 85-96% within the limit of detection varied from 10-30 ng/L in industrial effluent and from 0.1 to 2.9 ng/kg in solid samples. Thus, they concluded that the method is significantly sensitive for environmental analytical method when comparing with common methods such as gas chromatography-mass spectrometry (GCMS) and liquid chromatography-mass spectrometry (LCMS) (Krupadam *et al.*, 2009).

2.2.2 Electrophoretic Method

Capillary electrophoresis (CE) is the common name used for describing electrophoretic method. It appeared to be one of the most productive methods for separating charged components especially in mixture compounds (Sepaniak *et al.,* 1992). Although CE are the most efficient in separating charge components, modified CE was available for separating molecules that are hydrophobic and uncharged such as PAHs.The most familiar modified CE are micellar electrokinetic capillary chromatography (Terabe *et al.,* 1984), and cyclodextrin modified CE. The cyclodextrin type is the one that apply a mixture of neutral and charged CDs (Szolar *et al.,* 1995; Brown *et al.,* 1996). As mentioned before, CE has powerful and efficient separating ability with other advantages such as high resolution (Terabe *et al.,* 1984). Also, this method require less samples and solvents (Chiari *et al.,* 1996). Despite its powerful separating, there are a few drawbacks of CE when comparing with chromatography method. The injection of CE is not precise, it also have problem with capillary regeneration and adverse effect on reproducibility due to imbalance ratio of organic to solvent (Fifield and Kealey, 2000). Other than that, the detection of CE is not sensitive due to the small path length when applying the on-line absorbance detectors (Nguyen and Luong, 1997). Although the use of UV/ visible laser-induced fluorescence detection can successfully improve the sensitivity, but unreasonably high cost of UV lasers and stability of the laser still become a problem (Nguyen and Luong, 1997).

2.2.3 Chromatographic Method

There are two main phases involved in chromatographic method, namely mobile ad stationary phase. Liquid or gas may be applied as the mobile phase while solid or solid substrate coated with liquid film can be used as stationary phase. Basically, the sample that was injected into the mobile phase will pass through stationary phase. The partitioning process occurs during the movement of the component along the stationary phase. A component that requires longer time to pass through the system is normally attach with the stationary phase instead of mobile phase. This is due to the effect

of distribution ratio favours the stationary phase. As a result, it would be among the last component eluted to the detector. In 2010, a new method was introduced for the analysis of PAH compounds in ambient air dust through GCMS. This method adopting the conventional method solid- phase extraction with applying the use of a molecular imprinted polymer. They found that the limit of detection using GCMS is 0.15 ng/L for benzo[a] pyrene, a marker molecule of air pollution (Krupadam *et al.,* 2010). Another research related with the same compound using chromatography method was reported in the year 2014. In this research, they used solid phase microextraction method for the extraction of PAHs compounds from water samples before analyse it using GCMS. The limit of detection were less than 4.0 ng/L and the correlation coefficients (r) value is between 0.9940-0.9986 (Zhang *et al.,* 2014).

2.3 Sample Preparation

In analytical chemistry, the most crucial step is preparation of sample. It is needed because of the complexity and extremely low concentration of the target analytes present in most samples. Missed step in sample preparation may lead to inaccurate result. It is the most challenging step as stated by Major (1991), where a researcher had to spend approximately 60% of the time on sample preparation itself (Biparva and Martin, 2012). The main objective of this step is to pre-purify, concentrate and make it in the compatible form with the selected analytical system (Ulrich, 2000).

Over the years, several extraction methods have been developed. They can be divided into two major groups; conventional and new extraction method. Basically, the new extraction method is from conventional method, modified in certain aspects such as substitution of solvent, usage of emulsification and microwave.

2.3.1 Conventional Sample Preparation

The conventional sample preparation techniques is the earliest method that was developed by scientists for analysis. Below are the three conventional sample preparation techniques related to analysis of PAHs.

