การเตรียมตัวอย่างน้ำมันประกอบอาหารเพื่อการตรวจวัดพอลิไซคลิกแอโรแมติกไฮโดรคาร์บอนโดยไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี

นาง ทองสุข ปายะนันทน์

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SAMPLE PREPARATION OF COOKING OILS FOR DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Mrs. Thongsuk Payanan

A Thesis Submitted in Partial Fulfillment of the Requirements
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ทองสุข ปายะนันทน์ : การเตรียมตัวอย่างน้ำมันประกอบอาหารเพื่อการตรวจวัดพอลิไซคลิกแอโรแมติกไฮโดรคาร์บอนโดยไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี (SAMPLE PREPARATION OF COOKING OILS FOR DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร.พุทธรักษา วรานุศุภากุล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, 117 หน้า.

เทคนิคการเตรียมตัวอย่าง 2 วิธีคือ low-temperature cleanup และ sweep co-distillation ซึ่งเป็นเทคนิคที่ง่าย รวดเร็ว ราคาถูกและใช้ปริมาณตัวทำละลายอินทรีย์น้อยได้ถูกพัฒนาเพื่อใช้ในการตรวจวัดพอลิไซคลิกแอโรแมติกไฮโดรคาร์บอน (PAHs) 16 ชนิดในน้ำมันประกอบอาหารชนิดที่ยังไม่ผ่านการใช้งานและน้ำมันที่ผ่านการทอดซ้ำโดยไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีที่มีเครื่องตรวจวัดชนิดฟลูออเรสเซนต์ สำหรับวิธี low-temperature cleanup เวลาที่ใช้ในการแช่แข็ง ชนิดตัวทำละลายอินทรีย์ จำนวนครั้งของการสกัดและ SPE ที่ใช้ในการ cleanup มีผลต่อประสิทธิภาพการสกัด สภาวะที่เหมาะสมได้แก่การใช้ตัวทำละลายอินทรีย์ผสมอะซิโตไนไตรล์กับอะซิโตน (80:20, v/v) ทำการแช่แข็งที่อุณหภูมิระหว่าง -18 ถึง -25 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง จำนวน 2 ครั้งและทำการ cleanup ด้วย alumina N SPE cartridge วิธีนี้สามารถตรวจวัด PAHs ได้ทั้งหมด 16 ชนิด โดยมีร้อยละเฉลี่ยของการคืนกลับเท่ากับ 45.88-118.47 ขีดจำกัดของการวัดเชิงคุณภาพและขีดจำกัดของการวัดเชิงปริมาณเท่ากับ 0.13-3.13 และ 0.25-6.25 นาโนกรัมต่อกรัม ตามลำดับ สำหรับวิธี sweep co-distillation อุณหภูมิและเวลาที่ใช้ในการกลั่นมีผลต่อประสิทธิภาพการสกัด สภาวะที่เหมาะสมได้แก่การกลั่นที่อุณหภูมิ 235 องศาเซลเซียส เป็นเวลา 60 นาที แต่วิธีนี้สามารถตรวจวัด PAHs ได้เพียง 8 ชนิด โดยมีร้อยละเฉลี่ยของการคืนกลับเท่ากับ 47.48-119.47 ขีดจำกัดของการวัดเชิงคุณภาพและขีดจำกัดของการวัดเชิงปริมาณมีค่าประมาณสองเท่าของวิธี low-temperature cleanup สุดท้ายทั้ง 2 วิธีสามารถนำไปใช้เตรียมตัวอย่างน้ำมันมะกอกอ้างอิง (RM FAPAS T0631) และตัวอย่างน้ำมันประกอบอาหารจริงเพื่อการวิเคราะห์ PAHs ได้อย่างมีประสิทธิภาพ

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : PAHS / COOKING OILS / HPLC / LOW-TEMPERATURE CLEANUP / SWEEP CO-DISTILLATION

THONGSUK PAYANAN: SAMPLE PREPARATION OF COOKING OILS FOR DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. THESIS ADVISOR: PUTTARUKSA VARANUSUPAKUL, Ph.D. THESIS CO-ADVISOR: ASST. PROF. NATCHANUN LEEPIPATPIBOON, Dr.rer.nat., 117 pp.

Two sample preparation techniques, low-temperature cleanup and sweep co-distillation, which are simple, fast, inexpensive and using small amount of organic solvent have been developed for the determination of 16 polycyclic aromatic hydrocarbons (PAHs) in refined and used cooking oil samples by high-performance liquid chromatography with fluorescence detection (HPLC-FLD). For low-temperature cleanup method, freezing time, extraction solvent, number of extraction and SPE cleanup affected the extraction efficiency. Optimum extraction was achieved when using a mixture of acetonitrile and acetone (80:20, v/v) as extraction solvent, freezing between -18 °C to -25 °C for 24 hours twice and cleanup with alumina N SPE cartridge. This method was effectively determined all 16 PAHs. The recoveries were 45.88 – 118.47%. Limit of detections (LODs) and limit of quantitations (LOQs) were 0.13 to 3.13 ng/g and 0.25 to 6.25 ng/g, respectively. In sweep co-distillation method, distillation temperature and time had an effect on the extraction efficiency. Optimum extraction was attained when distillated at 235 °C for 60 minutes. However, this method was quantitatively analyzed only 8 PAHs. The recoveries were 47.48 – 119.47%. LODs and LOQs values were about two times higher than low-temperature cleanup method. Finally, both methods were successfully tested for determination of PAHs in reference materials of olive oil (RM FAPAS T0631) and real cooking oil samples.

Department : Chemistry Student's Signature .....
Field of Study : Chemistry Advisor's Signature .....
Academic Year : 2009 Co-Advisor's Signature .....

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CONTENTS

PAGE

PAGE

LIST OF TABLE

PAGE

LIST OF FIGURES

FIGURES **PAGE**

LIST OF ABBREVIATIONS

Ace	Acenaphthene
Ant	Anthracene
BaA	Benzo(a)anthracene
BaP	Benzo(a)pyrene
BbF	Benzo(b)fluoranthene
BeP	Benzo(e)pyrene
BkF	Benzo(k)fluoranthene
BghiP	Benzo(g,h,i)perylene
Chry	Chrysene
DiahA	Dibenzo(a,h)anthracene
Fl	Fluorene
Ft	Fluoranthene
Naph	Naphthalene
Phen	Phenanthrene
Pyr	Pyrene
123cd	Indeno(1,2,3-c,d)pyrene
%	percentage
AOAC	Association of Official Analytical Chemists
b.p.	boiling point
°C	degree celsius
cm	centimeter
RM	reference material
DACC	donor acceptor complex chromatography
EU	The Europian Union
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
F	female
FID	flame ionization detection
g	gram
g/cm^3	grams per cubic centimeter
GC	gas chromatography

GLC	gas-liquid chromatography
GC/MS	gas chromatography mass spectrometry
g/mL	gram per milliliter
HPLC	High performance liquid chromatography
HPLC-FLD	High performance liquid chromatography with fluorescence detector
HRGC	High-resolution gas chromatography
HRGC-MS	High-resolution gas chromatography mass spectrometry
hrs	hours
I.D.	internal diameter
K_D	distribution coefficient
Kpa	kilopascal
LC	liquid chromatography
LC-MS	liquid chromatography tandem mass spectrometry
LD_{50}	lethal dose 50%
LLE	liquid-liquid extraction
LODs	limit of detections
LOQs	limit of quantifications
LPG	liquified petroleum gas
LP-GC/MS	Low-pressure gas chromatography tandem mass spectrometry
LS	Light scattering
M	male
mm	millimeter
min	minute
mg	milligram
mg/kg bw	milligram per kilogram body weight
m^2/g	square meter per gram
mL	milliter
mL/min	milliliter per minute
MS	mass spectrometry
NMR	nuclear magnetic resonance

ng/g	nanogram per gram
ng/mL	nanogram per milliliter
nm	nanometer
PAHs	polycyclic aromatic hydrocarbons
PLE	pressurized liquid extraction
ppb	part per billion
ppm	part per million
RI	refractive index
R.S.D.	relative standard deviation
R^2	correlation of determination
SCD	sweep co-distillation
S.D.	standard deviation
SFE	supercritical fluid extraction
S/N	signal to noise ratio
SPE	solid-phase extraction
TCPI	tetrachlorophthalimidopropyl
TLC	thin-layer chromatography
UNITREX	universal trace residue extraction instrument
USEPA	United States Environmental Protection Agency
UV	ultraviolet
v/v	volume by volume
V_{aq}	volume of aqueous solution
V_{org}	volume of organic solvent
µg/kg	microgram per kilogram
µg/L	microgram per liter
µL	microliter
µm	micrometer

CHAPTER I

INTRODUCTION

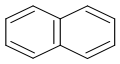
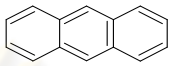
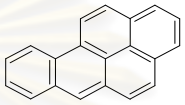
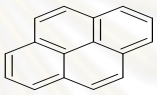
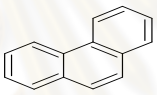
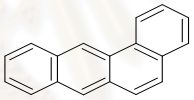
1.1 Problem Definition

Nowadays, more and more people are interested in good health and becoming more selective in their food selection. One of those common attracted foods are cooking oils because they contain many essential nutrients for example, oleic acid, erueic, linoleic, palmitic, omega-6 fatty acids, omega-3 fatty acids, vitamin E and co-enzyme Q10 etc. *(1)*. Thus, the quality of cooking oils in many applications should be controlled. The control of overused cooking oil leads numerous problems. It is well known that when heated with extended time, the overheating or overusing of frying oil lead to form were changed oxidation products. Many of these oxidation products such as aldehydes, polycyclic aromatic hydrocarbons, and polymeric substances have shown adverse effect to health and biology such as increase in liver and kidney size as well as a kind of cancer *(2, 3)*. Due to these problems, in 2005, the European Union has set a limit of 2 μg/kg benzo(a)pyrene in oils and fats intended for direct human consumption on use as an ingredient in food *(4)*. There have several works attempted to determine PAHs with the highest effective analysis methods *(5)*. Analysis of PAHs in oil sample is challenging because of matrix complexity. Therefore, sample preparation is an essential step in the analysis. It is widely known that many methods have been applied for the extraction of PAHs from fats and oils such as liquid-liquid extraction, supercritical fluid extraction and pressurized liquid extraction. However, all of those extraction methods are tedious and still needed a clean-up process before chromatographic analysis as well as large amounts of solvent are usually required.