2.3.1.1 Liquid-Liquid Extraction

Solvent extraction (SE) was the first method introduced in sample preparation. This method was also known as liquid-liquid extraction (LLE). Several works have reported the extraction of PAHs compounds in water by LLE technique (Doong *et al.*, 2000; Popp *et al.*, 2003; Popp *et al.*, 2000). This method was performed by the used of buffer, hot water and organic solvents. The commonly used organic solvents were chloroform, acetonitrile, methanol and ethyl acetate. During trace analysis, this method requires too much sample and toxic solvents which may result to environmentally unfriendly. LLE method is labour intensive and the process is time consuming (He and Lee, 1997; Hosseini *et al.*, 2012). Several other techniques have been developed to replace LLE due to its disadvantages.

2.3.1.2 Solid Phase Extraction

Solid support was introduced for concentrating and isolating target analytes and this method was known as solid phase extraction (SPE). It had been used widely in various areas of chemistry including clinical, environmental and applications of pharmaceuticals (Hennion, 1999). The development of SPE is a complimentary to liquid-liquid extraction (LLE). This is due to several drawbacks related to LLE such as it required labour intensive, difficult to operate and required high volume of toxic organic solvent (He and Lee, 1997). Thus, SPE has successful in optimising the amount of solvent used. When conducting SPE method, the solvent used is less, and the duration of experiment also shorter as compare to LLE technique. Although SPE was successfully decreased the

consumption of organic solvent and shortened the experimental duration, it required column conditioning and this was relatively expensive (Junk and Richard, 1988).

2.3.1.3 Cloud Point Extraction

This technique uses surfactants for extraction of target analytes. Surfactants for extraction have been known for their capability to enhance the solubility of hydrophobic materials (Bai *et al.,* 2001).The benefit of cloud point extraction (CPE) is that the solvent used in the micellar solution is water. However, difference among surfactants poses a challenge to the analysis of analyte when using gas chromatography and high performance liquid chromatography (Carabias-Martinez *et al.,* 2000). In addition, the use of anionic surfactants as a prodcutive extractant during separation phase often require salts and pH alteration (Casero *et al.,* 1999).

2.3.2 Microextraction Method

Current aims of analytical techniques is to overcome all limitations presence in conventional extraction techniques, and to simplify and miniaturize the analytical procedure (Cunha and Fernandes, 2013). Thus, microextraction technique was introduced. Microextraction is a non-exhaustive sample preparation. The word micro describing that, it require a minute amounts of extracting phase (microliter range or smaller) relative to the sample volume. This method is a simple, clean and quick technique which requires low quantities solvent (Cacho et al., 2016).

2.3.2.1 Liquid Phase Microextraction

A novel technique for sample preparation is the Liquid phase microextraction (LPME) (Jeannot and Cantwell, 1996). There are many method in LPME technique and one of them is single drop microextraction method. Those two researchers introduced LPME technique with the use of Teflon

rod. A minute amount of organic solvent that is immiscible in water was held on a hold at the end of the Teflon rod. Then the rod will be immersed in the stirred aqueous sample solution. After a year, they made an improvement to the original method by replacing the solvent holder which is from Teflon rod to microsyringe. Therefore, LPME was carried out using a 1µL solvent drop at the tip of a microsyringe needle (Jeannot and Cantwell, 1997). The advantages of this technique is, it is cheap, required a simple equipment, the operation system is easy and it is environmentally friendly due to the use of less amount of solvents (Biparva and Martin, 2012). There are also disadvantages of this technique include slow movement of extraction, drop instability and small surface of the drop (Biparva and Martin, 2012).

The liquid phase microextraction technique was further modified by Lee and his group between the years 1997 to 2001. They hypothesized the concept of static (S-LPME) and dynamic microextraction (D-LPME) (He and Lee (1997); Wang *et al.*, (1998); Zhao and Lee (2001)). The technique that was introduced is cheap and requires minimal exposure to hazardous solvents (Zanjani *et al.*, 2007). However, a major drawback is that it is not suitable when applying for dirty samples. The reason is, the particles that present in most of the dirty samples may affect the solvent droplet stability during extraction process. Dirty samples also harmful to the analytical instrument (Zhao and Lee, 2000). Furthermore, another setback is the microdrop that suspended on the microsyringe needle during stirring of the aqueous sample may remove easily (Zanjani *et al.*, 2007).

In order to solve the drawback that present in the static liquid phase microextraction technique, a porous hollow-fiber membrane was introduce. The main objective of this particular substitution is to solve the problem of unstable solvent drop and improve static-LPME sensitivity (Rasmussen and Pedersen-Bjergaard, 2004; Shen and Lee, 2002). Organic solvent was then injected to the system. In the lumen of the porous hollow fiber, there is also organic solvent that function as an interface between the sample solution and the extracting phase. The harzadous and harmful level of this technique is low due to low requirement for toxic organic solvent, In addition, this techniques

is very special because it combines extraction, concentration and sample introduction into one step (Eshagi, 2010). As reported previously by many researcher, this technique has been successfully applied for detection of insecticides (Chen *et al.,* 2009), dichlorophenol isomers (Ziagova *et al.,* 2009) and polycyclic aromatic hydrocarbons (De La Torre-Roche *et al.,* 2009).

2.3.2.2 Dispersive Liquid-Liquid Microextraction (DLLME)

A new microextraction method namely dispersive liquid-liquid microextraction was introduced by Assadi et al. in 2006. (Rezaee *et al.,* 2006). The injection of extraction solvent and disperser solvent into an aqueous sample will cause the formation of fine droplets emulsion that lead to cloudy solution. The formation of cloudy solution fine droplets is occurs after undergo centrifugation process. This may lead to form sediments at the bottom of a conical flask. (Miri and Jalali, 2013). This technique give us many benefit includes simplicity, rapidity, low cost, high recovery and enrichment factor (Miri and Jalali, 2013; Rezaee *et al.,* 2006). Although there are many benefit from this technique, but it is not environmentally friendly due to the consumption of very toxic extraction solvents and also require large amount of dispersive solvent. (Xu *et al.,* 2009). A conventional DLLME was used solvents that is denser than water. This solvents commonly chlorinated solvents which is environmentally unfriendly. However, the uses of an extraction solvents that is lighter than water were reported (Farajzadeh *et al.,* 2009). A combination of two methods, DLLME and LLME-SFO was successfully formed a new microextration method named dispersive liquid-liquid microextraction based on solidification of floating organic droplet (DLLME-SFO). This method was development by Leong and Huang in 2008 (Wang *et al.,* 2010). In this method, it is not required the use of microsyringe, or hollow fiber as a holder because they used low density of organic solvent with suitable melting point (Xu *et al.,* 2009). Extraction solvents which have low densities below than 1g mL^{-1} and low toxicity can be used to this method (Wang *et al.,* 2010). Then, the solidification process required the help of an ice bath. Advantage of using this method is the mass transfer from the aqueous phase to the organic phase become fast as the contact area between the

organic droplets and sample solution is large (Xu *et al.,* 2009). Other than that, this method can easily transfer solidified phase from aqueous phase (Xu *et al.,* 2009).

2.3.2.3 Ultrasound Assisted Surfactant-Enhanced Emulsification Microextraction (UASEME)

Ultrasound assisted surfactant- enhanced emulsification microextraction is the new method that previously adopted from a novel microextraction technique namely ultrasonic-assisted emulsification microextraction. This technique was developed by Rugueiro *et al.,* in 2008 (Rugueiro *et al.,* 2008). Ultrasound- assisted emulsification microextraction is the technique that not require any dispersive solvent. The microvolume of the extraction solvent is dispersed into aqueous sample solution by ultrasound– assisted emulsification (Cheng *et al.,* 2011). This is one of the efficient technique used to facilitate the emulsification phases and increase the rate of mass-transfer process between two immiscible phase (Fontana *et al.,* 2009). Ultrasonic radiation have been shown to be an efficient tool for solid and liquid samples. However, a new method was developed by adopting the previous novel technique. Wang et al. were introduced the uses of surfactant as an emulsifier. The first experiment using this method is the detection of certain carbamates in water samples. Combination of both surfactant and ultrasound greatly shortened the time of extraction (Cheng *et al.,* 2011). Surfactants also known as surface active agent are amphiphilic molecules. They have both hydrophilic and hydrophobic areas which is advantageous for water and immiscible phase reaction. As mentioned before, surfactant act as emulsifier in order to enhance the dispersion process of water-immiscible phase into the aqueous phase. Therefore, the application of a surfactant as an emulsifier in UASEME combines the advantages of both DLLME and UAME technique.