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds consist of two or more aromatic rings in linear, angular, and cluster arrangement *(6)*. Ring arrangements of PAHs are given in Table 1.1. There are several sample preparation techniques for PAHs determination in fats and oils. Hence, the purposes of all methods intend to

eliminate sample interferences, lower amount of solvent consumption and shorter analysis time. Previously, extracting PAHs from fat and oil samples, that are dissolved by the solvent as selectively as possible. The procedures mostly used for extraction is liquid-liquid extraction and one of common organic solvents used are cyclohexane(CH), solution of dimethylformamide (DMF):water, and dimethyl sulfoxide (DMSO) *(7)*. Some purification processes were performed by solid-phase extraction (SPE) cleanup, thin-layer chromatography (TLC) and column chromatography with different adsorbent materials. Most of these procedures rely on tedious steps, time consuming, require large volume solvents and always giving low reproducible results. Moreover, modern instrumental analysis is sensitive that strongly required high effective sample cleanup method. The sample matrix from oils and fats regularly interfere the measurement due to the complexity of sample and the low level of PAHs in sample. Therefore, enrichment and cleanup procedures are necessary before analysis with specific instrument such as HPLC, GC and especially when hyphenated with mass spectrometry technique. From these reasons, sample preparation is an unavoidable step in order to extraction and purification the oils and fats matrix before analysis by HPLC or GC.

Table 1.1 Ring arrangement of PAHs

Ring arrangement	Description	Examples
Linear	All rings in line	 naphthalene  anthracene
Cluster	At least one ring surrounded on the three side	 benzo(a)pyrene  pyrene
Angular	Rings in step	 phenanthrene  benzo(a) anthracene

1.2 Literature Review

In 2005, the European Union has set a limit of 2 μg/kg for benzo(a)pyrene in oils and fats intended for direct human consumption on use as an ingredient in food *(4).* Furthermore, some European countries such as Germany, Austria and Poland have set a legal limit of 1 μg/kg of benzo(a)pyrene content in smoked foodstuff, but there is no legal limits for PAHs content in oils and fats *(8).*

In food matrix, the determination procedures of PAHs consisted of extraction, preconcentration, cleanup and quantification steps. PAHs are regularly extracted by liquid-liquid extraction. In some cases, it may be preceeded by a saponification step or even by caffeine complexation, pressurized liquid extraction (PLE), supercritical fluid extraction (SFE) and microwave assisted extraction (MAE) *(3,17).* These

techniques provide many advantages for the extraction of lipophilic organic compounds. Soxhlet and sonication extraction have also been described *(9)*. Purification is performed through one or more procedures with column chromatography, thin-layer chromatography (TLC) and solid-phase extraction (SPE). The determination of PAHs can be carried out by high-performance liquid chromatography (HPLC) with spectrofluorometric detection or by high-resolution gas chromatography (HRGC) coupled to flame ionization detection (FID) or mass spectrometry (MS). The best analysis techniques in terms of sensitivity and selectivity are HPLC with fluorescence detection and HRGC-MS, because it is able to reach detection limits below 1 μg/kg *(10)*.

According to the preliminary study, Grimmer and Böhnke *(7)* reported that liquid-liquid extraction with methanol:water:cyclohexane, N,N-dimethylformamide:water: cyclohexane and concentration by column chromatography on Sephadex LH20 were applied prior to detection with high-performance gas-liquid chromatography (GLC) analysis. The sensitivity was significantly higher than by which obtained with ultraviolet spectroscopic methods. However, the large amounts of organic solvent were consumed.

Few years later, Diletti et al. *(10)* employed liquid-liquid extraction method with dimethyl sulphoxide (DMSO), furthered cleanup by thin-layer chromatography on silica gel, and then analysis by gas chromatography-mass spectrometry. This method has been developed for the separation of eight polycyclic aromatic hydrocarbons in olive pomance oils. Limit of detection was 0.1-0.43 μg/kg. The average recovery ranged from 69.0 to 97.5%. Even though, this work shown the high recovery rates but the large amount of organic solvent and time consuming were still required. The method based on liquid-liquid extraction also appeared by Guillén et al. *(11)*. Five samples of olive pomance oil were determined by gas chromatography-mass spectrometry.

Sample preparation is a very important and essential step to improved analytical performance of method. Based on the limited available condition, there have many

researchers tried to extract and cleanup PAHs from complex sample matrices. As previously described, on-line method was investigated by Moret et al. *(8)* with LC separation on a large column, evaporated of a 6 mL fraction in an on-line solvent evaporator, a second LC separation used a different mobile phase, fractionated the components of interest and transferred to GC through the in-line vaporize over interface. This method modified the sample preparation step with the injection of a large amount of food extract. Another study of on-line method for the determination of PAHs in vegetable oils is on-line coupling of liquid chromatography with capillary gas chromatography combines with mass spectrometry, it was developed by Vreuls et al. *(12)*. This method is a sample pretreatment of oil samples from different origins with liquid chromatography by appropriate LC fraction whereby transferred polycyclic aromatic hydrocarbons to the gas chromatograph using a loop-type interface. Then, solvent was evaporated through the solvent vapour exit and subsequent GC separation, and then the compounds were introduced into the mass spectrometer for detection and identification. The advantage of using on-line technique is total analytical set-up allowed the direct analysis of sample without any sample cleanup.

For the extraction of trace PAHs in edible oils and fats, Perrin et al. *(13)* investigated by donor acceptor complex chromatography (DACC) as a cleanup step for multi PAHs analysis with a tetrachlorophthalimidopropyl (TCPI) modified silica. As a result of the low level of individual PAHs, the determination of PAHs in lipids is beset with many difficulties. The purpose of this report was to describe a new method for trace multi PAHs analysis from complicated sample such as oils or fats. Even though DACC is an environmental friendly technique, they may be interfered with PAHs compounds. Barranco et al. *(14)* studied the two methods for cleanup and sample enrichment for the analysis of polycyclic aromatic hydrocarbons (PAHs) in edible oils. A donor-acceptor complex chromatography (DACC) column was used as cleanup technique. A standardized method was used in a low pressure column chromatography with alumina as stationary phase. Both methods are followed by a reversed phase high performance liquid chromatography with fluorescence detector.

The limits of detection were lower than 1 ng/g. On-line method using LC-LC coupling with fluorescence was applied to identify trace of 15 PAHs in edible oils by Stijn et al. *(15)* DACC column cleanup is fast and carried out during the HPLC run of the previous sample.

Many recent studies have focused on new traditional methods for extraction of several compounds. Using solid-phase extraction (SPE) has been reported. Haifang et al. *(16)* employed SPE method using triacontyl bonded silica (C_{30}) as sorbent for the determination of 16 PAHs in airborne particulate matters and quantified by gas chromatography-mass spectrometry (GC/MS). This method were investigated in SPE procedures including the concentration of organic modifier, flow rate of sample loading, elution solvents, ultrasonication time, and solvent types were investigated. Recoveries were in the range of 68-107% for standard PAHs aqueous solution and 61-116% for real spiked sample. Limits of detection (LODs) and limits of quantification (LOQs) were in the range of 0.0070-0.21 μg/L and 0.022-0.67 μg/L, respectively. However, C_{30} has longer carbon chain; it could provide adequate hydrophobic space to enhance the interaction with analytes. Since PAHs are almost nonpolar compounds. Thus, it could be suitable only for 5-6 ring PAHs and inappropriate for 2-3 ring PAHs.

Barranco et al. *(17)* compared the efficiency of various SPE sorbent such as C_{18}, C_8, C_2, CH, PH and NH_2 for sample cleanup. This method utilised a solid-phase extraction for sample cleanup, followed by reversed phase high performance liquid chromatography (HPLC) with fluorescence detector. The effects of experimental variables such as washing and elution solvents, sample solvent and drying time have been studied using C_{18} compared with various SPE sorbent. The recoveries range between 50 and 103% depending on the molecular mass of the PAH. The limits of quantitation were lower than 1 ng/g. In this work, liquid-liquid extraction was used for sample preparation and then cleanup with SPE, thus the whole procedure requires about 80 min. However, this method still used large amounts of organic solvent for liquid-liquid extraction procedure.

Many investigators have recently interested in rapid determination of PAHs with SPE such as Bogusz et al. *(18)*, the sample was extracted using solid-phase extraction on column filled with florisil and nucleoprep C_{18}, and the extracts were analyzed with GC/MS using standard capillary column and low-pressure wide-bore column (LP-GC/MS), as well as with HPLC on standard column and short donor-acceptor complex chromatography (DACC) column. Quantitation was done with isotope dilution method such as GC/MS and LP-GC/MS. Limits of detection were 1 ng/g for GC/MS on standard column, 1.6 ng/g on LP colimn, 0.5 ng/g for HPLC on standard column, and 0.3 ng/g on DACC column, respectively. The recoveries are over 80%.

In fat and oil samples analysis, Lentza-Rizos et al. *(34)* present a method for trace analysis of organophosphorus insecticides, by low-temperature cleanup method and GC with nitrogen-phosphorus detection. The method gives good cleanup by using low-temperature for lipid precipitation. They reported quantitative recoveries of these compounds ranging from 77 to 104%, with RSD values of 7-16%. In 1984, Luke et al. *(35)* used UNITREX for extraction of organochlorine residues in fat and oil samples, then analyzed by gas chromatography using an electron capture detector with a vitreous silica capillary column containing a medium polarity bonded phase. Recovery of several of organochlorine residues are 83-105%, with coefficient of variation between 4 and 6%.

1.3 Purpose of The Study

Many countries have been set a legal limit for polycyclic aromatic hydrocarbons contaminated in a various kind of cooking oils for benzo(a)pyrene because they are related with health risk assessment. From the proposed literature reviews, many researchers were paid attention to develop a new method to determine the polycyclic aromatic hydrocarbons in edible fats and oils. Most reported analysis methods were analyzed with various sample extraction, cleanup, and quantitation procedures. Unfortunately, some of these methods are unable to determine PAHs from a weakness of sample preparation and detection because of the food matrices complexity. Therefore, the analysis of PAHs in oil sample is challenging. Sample preparation is an

important step in the analysis procedure. It is widely known that many classical methods have been applied for the extraction of PAHs from fats and oils such as liquid-liquid extraction, supercritical fluid extraction and pressurized liquid extraction *(19)*. However, all of these extraction methods are tedious and still needed an efficient cleanup process before chromatographic analysis because large amounts of solvent are usually required.

Besides the detection of trace amounts of PAHs residues in complex matrices, all steps are very importance. It should pay attention to an alternative extraction technique to obtain high preconcentration level with low organic solvent consumptions. The comparison between low-temperature cleanup and sweep co-distillation method were tested. An advantage of these powerful methods are take a short time and less expensive than previous methods. Thus, low-temperature cleanup and sweep co-distillation method are the techniques that have been used in sample preparation for fat and oil samples. Both methods have many advantages with 1) high sample throughput; 2) no clean-up step (for sweep co-distillation method); 3) a closed extraction system; 4) using small volume of extracted solvent; 5) taking short analysis times; and 6) good behavior in elimination of interferences. According to these advantages, the method was applied to extract 16 PAHs from purified and used cooking oils. Low-temperature cleanup method based on fat precipitation using very low-temperature was utilized in combination with sweep co-distillation method based on volatilization by temperature controlled. Thus, the factors such as freezing temperature, extraction time, extraction temperature and extraction solvent were studied and optimized by high performance liquid chromatography with fluorescence detector (HPLC-FLD). The development of this new sample preparation technique proved that both methods provide simple, compact, fast, and cost-effective sample preparation techniques using only small amount of organic solvent. The technique allows a simultaneous and convenient treatment of multiple samples. The methods were developed to analyze sixteen target priority polycyclic aromatic hydrocarbons in purified and used cooking oils. All procedures were validated for the determination of PAHs in order to correspond with European Union regulation.

CHAPTER II

THEORY

2.1 Physical and Chemical Properties of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) is a group of chemical compounds consisting of two or more aromatic rings. In general, it is originated from incomplete combustion of organic compounds such as coal, oil, gas, garbage and geochemical processes. Food processing is one of common source for PAHs contamination. The concentration of PAHs in food varies depending not only on the location where raw material was grown, but also on the manner of food preparation including exposure time, the distance between heat sources and food, and the fat composition in food. PAHs have more than 100 compounds and can be classified into two main groups that are light PAHs which contain up to four aromatic rings and heavy PAHs which consist of five or more than six rings. Most of PAHs are not carcinogen but some of them are. The most effective carcinogens are the group of five or six fused rings, and these tend to be less prevalent in mixtures than the three and four ring hydrocarbons, which are not carcinogenic.

The significant property of PAHs is lipophilic and can accumulate in lipid layer of animal tissue but not in plant tissues. Most of PAHs tend to adsorb on organic matter while some of them are semi-volatile. Therefore, the content of PAHs in the atmosphere depends on their structure. Light PAHs are always retained in vapor phase. Four rings PAHs are stored in intermediate position and heavier PAHs are normally found on particles. The structure, physical and chemical properties of PAHs are shown in Figure 2.1 and Table 2.1 *(20)*.

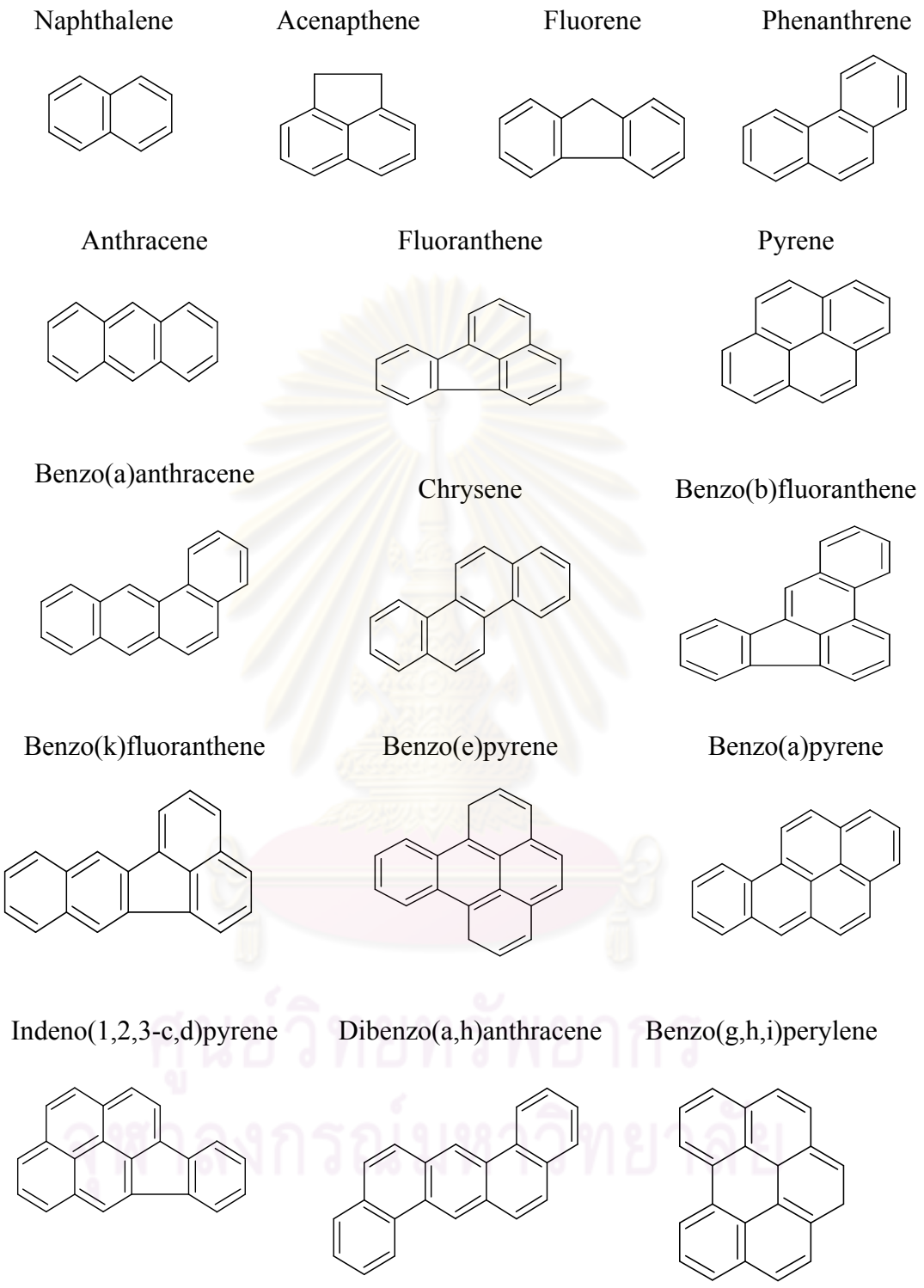


Figure 2.1 The molecular structure of 16 polycyclic aromatic hydrocarbons

Table 2.1 Physical and Chemical Properties of Polycyclic Aromatic Hydrocarbons.

Compounds	MW	Colour	MP (^oC)	BP (^oC)	Density (g/cm^3)	Solubility
Acenaphthene	154.21	White	93.4	279	1.225 at 0 ^oC	Soluble in alcohol, methanol, propanol, chloroform, benzene, toluene, glacial acetic acid
Anthracene	178.2	Pure: colorless with violet fluorescence Impure: yellow with green fluorescence	215	340	-	Soluble in acetone, benzene, carbon disulphite,carbon tetrachloride, toluene, chloroform, ether, ethanol, methanol
Benzo(a)anthracene	228.29	Yellow with blue fluorescence	167	435	1.274 at 20 ^oC	Slightly soluble in acetic acid and hot ethanol; soluble in acetone and diethyl ether; very soluble in benzene
Benzo(a)pyrene	252.32	Pale yellow	177	495	1.351at 20 ^oC	Sparingly soluble in ethanol and methanol; soluble in benzene, toluene, xylene, and ether
Benzo(e)pyrene	252.32	Colorless	178	311	-	Soluble in acetone

Note: MW – Molecular weight MP – Melting point BP – Boiling point

Table 2.1 (cont.) Physical and Chemical Properties of Polycyclic Aromatic Hydrocarbons.