2.4 Sample Preparation of Polycyclic Aromatic Hydrocarbons

As mentioned earlier, several sample preparation techniques have been developed for detection of polycyclic aromatic hydrocarbon in samples such as water, spirits, vegetables oil, milk sample, and soil sample examples are flotation-assisted homogenous liquid-liquid microextraction (FA-HLLME) (Hosseini *et al.*, 2012), agarose film liquid phase microextraction (AF-LPME) (Sanagi *et al.*, 2012), Solvent-impregnated agarose gel liquid microextraction of polycyclic aromatic hydrocarbon in water (Loh*et al.,* 2013) and Polydimethylsiloxane/ metal-organic frameworks coated fiber for solid-phase microextraction (Zhang*et al.,* 2014). Summary of sample preparation techniques and GC determination of polycyclic aromatic hydrocarbon is shown in Table 2.2.

Table 2.2: Summary of sample preparation methods and GC determination of polycyclic aromatic hydrocarbon.

Instrument	Analyte(s)	Matrices	Sample preparation	Linear range (µg/L)	LOD/LOQ (µg L^{-1})/ (ng g^{-1})	Precision (%RSD)	Recovery (%)	References
GC-FID	4 PAHs	Soil sample	Flotation-assisted homogenous liquid-liquid microextraction (FA-HLLME)	40-1000	24-30	7.4-11.2	-	Hosseini *et al.*, 2012
GC-MS	4 PAHs	Water sample	Agarose film liquid phase microextraction (AF-LPME)	0.1-200	0.01-0.04	6.1-9.2	92.9-104.7	Sanagi *et al.*, 2012
GC-MS	4 PAHs	Water sample	Solvent-impregnated agarose gel liquid microextraction	-	9-14 ng L^{-1}	3.4-9.4	93.3-108.2	Loh *et al.*, 2013
GC-MS	5 PAHs	River and lake water sample	Polydimethylsiloxane/ metal-organic frameworks coated fiber for solid-phase microextraction	0.01-2.0	40ng L^{-1}	9.3-13.8	78.2-110.3	Zhang *et al.*, 2014

CHAPTER 3

MATERIALS AND METHODS

33.1 Chemicals and Reagents

Polycyclic aromatic hydrocarbons (PAHs), phenanthrene (PHE) and fluoranthene (FLU) were obtained from Toronto Research Chemicals INC (Toronto, Canada). Toluene, n-hexane, iso-octane, and 1-octanol were HPLC grade and purchased from QReC Asia Sdn. Bhd. (Selangor, Malaysia). Analytical grade surfactants (Tween 80, Tween 20 and DC193) were purchased in chemically pure form from Fisher Scientific (Selangor, Malaysia). Ultrapure water of 18.2 MΩ was purified by a Sartorious system, model Arium 611 D1 (Stedim Biotech, Gottingen, Germany).

3.2 Preparation of Standard Solutions

Standard stock solution (1000 µg L^{-1}) was prepared by weighing 0.01g PHE and FLU into separate amber vial. Each standard was dissolved in solvent mixture of methanol and acetonitrile (50:50). Then, the stocks were stored in amber vial for less than a week in fridge at temperature 4˚C. Working solutions of PAHs were prepared in ultrapure water daily prior to extraction and analysis.

3.3 Sample Collection and Pre-treatment

Tap water was collected from Integrative Medicine Lab, AMDI, USM. Sugarcane juice samples were purchased and collected from street vendors and local stores in Kedah and Penang, Malaysia. Samples were stored at 4˚C before use and defrosted at room temperature before analysis. The sugarcane juice samples were centrifuged for 15 minutes at 3500 rpm prior to extraction. The pellet layer was discarded before microextraction procedure.

3.4 UASEME Procedure

Several simple steps were involved in UASEME procedure. Firstly, 15 µL of Tween 20 was injected into 10mL volumetric flask. Then followed by addition of 30 µL toluene. Then, immediately the sample solution was added into the volumetric flask. The upper part of the volumetric flask was covered with cap and turned up-side down. Then, the sample was ultrasonicated for 2 min and the solution turned into cloudy mixture. After extraction process, two immiscible phases were formed and 20 µL of the upper layer was taken out using microsyringe. Finally the clean extract was diluted with 80 µL of methanol prior to GC-MS analysis.