Compounds	MW	Colour	MP (^oC)	BP (^oC)	Density (g/cm^3)	Solubility
Benzo(b)fluoranthene	252.32	Colorless	168.3	480	-	Slightly soluble in benzene, acetone
Benzo(k)fluoranthene	252.32	Pale yellow-green	217	480	-	Soluble in benzene, acetic acid, ethanol
Benzo(g,h,i)perylene	276.34	Pale yellow-green	278	550	-	Soluble in benzene, dichloromethane, acetone
Dibenzo(a,h)anthracene	278.35	Colorless	270	524	1.282 at 20 ^oC	Slightly soluble in ethyl alcohol; soluble in acetone, acetic acid, benzene, toluene and xylene
Chrysene	228.29	Colorless with blue or red-blue fluorescence	258	448	-	Slightly soluble in acetone, carbon disulphite,diethyl ether, ethanol glacial acetic acid toluene hot xylene; soluble in benzene
Fluoranthene	202.26	Pale yellow	108	384	-	Soluble in alcohol, ether, benzene, acetic acid

Note: MW – Molecular weight MP – Melting point BP – Boiling point

Table 2.1 (cont.) Physical and Chemical Properties of Polycyclic Aromatic Hydrocarbons.

Compounds	MW	Colour	MP (^oC)	BP (^oC)	Density (g/cm^3)	Solubility
Fluorene	166.22	White	115	295	-	Soluble in acetic acid, acetone, benzene, carbon disulphite, carbon tetrachloride diethyl ether, ethanol, pyrimidine solution, toluene
Indeno(1,2,3-c,d) pyrene	276.3	Pale yellow or needles with greenish	164	530	-	Soluble in organic solvents
Naphthalene	128.17	White	80.2	218	1.14 at 0 ^oC	Soluble in ether, chloroform, carbon tetrachloride
Phenanthrene	178.23	Colorless	99.2	340	0.980 at 4^oC	Soluble in glacial acetic acid, benzene, carbondisulphite, carbon tetrachloride, toluene, anhydroustoluene, diethyl ether, ethanol
Pyrene	202.26	Colorless, pale yellow or slight blue fluorescence	151	404	1.271 at 23 ^oC	Soluble in alcohol benzene, carbon disulphite, diethyl ether, ethanol, petroleum ether, toluene

Note: MW – Molecular weight MP – Melting point BP – Boiling point

2.1.1 Sources of PAHs

There are many pathways of PAHs contamination. Domestic sources, industrial sources, mobile sources and natural sources are four major sources of PAHs components contamination. Firstly, PAHs influence on ambient air quality inhousehold. Heating and cooking are the main process of fuel combustion in which level varies from the consumption of natural gas, liquefied petroleum gas (LPG), wood or charcoal. The burning procedure can release PAHs into environment. Hence, the domestic sources should be controlled to prevent PAHs pollution. For industrial sources, PAHs are emitted into the atmosphere from industries such as aluminium production, coke production (the part of the iron and steel production), creosote and wood preservation, waste incineration, cement manufacture, petrochemical and related industries, bitumen and halt industries, rubber and tire manufacturing, and commercial heat and power origin. The particles occurred from these sources are less than 2.5 μm *(21)*. Thirdly, mobile sources are related to transport reliant on a combustion engine. Not only motor vehicles but also aircraft, shipping, railway and automobiles are the major sources of PAHs production. There has been found that the amount of PAHs in this kind of sources is increasing every year. The factors affecting on PAHs emission are engine temperature, load, fuel quality and speed of combustion and diesel vehicles that have particulate emission higher than gasoline vehicles. Therefore large city has higher PAHs contamination than urban area. From these problems, new technology and effective measurement will be considerate to reduce the pollutions. Finally, natural sources of PAHs also include the accidental burning of forests, woodland and moorland.

Polycyclic aromatic hydrocarbons (PAHs) existing in the atmosphere derived from the combustion and volatilization. They are presented in the ambient air as vapors or adsorbed into airborne particulate matter *(22-26).* The high concentration of PAHs in the atmosphere depends on the molecular weight of the PAHs compounds, temperature, humidity and precipitation *(27, 28)*. The lower-molecular weight compounds with 2-3 rings, exhibiting low temperatures of condensation, are more abundant in the gas phase *(29, 30)*. The aromatic compounds having more than five rings, low-volatile and exhibiting high temperatures are adsorbed on the airborne

particles. For the semi-volatile, four ring PAHs can be found in both gas phase and airborne.

The other major sources of PAHs compounds generally come from food processing. Polycyclic aromatic hydrocarbons can be presented in food by a variety of circumstances, including cooking, smoking and frying. The factors that may cause PAHs in food consist of high temperature, long period cooking, and direct frying for food production. The contamination of PAHs can also be occurred by raw material in which come from some operation during their processing, such as seed drying or using solvent extraction. Hence, the quality of cooking oil should be controlled because PAHs are produced from some of these materials.

2.1.2 Degradation

PAHs decompose via two main processes: degradation and biodegradation that can be examined by both aerobic and anaerobic conditions. The biodegradation process related with bacteria, fungi, and algae *(31)*. For degradation, PAHs are chemically stable, very poor degraded by hydrolysis and susceptible to oxidation and photo-degradation. PAHs half life in the air ranges from a few hours to several days and the estimation of PAHs half life in soil was varied from several months to several years. The PAHs half life in soil and air depends on various parameters such as type of adsorption onto particles, molecular weight. A biotic degradation may remove 2-20% of two and three ring PAHs in contaminated soils. PAHs with four or more aromatic rings persist in the environment but they may strongly adsorb on organic matter. Following degradation process, oxidized products may form and tend to react with biological components. The reaction between nitrogen oxides and nitric acid in the atmosphere can form nitro derivatives, which could contaminate in foods. Although parent compounds cannot be detected in PAHs contaminated foods, the degradation products or derivatives that have significant toxicity may be present.

2.1.3 Toxicology and Regulation

PAHs toxicity is very structurally dependent with isomers varying from being nontoxic to extremely toxic. Thus, highly carcinogenic property of PAHs may be low or high. One of PAH compound, benzo(a)pyrene, is notable for being the first chemical carcinogen that has ever discovered. The experimental laboratory in case of animal studies which the carcinogenicity of PAHs compound is distinctly expressed, irrespective of administration route, have been reported. The studies indicate that the amounts of different PAHs are necessary to induce cancer in 50% of treated animal (LD_{50}), *(31)* the acute oral toxicity are shown in Table 2.2.

Table 2.2 Acute oral toxicity of PAHs

Compounds	Species	LD_{50}(mg/kg)	Reference
Anthracene	Mouse	18000	Montizaan et al. (1989)
Benzo(a)pyrene	Mouse	>1600	Awogi and Santo (1980)
Fluoranthene	Rat	2000	Smyth et al. (1962)
Naphthalene	Rat	1250	Sax and Lewis (1984)
	Rat (M)	2200	Gaines (1969)
	Rat (F)	2400	Gaines (1969)
	Rat	9430	US EPA (1978a)
	Rat	1110	Montizaan et al. (1989)
	Mouse (F)	354	Plasterer et al. (1985)
	Mouse (M)	533	Shopp et al. (1984)
	Mouse (F)	710	Shopp et al. (1984)
	Guinea-pig	1200	Sax and Lewis (1984)
Phenanthrene	Mouse	700	Montizaan et al. (1989)

Besides considering about health issues, the assessment of degradation level required tedious time. It is well known that when heating with extended time, overheating or over-using the frying oil lead to form were changed oxidation products. Many of these

oxidation products such as aldehydes, hydroperoxides, polycyclic aromatic hydrocarbons, and polymeric substances cause adverse health and biological effects such as increase in liver and kidney size as well as a kind of cancer. Due to their toxicity, many organizations have set a limit value of PAHs in a various kind of food, such as Spain has set a maximum level in olive oil of 2 μg/kg for each compound and the amount should not exceed 5 μg/kg, and German Society for Fat Science proposed a value of 5 μg/kg in fat and oil for total heavy PAHs and 25 μg/kg for both light and heavy PAHs *(17)*. Another legislation in 2005, the European Union has legislated a limit for PAHs in foods (only benzo(a)pyrene) which related with human consumption or use as an ingredient in foods (excluding cocoa butter until 01/04/07) of 2 μg/kg.

2.2 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a chromatographic technique normally used in analytical chemistry to separate, identify, and quantify compounds. The technique is based on the polarities and interactions between analytes and the stationary phase in column as well as mobile phase. In general, a HPLC system consists of five major components i.e. mobile phase reservoirs, pump, column, injection unit, and detector. A schematic diagram of a typical HPLC instrument is shown in Figure 2.2. A system of chromatographic process begins with sample injection at an injector, then mobile phase carries a sample solution through the column by a pumping system while the separation is occurred. Finally, analyte is detected by detector which provides a characteristic retention time for the analyte. The retention time of analyte is varied depending on its interaction strength with the stationary phase, the ratio or the composition of solvents used, and the flow rate of the mobile phase *(11, 32)*. Finally, the separation of each individual component is shown in forms of chromatogram.

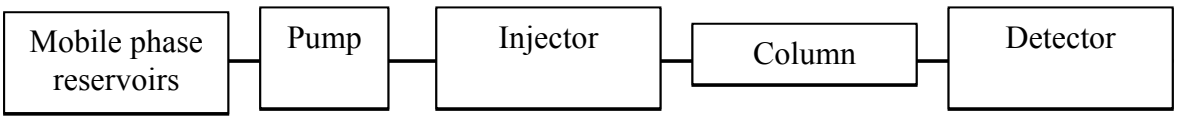


Figure 2.2 Schematic diagram of a typical HPLC instrument.

2.2.1 Mobile Phase System

The mobile phase is the part of the chromatographic system which carries the solutes through chromatographic column where the sample interacts with the stationary phase. The mobile phase can be a non-polar solvent (normal phase) or polar solvent (reverse phase). In HPLC, the mobile phase type depends on chromatographic method and type of detector. Moreover, all liquid entering the HPLC system should be filtered due to the small particles and the solvent used as mobile phase is generally HPLC grade because impurities in solvent can react with solute *(33)*.

2.2.2 Pumping System

Passing mobile phase through the column at high pressure and controlling flow rate is the function of the pump. There are a number of different types of pumps that can provide the necessary pressures and flow-rates required by the modern liquid chromatograph. Constant pressure pump applies a constant pressure to the mobile phase but flow rate will changes with the flow resistance while constant flow pump generates steady flow of liquid but a pressure will vary depending on the flow resistance. In general, HPLC pump configuration consists of pistons, seals, check valves pulse dampers, prime/purge valves. In the early years of the LC renaissance, there had two types of pump that commonly used, pneumatic pump and syringe pump. The pneumatic pump was achieved high pressures by pneumatic amplification, and the syringe pump was simply a large, strongly constructed syringe with a plunger that was driven by a motor. Today the majority of modern chromatographs are fitted with reciprocating pumps that fitted with either pistons or diaphragms.

2.2.3 Sample Introduction System

Samples are injected into the HPLC at injection port which are commonly consisting of an injection valve and a sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop, then the sample is drawn into a syringe and injected into the loop via the injection valve. Valve rotor will closes and opens the loop in order to inject the sample into the stream of the mobile phase.

Normally, loop volumes can range between 10 μL to over 500 μL. Injector has both a manual injector and an auto injector. The manual injector can be done with only single injection and auto injector can be programmed to do up to 99 injections in a sequence but in modern HPLC systems, the sample injection is typically automated. The function of the injector is to place the sample into the high-pressure flow in as narrow volume as possible so that the sample enters the column as a homogeneous, low-volume plug. To minimize spreading of the injection volume during transport to the column, the shortest possible length of tubing from the injector to the column should be used.

2.2.4 Column (Stationary Phase)

The column is normally a stainless steel tube containing particle of stationary phase. Generally, the column has a number of alternative lengths and diameters such as 10, 12.5 or 15 cm for lengths and internal diameters of 3, 6.2 or 9 mm. Column can also be packed with 10, 5 or 3 μm diameter particles. There are several types of phase i.e. normal bonded phase, reversed phase, size exclusion, ion exclusion, and ion exchange. The majority of HPLC analyses using reversed phase systems is the columns containing chemically modified silica stationary phase (non polar). Inside the column, the mixture is resolved into its component parts. This method separates analytes based on adsorption to a stationary surface chemistry and by polarity. Reversed phase uses a non polar stationary phase and a polar mobile phase, and effectively work for separating analytes soluble in polar solvents. The analyte associates with the mobile phase and retained by non polar stationary phase. Adsorption strengths will increase when analyte polarity decrease, and the interaction between non polar analyte and non polar stationary phase increases the elution time. The use of non polar solvents in the mobile phase will decrease the retention time of the analytes.

2.2.5 Detector

The detector for HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is

positioned immediately posterior to the stationary phase in order to detect the compounds when they elute from the column. There are many types of detectors that can be used with HPLC. Some of the common detectors include: Refractive Index (RI), Ultra-Violet (UV), Fluorescent, Radiochemical, Electrochemical, Near-Infra Red (Near-IR), Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Light Scattering (LS). The most popular HPLC detectors based on spectroscopic measurements are UV/visible and fluorescence detectors. The resulting chromatogram of UV/visible is a plot of absorbance as a function of elution time and fluorescence is a plot of intensity as a function of time.

2.3 Sample Preparation Techniques

Sample preparation is the most essential step in analytical techniques, because they are often not responsive to the analyte in various matrices or the results are distorted by interfering of samples. More than a half of the work activity and operating cost in an analytical technique is spent for preparing samples in the introduction into an analytical device. In general, the component of target analyte is present in the level that is too low for detection. Thus, sample preparation can concentrate the component into adequate levels for measurement; however, it depends on the type of sample. Sample preparation may involve dissolution, reaction with some chemical species, or many other techniques. Many recent studies have focused on how to develop sample preparation procedures to remove interferences, increase the concentration of analytes, and provide a simple, inexpensive, robust, and reproducible method. There are many traditional sample preparation methods such as liquid-liquid extraction (LLE), saponification step with KOH, supercritical fluid extraction (SFE), which are still used large amounts of toxic organic solvent, tedious time consuming. These problems may result in environmental impacts and potential health hazards.

2.3.1 Low-Temperature Cleanup

Low-temperature cleanup method was introduced to analyze organophosphorus pesticide residues in fat and oil samples in 2007 by Zhiqiang et al. *(32)* and in 2001 by Lentza-Rizos. et al. *(34)*. This procedure was developed to separate analytes within

one operation by using some less polar and non polar analytes from lipid or oil samples. The extraction principle is extraction of sample with pure or mixture of organic solvent and the selection depends on polarity of target analyte, and then prepare fat precipitation with very low temperature. Thus, the polarity of organic solvents must be strong enough to solubilize target analyte. At very low temperatures, fat was frozen out and organic solvent can be simply separated. Then, the solution was furthered cleanup by solid-phase extract (SPE) such as alumina N cartridge, florisil, and SPE C_{18}. Then, the eluting solution was evaporated to dryness in a rotary evaporator to increase sample concentration. However, one consideration of this method are fat precipitation and percentage of fat remaining after freezing step with the selected organic solvent which was allowed fat to frozen out. Therefore, the freezing time is a parameter that has to be optimized to determine the completeness of fat precipitation. According to this preliminary mention, this technique is a simple, compact, fast, and cost-efficient sample preparation technique using only small amount of organic solvent. The technique allows for convenient treatment of multiple samples simultaneously.

2.3.2 Sweep Co-Distillation

Storherr and Watts reported sweep co-distillation (SCD) methods for fats in 1965 and Dingle, Heath and Black *(35)* used sweep co-distillation equipment designed and produced to monitor meat fats for organochlorine and organophosphorus pesticide residues. Sweep co-distillation is a sample preparation technique that is simple, fast, cost-efficient and using only small amount of organic solvent. The technique allows a treatment of multiple samples simultaneously. The basic principle of this technique is operated by Universal Trace Residue Extraction instrument (UNITREX). There has melted the sample in order to prevent the sample solidify in the needle before injection. The sample is distillated with controlled temperature and purged by a gas in order to trap the target analyze in the sorbent. Then, the sorbent was eluted by a mixture of organic solvent. Sweep co-distillation is one of the techniques that have been used in sample preparation for oil and fat samples *(36).* This method has many advantages such as 1) high sample throughput; 2) no clean-up step; 3) a closed

extraction system; and 4) using small volume of extracted solvent. The temperature and distillation time on extraction was performed and analyzed by HPLC.

2.3.3 Solid-Phase Extraction (SPE)

Solid-phase extraction (SPE) has currently come to be an increasingly useful tool for sample preparation of a wide variety of samples. SPE products are now widely used for sample extraction, preconcentration and cleanup of analytical samples. SPE is an extraction technique based on the selective partitioning of one or more components between two phases, one of which is a solid sorbent and the second phase typically is a liquid. With SPE, many of the problems associated with liquid-liquid extraction can be prevented , such as incomplete phase separations, less-than-quantitative recoveries, use of expensive, and disposal of large quantities of organic solvents. SPE is more efficient than liquid-liquid extraction, yields quantitative extractions that are easy to perform. Furthermore, SPE extraction technique can be classified into two strategies The first strategy is an extraction technique used to prepare samples for subsequent analysis by removing interfering substances which is perform by retaining the substance of interest and washing off everything else. The second strategy is called pass-through cleanp by retaining the interfering substances and eluting the product of interest. It is usually used to cleanup a sample before using a chromatographic to quantify the amount of analyte in the sample. The general procedure is to load a solution onto the SPE phase, wash away the undesired components, and then wash off the desired analytes with another solvent into a collection tube. The extraction procedure of SPE system is shown in Figure 2.3. A simple SPE tube has 2-4 mm I.D. and 2-4 cm long and made from stainless steel or a suitable inert polymer. The extraction tube is usually packed with an appropriate bonded phase *(37, 38)*. The separation mechanisms of SPE can be categorized into four types: normal phase, reverse phase, ion exchange, and mixed mode *(38, 39)*

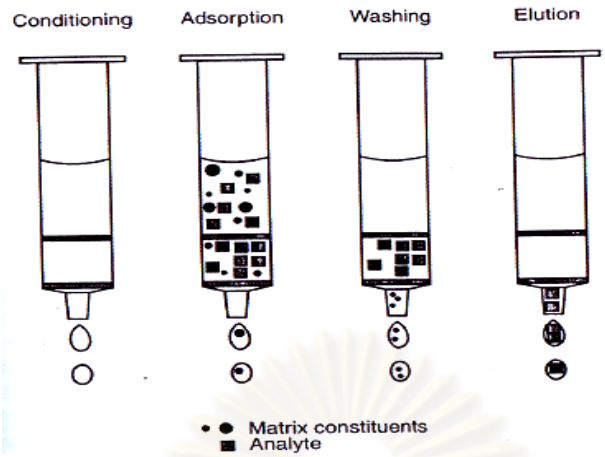


Figure 2.3 The basic procedure of extraction in SPE.

2.3.3.1 Mode of Solid-Phase Extraction

Normal phase

Normal phase SPE procedures typically involve a polar analyze and a polar stationary phase. The mechanism between polar functional groups of the analyze and polar groups on the sorbent surface were occurred with hydrogen bonding, pi-pi interactions, dipole-dipole interactions, and dipole-induced dipole interactions. Polar-functionalized bonded silicas (LC-CN, LC-NH_2, and LC-diol), and polar adsorption media (LC-Si, LC-florisil, ENVI-florisil, and LC-alumina) are typically used under normal phase conditions. Polar compounds are adsorbed by this mechanism and then eluted with a more polar than the original sample matrix.

Reverse phase

A polar or moderately polar sample matrix and a nonpolar stationary phase are involved with reverse phase procedures. Several types of stationary phase are C-18, C-8, C-4, cyano, and amino groups. Retention of organic analytes from polar solutions onto SPE sorbents is primarily occurred due to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. This hydrophobic interaction between nonpolar and nonpolar attractive forces is van der waals or dispersion forces. To elute an adsorbed compound from a reversed

phase, SPE uses a nonpolar solvent to disrupt the forces that bind the compound to the packing.