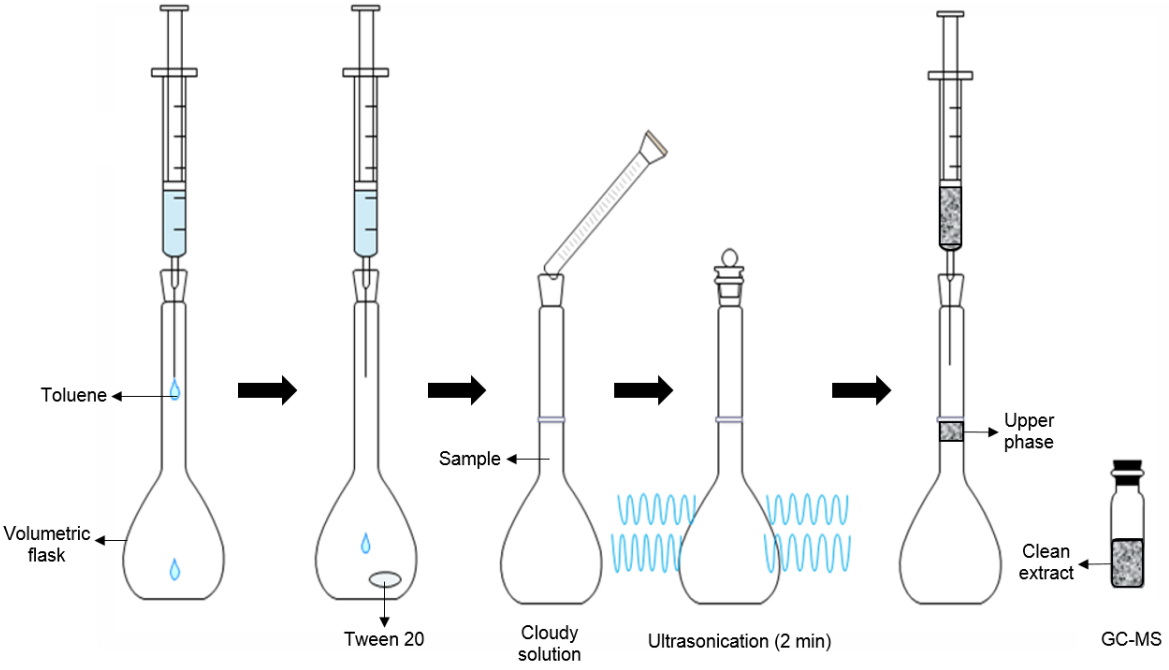


Figure 3.1: Schematic of UASEME procedure.

3.5 Chromatographic Conditions

All analyses were performed on Agilent GC system Model 7890A with mass spectrometry analyser Model 5975C (Palto Alto, USA). Helium gas was used with a constant flow rate of 1mL min^{-1}. Splitless injections were performed at 250 ˚C and volume of injection was 1µL. The chromatographic condition was carried out on an Agilent HP 5MS column (30 m x 0.25 mm ID, 0.25 µm film thickness). The oven temperature applied was initially programmed from 150 ˚C for 3 minutes. After that, the temperature rose up to 250 ˚C at 10 ˚C per minute. The transfer line and the ion source temperature were fixed at 280 ˚C respectively. Data were collected and quantified in the scan of mass spectrometer analyser. The retention times of PHE and FLU recorded under the optimised chromatographic conditions were 8.90 and 11.75 min, respectively. Electron impact mass spectra were recorded at 70 eV and the detector voltage was set at 1.0 kV. Chromatographic data were processed using MSD Chem Station E.01.01 Agilent Technology software.

3.6 Optimisation of UASEME Method

The goal of optimisation in analytical chemistry is to obtain the best response, such as signal of detector, enrichment factor and chromatographic resolution. The common practice for optimisation is changing one parameter and at the same time keeping all other parameters at a constant level then the response was monitored by an appropriate detector. This procedure is used in UASEME method. Six UASEME parameters were optimised in this study including type of extraction solvent, type of surfactant, volume of toluene, volume of tween 20, extraction time and salt addition. Optimisation was carried out in triplicate using ultrapure water spike with concentration of 1000 µg L^{-1}.