Ion exchange

Ion exchange SPE can be used for compounds that are charged in a solution. The sorbent contains ionized functional groups as primary, secondary amines or carboxylic acid, or associates with oppositely charged. The mechanism of the compounds is mainly based on the electrostatic attraction of the charged functional group on the compound to the charged group that is bonded to the silica surface. Thus, ion exchange sorbent contains both weak and strong cation and anion functional groups. Anion exchange was occurred when the sorbent contains a positively charged functional group and the exchangeable counter ion on the analyte is negatively charged. On the other hand, if the sorbent surface is negatively charged and the exchangeable counter ion on the analyte is positively charged, it is called "cation exchange". In order for a compound to retain by ion exchange from an aqueous solution, the pH of both the compound of interest and the functional group on the bonded silica are very important. Therefore, a solution having a pH that neutralizes either the compound's functional group or the functional group on the sorbent surface is used to elute the compound of interest.

Mixed – mode

Mixed-mode stationary phase contains two different functional groups on the same sorbent as hydrophobic and strong cation exchange ligands. This sorbent is useful for the separation of complex samples by chemically co-bonding of strong propylsulfonic acid and octadecyl groups onto the silica surface. Moreover, mixed-mode SPE extends pH range for the good retention of acids or bases by reversed phase, ion-exchange or both phases.

2.3.3.2 Process of Solid-Phase Extraction

In general, SPE process can be divided into four main steps: conditioning, loading sample or adsorption, washing and eluting analytes *(39, 40)*. Figure 2.3 illustrates the basic procedure of extraction in SPE.

In the conditioning step, the purpose of this step is to make the sorbent compatible with sample to closely contact inside small channels. Afterwards, a few volumes of solvent are passed through the SPE cartridge, typically acetonitrile or methanol. In this step, the sorbent should not be dry at any stage because it tends to be coiled up.

The next step is loading a sample into the SPE cartridge. Overall, samples need to be dissolved in an appropriate solvent before loading. After the liquid sample is passed through the packed column, the flow rate of a sample should be controlled which depend on the column dimensions and on the particle size of the solid extraction. Moreover, the column is not allowed to dry because some sample matrix may retain the sorbent.

In the washing step, the removal of the interferences coadsorbed in the SPE column is the purpose of this step, so the selection of appropriate solvent is concerned that it must not be too strong to partially eluteing the analyze of interest.

In the last step, the adsorbed analytes were removed from the sorbent by appropriate eluting solvent and returned into liquid phase that is suitable for analytical measurement. Solvent should be studied to completely elute the analytes from the sorbent as small volume as possible. Furthermore, the eluting solvent should have a low boiling point, impurity free, low cost and nontoxic.

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 High performance liquid chromatography (HPLC) with fluorescence detector: A module 1200 ^{TM}series consists of automatic vacuum degasser, binary pump, autosampler and column thermostat compartment, Agilent Technologies, Palo Alto, U.S.A.

HPLC column: Water PAH C_{18}, 4.6 x 250 mm I.D., 5 µm, Waters-Corporation, Milford, Massachusetts, U.S.A.

C_{18} high performance guard column, Agilent Technologies, Pola Alto, U.S.A.

3.1.2 Universal Trace Residue Extractor (UNITREX): consists of an insulated, cylindrical, aluminum blocks, heater controls and the pneumatics for the carrier gas system, Scientific Glass Engineering (SGE) Pty Ltd, Australia

UNITREX packing: consists of

- UTX-C Fractionation tubes
- UTX-T Traps
- UTX-R Solvent Reservoir
- Syringe 1.2 MR-U-GT

3.1.3 Milli-Q, Ultrapure W-Q, water systems with Millipak® 40 Filter unit 0.22 µm, model ZFMQ050RG, Millipore, Billerica, MA, U.S.A.

3.1.4 A rotary evaporator (Büchi, Flawil, Switzerland)

3.1.5 Analytical balance (5 digits), Model MC 210 S, Max 210 g, Sartorious AG Gottingen, Germany.

3.1.6 Analytical balance (3 digits), Model LP 620S, Max 650 g Sartorious AG Gottingen, Germany.

3.1.7 Vacuum pump with pressure regulator, Model SUE 300E, Heto-Holten A/S 17-19 DK-3450 Allerod, Denmark.

3.1.8 Vortex mixer, Model KMS1, IKA-works Industries, Willmington, U.S.A.

3.1.9 Shaker, Model SA 300 (2-way Shaking Method), Yamato Scientific Co., Ltd., Tokyo, Japan.

3.1.10 Micro-pipettes 10-100 µL, 20-200 µL, 100-1000 µL and tips, Eppendorf, Hamburg, Germany.

3.1.11 Syringe filters, PTFE 13 mm, 0.2 µm, Vertical Chromatography Co., Ltd.

3.1.12 Refrigerator, SANTO Medical Freeze Coperation, Scientific, Co., Ltd., Tokyo, Japan.

3.1.13 HPLC amber vials 2 mL with PTFE caps, Agilent technologies, Pola Alto, U.S.A.

3.1.14 Round bottle flasks 25 mL, 50 mL.

3.1.15 Beakers 10 mL, 50 mL, 150 mL, 250 mL.

3.1.16 Graduated cylinders 20.0 mL, 25.0 mL, 50.0 mL, 100.0 mL, 250.0 mL.

3.1.17 Volumetric flasks 10.00 mL, 25.00 mL, 50.00 mL.

3.1.18 Volumetric pipettes 1.00 mL, 2.00 mL, 5.00 mL.

3.1.19 Oak Ridge Centrifuge Tubes, polypropylene copolymer; polypropylene screw closure, NALGENE®

3.1.20 Glass syringe 10.0 mL, TOP Surgical Manufacturing Co., Ltd.

3.1.21 Solid-Phase Extraction

- Sep-Pak®Plus Alumina N Cartridges, 1710 mg, 1.2 mL, Waters Corporation, Milford, Massachusetts, U.S.A.
- Sep-Pak®Florisil Cartridges, 1710 mg, 1.2 mL, Waters
- Sep-Pak®C_{18} Cartridges, 500 mg, 3.0 mL, Waters Corporation, Milford, Massachusetts, U.S.A.

All experimental glasswares were washed with detergent and rinsed with deionized water, then allowed to dry at room temperature and rinsed with hexane before use.

3.2 Chemicals

3.2.1 PAHs Standard

Dibenzo(a,h)anthracene (DiahA), benzo(g,h,i)perylene (BghiP), indeno(1,2,3-c,d)pyrene (123cd), fluorene (Fl), fluoranthene (Ft), and chrysene (Chry) purity were

99.0%. Acenaphthene (Ace), phenanthrene (Phen), anthracene (Ant), benzo(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(e)pyrene (BeP), benzo(k)fluoranthene (BkF), and benzo(a)pyrene (BaP) purity were 99.5%. Naphthalene (Naph) purity was 99.8%, and pyrene (Pyr) purity was 98.0%. All standard compounds were purchased from Dr. Ehrenstorfer (Augsberg, Germany).

3.2.2 Organic Solvents

All solvent used (e.g. acetonitrile, methanol, acetone, dichloromethane and hexane) were HPLC grade. Acetonitrile, methanol and acetone were supplied by Burdick & Jackson (SK Chemicals, Ulsan 680-160, Korea). Dichloromethane was purchased from Merck (Darmstadt, Germany) and hexane was purchased from Fisher Scientific (UK Limited).

3.2.3 Reagents

Sodium sulphate anhydrous* was analytical reagent grade from Fisher Scientific (UK Limited). Florisil for pesticide residue analysis** was purchased from Sigma-Aldrich (Chemie GmbH, CH-9471 Buchs). Glasswool for silane treatment was purchased from Supelco, INC (Bellfonte, Pennsylvania).

*Sodium sulphate anhydrous was treated before use by heating at 650 ^{o}C for 3 hours, and allowed to cool down to room temperature for overnight. After that, it was transferred to a sealed glass container.

**Florisil was first activated by heating at 600 ^{o}C for 2 hours and allowed to cool down to room temperature. After that, the florisil was transferred to a sealed glass container and allowed to equilibrate for 48 hours. This material can be used for up to 2 weeks. Before use, florisil is deactivated by adding 1.0 mL water into 99.0 g of activated florisil. (The optimum amount of water deactivation should be in range 0.5% to 2.0%.) Hand shaking was intermittently taken for 15 minutes and left it overnight. Again, it was shaken over 15 minutes only before use. This deactivated florisil was suitable for use up to a week.

3.3 Preparation of PAHs Standard Solutions

3.3.1 Stock Standard Solutions

There were two types of PAHs standard solution. The first type was prepared by dissolving PAHs with methanol and employed for calibration curve. The second type was prepared by dissolving PAHs with hexane, and used as the analyte spiking solution. Individual PAHs standard solution of 100 µg/mL was prepared by weighing 2.5 mg of each standard and dissolving with methanol in 25.00 mL volumetric flask. These stock standard solutions were kept in brown glass bottle with screw cap and stored at 4 ºC.

3.3.2 Intermediate Standard Solutions

The intermediate standard mixture solutions of 16 PAHs was prepared by diluted stock standard solution into 50.00 mL volumetric flask with methanol or hexane which contained 0.1 µg/mL of fluorene, anthracene, benzo(k)fluoranthene and benzo(a)pyrene, 0.2 µg/mL of acenaphthene, chrysene and benzo(a)anthracene, 0.3 µg/mL of dibenzo(a,h)anthracene, 0.4 µg/mL of benzo(b)fluoranthene, 0.5 µg/mL of naphthalene, and pyrene, 0.8 µg/mL of benzo(e)pyrene, 1.0 µg/mL of phenanthrene, and benzo(g,h,i)perylene, 1.5 µg/mL of indeno(1,2,3-c,d)pyrene, and 2.5 µg/mL of fluoranthene. (In cases of hexane, the PAHs mixed solution was prepared into hexane after evaporation of methanol). This intermediate standard solution was kept in brown glass bottle with screw cap and stored at 4 ºC.

3.3.3 Working Standard Mixture Solutions

The working standard mixture solutions of 16 PAHs were prepared as followed;

A standard mixture solution at a concentration level of 10.0 ng/mL of benzo(a)pyrene was prepared by diluting 1 mL of the intermediate standard mixture solutions into a 10.00 mL volumetric flask with methanol. The standard mixture solution was stored in brown glass bottle with screw cap and stored at 4 ^{o}C.

A standard mixture solution at a concentration level of 1.00 ng/mL of benzo(a)pyrene was prepared by diluting 1 mL of standard mixture at a concentration level of 10.0 ng/mL of benzo(a)pyrene into a 10.00 mL volumetric flask with methanol. The standard mixture solution was stored in brown glass bottle with screw cap and stored at 4 ^{o}C.

3.4 The Optimization of HPLC Conditions for PAHs Analysis

In this research, the PAHs analysis was performed using Agilent, HPLC module 1200 series, with a solvent degassing unit, a binary pump, an automatic sample injection, column thermostat and fluorescence detector.

The gradient program was developed by varying percentage of acetonitrile and water as mobile phase. The HPLC conditions were summarized in Table 3.1. In addition, the gradient program used for HPLC optimization was presented in Table 3.2 and fluorescence detector conditions were described in Table 3.3.

Table 3.1 The HPLC condition for separation of PAHs

HPLC Parameter	Conditions
Analytical column	PAHs C_{18}, 5 μm, size 250 mm X 4.6 mm id. (Waters)
Guard column	C_{18}, 5 μm
Injection volume	10 μL
Detector	Fluorescence
Column temperature	40 ^{o}C
Mobile phase flow rate	1.5 mL/min

Table 3.2 Gradient program used for HPLC analysis

Time (min.)	% Acetonitrile
0.0	45.0
35.0	90.0
45.0	90.0
55.0	45.0

Table 3.3 Fluorescence detector condition

Time(min.)	Excitation (nm)	Emission (nm)	PMT-Gain
0.0	280	330	11
13.0	280	330	11
13.5	264	410	11
33.0	264	410	11
33.5	290	410	11
35.5	290	410	11
36.0	300	500	11
39.0	300	500	11
40.0	280	330	11

3.5 Sample Preparation of Cooking Oil by Low-Temperature Cleanup Method

3.5.1 Extraction of PAHs in Cooking Oil by Low-Temperature Cleanup Method

The procedures for extraction of PAHs in cooking oil can be described as follows:

3.5.1.1 1.00 g of cooking oil sample (W_{oil}) was weighed into a teflon container. Adding extracted solvent (V_1) and vortexed for 2 minutes.

3.5.1.2 The sample solution was shaked by a shaker for 10 minutes.

3.5.1.3 Kept the solution in the refrigerator under temperature between -18 ^{o}C to -25 ^{o}C.

3.5.1.4 After a certain period, the extracted solution was immediately emptied into a new teflon container leaving the solids which included the frozen oil, behind as far as possible.

3.5.1.5 The residue was re-extracted in a similar way as 3.5.1.2-3.5.1.4.

3.5.1.6 The total extracted solution was transferred from a teflon container to 25 mL round bottom flask. (In case of %fat remaining, weighing round bottom flask before placing 5 mL of the extracted solution (V_2) and recorded as W_1)

3.5.1.7 The extracted solution was evaporated to dryness using a rotary evaporator under reduced pressure at 40 ^{o}C.

3.5.1.8 Weighing round bottom flask again and recorded as W_2.

3.5.1.9 The efficiency of fat removal by this method was evaluated by a weight percentage of fat remaining which calculated from

$$\% \text{ Fat remaining} = \left[\frac{((W_2 - W_1) \times V_1)/V_2}{W_{oil}} \times 100 \right]$$

3.5.1.10 Dissolving the residue from 3.5.1.8 in 1 mL of mixed solvent of hexane/dichloromethane (1:1, v/v) and loading the sample solution to SPE cartridge which was Sep-Pak Alumina N*.

**Activated the Sep-Pak Alumina N cartridge before loading the sample by sequentially added 5 mL of dichloromethane and 5 mL of hexane.*

3.5.1.11 The analytes were eluted from SPE cartridge with 10 mL of mixed solvent of hexane/dichloromethane (1:1, v/v).

3.5.1.12 The solvent was evaporated to dryness in a rotary evaporator again (40 ^{o}C, reduced pressure) and re-dissolved in 1 mL of acetonitrile. Filtered the solution through 0.2 μm syringe filter and analyzed PAHs by HPLC.

3.5.1.13 The extraction of PAHs in cooking oil was evaluated as percent recoveries of analyte.

Spiked samples were prepared by adding the standard mixture solutions into cooking oil sample at concentration level of 5.0 ng/g of benzo(a)pyrene.

3.5.2 The Study of Freezing Time

Freezing step in low-temperature cleanup method will allow a fat to be frozen and precipitated out from the sample solution. Therefore, freezing time has to be optimized to get rid of all fat in oil sample. The optimization of freezing time was performed as the procedure in 3.5.1 and compared the percentage of fat remaining. The extracted solvent used in this study was 8 mL of mixed solvent of acetonitrile/acetone (80:20, v/v) and the freezing time was varied as 4, 8, 12, 24 and 36 hours.

3.5.3 Selection of Extraction Solvent

The type of solvent affects the extraction efficiency; hence, the organic solvent chosen must strong enough to solubilize both light and heavy PAHs. Consequently, the polar and non-polar solvents were optimized including acetonitrile and mixture of acetonitrile/acetone. The optimization of extraction solvent was performed as the procedure in 3.5.1 at optimum freezing time from 3.5.2 and compared the percentage of recovery. The volume of 4, 8, 10 mL of pure acetonitrile and mixture solvent of acetonitrile/acetone at ratio 90:10 and 80:20 (v/v) were studied.

3.5.4 The Study of Number of Extraction

To improve the extraction of analytes in low-temperature cleanup system**,** number of extraction was investigated. The optimization of number of extraction time was performed as the procedure in 3.5.1 and compared the percentage of recovery. The extraction time was varied as 1, 2, and 3 times and the freezing time and extraction solvent was used at the optimum value from 3.5.2 and 3.5.3.

3.5.5 Comparison of Solid-Phase Extraction (SPE) Cartridges

Three types of SPE cartridges (Sep-Pak alumina N, florisil, and SPE C_{18}) were tested. The optimum conditions of freezing time and extracted solvent were performed which were 24 hours and 8 mL of acetonitrile/acetone (80:20, v/v), respectively. The procedure of low-temperature cleanup method was followed section 3.5.1. In addition, the activation and elution of analyte from the cartridge were described as followed.

SPE C_{18} cartridge

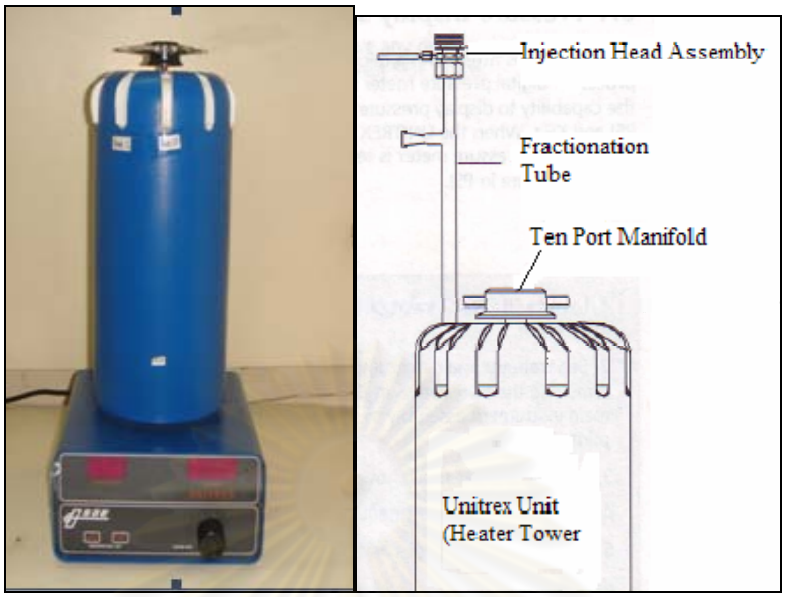
The cartridge was conditioned by washing sequentially with 5 mL of acetonitrile and 5 mL of water. After sample loading (the sample extracted from 3.5.1.10 was re-dissolved with 5 mL of mixture of acetonitrile:water (1:4, v/v)), the cartridge was washed with 5 mL of 10 % acetonitrile in water. Then, the cartridge was allowed to dry by drawing air through for a minute. After that, the analytes were eluted with 10 mL of mixture of acetonitrile/acetone (1:1, v/v).

Florisil cartridge

The cartridge was activated by sequentially washing with 5 mL of dichloromethane and 5 mL of hexane. After sample loading, the analytes were eluted from the cartridge with 10 mL of mixed solvent of hexane/dichloromethane (1:1, v/v).

3.6 Sample Preparation of Cooking Oil by Sweep Co-Distillation

Sweep co-distillation was performed on the Universal Trace Residue Extractor (UNITREX ^{TM}) II system *(41)*. The components and setup of the system were shown in Figure 3.1 and 3.2.



(a) **(b)**

Figure 3.1 (a) Universal Trace Residue Extractor (UNITREX) II system *(41)*.
(b) Assembly of fractionation tube and heater tower

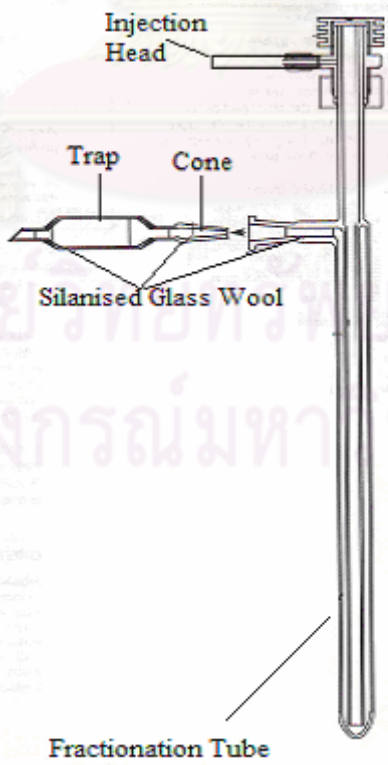


Figure 3.2 Setting up of fractionation tube

3.6.1 Operation of Universal Trace Residue Extractor (UNITREX)

The operation of UNITREX was done by turn on the heater unit and let the temperature in heater tower to stabilize at required temperature. Afterwards, the fractionation tubes assembled as shown in Figure 3.2 were placed into the unit and connecting a nitrogen gas line to each tube. The traps connected with the fractionation tube must be freshly packed with 0.80 ± 0.05 g deactivated florisil and 1.5 g granular anhydrous sodium sulphate as illustrated in Figure 3.3. Then, adjusting the nitrogen gas to given a flow of 230 mL/min to each fractionation tube. A part of the extraction with UNITREX allows a treatment of ten samples simultaneously.

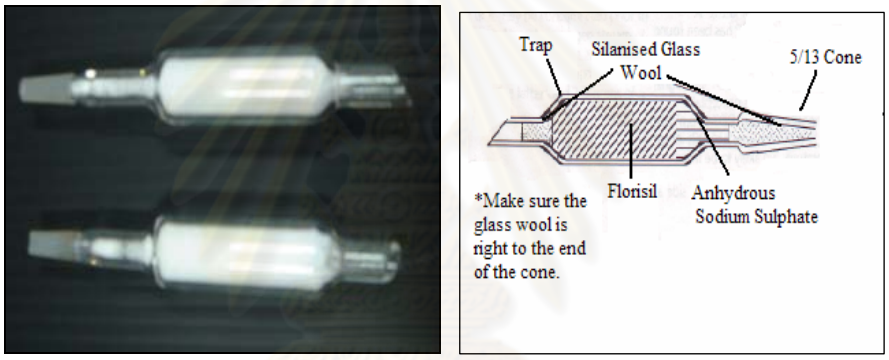


Figure 3.3 Packing of trap unit

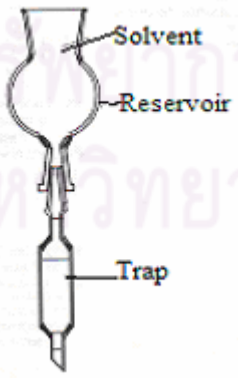


Figure 3.4 Setting up the reservoir

3.6.2 Extraction of PAHs in Cooking Oil by Sweep Co-Distillation Method

The procedures for extraction of PAHs in cooking oil can be described as follows:

3.6.2.1 The sample was warmed at 50 ^{o}C before introducing into the fractionation tube.

3.6.2.2 Draw up 1.14 mL (1.00 g) of cooking oil sample into the syringe and inject through the pre-punctured septum into the fractionation tube. (Rinse the syringe with hexane immediately after use.)

3.6.2.3 The sample was distilled by setting up temperature and time as investigation.

3.6.2.4 After a certain period, carefully remove the fractionation tube with the trap attachment from the heated block.

3.6.2.5 Elute the analytes from the trap with 12 mL of mixed solvent of hexane/dichloromethane (70:30, v/v) by immediately connect the florisil trap to the solvent reservoir illustrated in Figure 3.4.

3.6.2.6 The extract solution was evaporated to dryness in a rotary evaporator (40 ^{o}C, reduced pressure) and re-dissolved with 1.0 mL of acetonitrile. Filtered the solution through 0.2 μm syringe filter and analyzed PAHs by HPLC.

3.6.2.7 The extraction of PAHs in cooking oil was evaluated as percent recoveries of analyte.

Spiked samples were prepared by adding the standard mixture solutions into cooking oil sample at concentration level of 10.0 ng/g of benzo(a)pyrene.

3.6.3 The Study of Distillation Temperature and Time

Sweep co-distillation is a technique used for cleanup of sample extraction by volatilized analytes in a controlled temperature chamber and in the presence of an inert gas purging. Then, volatilized analytes were collected by a trap. The less volatile analytes were still retained in the fractionation tube. Thus, the temperature and time should optimize to enrich all PAHs in oil sample. The optimization of temperature

and time were performed as the procedure in 3.6.2 and compared the percentage of recovery. The distillation temperature and time were varied at 230 and 235 ^{o}C for 30, 45 and 60 minutes, respectively.

3.7 Method Validation

Method validation is a process to verify that an analytical test system is suitable for its intended purpose and is capable of providing useful and valid analytical data. Thus, in this research, both developed methods were evaluated for standard calibration curve, limit of detections (LODs), limit of quantifications (LOQs), linearity and working range, accuracy, precision, and trueness.

3.7.1 The Study of Standard Calibration Curve

The procedure for the study of calibration curve can be described as follows:

Mixed standard solution of 16 PAHs were prepared at 6 concentration levels as present in Table 3.4 and analyzed by HPLC under optimal conditions (Table 3.1, 3.2, and 3.3). Each concentration level was analyzed in triplicate. The intercepts, slopes and coefficient of determination (R^2) of calibration curves were presented.

Table 3.4 Concentration of mixed standard solution (ng/g) for the study of calibration curve, linearity and working range.

Compounds	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Naph	1.25	2.50	5.00	10.0	20.00	30.00
Ace	0.50	1.00	2.00	4.00	8.00	12.00
Fl	0.25	0.50	1.00	2.00	4.00	6.00
Phen	2.50	5.00	10.00	20.00	40.00	60.00
Ant	0.25	0.50	1.00	2.00	4.00	6.00
Ft	6.25	12.50	25.00	50.00	100.00	150.00
Pyr	1.25	2.50	5.00	10.00	20.00	30.00
BaA	0.50	1.00	2.00	4.00	8.00	12.00
Chry	0.50	1.00	2.00	4.00	8.00	12.00
BeP	2.00	4.00	8.00	16.00	32.00	48.00
BbF	1.00	2.00	4.00	8.00	16.00	24.00
BkF	0.25	0.50	1.00	2.00	4.00	6.00
BaP	0.25	0.50	1.00	2.00	4.00	6.00
DiahA	0.75	1.50	3.00	6.00	12.00	18.00
BghiP	2.50	5.00	10.00	20.00	40.00	60.00
123cd	3.75	7.50	15.00	30.00	60.00	90.00

3.7.2 The Study of Linearity and Working Range

Linearity or working range is the degree of proportionality between the measurement taken during the method and the concentration of the compound of interest in the sample *(42)*. Linearity and working range were studied by spiked the sample at concentration level as presented in Table 3.4. Extractions of the PAHs were performed using the developed methods and analyzed by HPLC. Triplicate analysis were done for linearity and working range.

3.7.3 The Study of Limit of Detections (LODs)

The limit of detection is the lowest concentration of analytes that can be distinguished from base line noise. The limits of detection were calculated as three times of signal-to-noise ratio (S/N=3) *(43).* These methods were determined by analyzing samples containing low concentration of analytes that provided a peak height of signal-to-noise ratio equal to 3. Ten replicates analysis was done. The experimental procedure can be described as follows:

3.7.3.1 Blank sample was prepared by using the extraction method described in 3.5.1 for low-temperature cleanup at optimum condition (Freezing time of 24 hours, 8 mL of mixture of acetonitrile/acetone (80:20, v/v) and cleanup with alumina N cartridge and 3.6.2 for sweep co-distillation method at optimum condition (Temperature 235 ^{o}C for 60 minutes).

3.7.3.2 Spiked sample was prepared by spiking standards mixture solution into cooking oil sample and extracted in the same way as blank sample.

3.7.3.3 The blank and spiked samples were analyzed under the optimum HPLC conditions. The peak signals of each compound were measured from chromatograms.

3.7.3.4 The limit of detection of each compound was obtained from the concentration that gave peak height at 3 times over the baseline.

3.7.4 The Study of Limit of Quantifications (LOQs)

The limits of quantifications were calculated as ten times of signal-to-noise (S/N=10) *(44).* These parameters were determined by analyzing samples that provided a peak height of signal-to-noise ratio equal to 10. Ten replicates analysis was done. The experimental procedure was similar to the study of LODs (section 3.7.3) and the limit of quantitation of each compound was obtained from the concentration that gave peak height at 10 times over the baseline.

3.7.5 The Study of Accuracy and Precision

The procedure for the study of method accuracy and precision was carried out in the same way as LODs study. The extractions of spiked sample in 5 levels of concentrations as present in Table 3.4 (level 2 to level 6) were evaluated. Each concentration level was analyzed in ten replicates. The accuracy and precision of the method was calculated and reported in term of percent relative standard deviation (%R.S.D.), standard deviation, and percent recoveries.

The acceptable value for %R.S.D. was calculated from Horwitz equation as followed:

$$RSD_r = 0.67 \times 2^{(1-0.5\log C)} = 0.67 \times 2C^{-0.1505}$$

3.7.6 The Study of Method Trueness

Trueness is the closeness of agreement between the average value obtained from a large set of test results and an accepted reference value *(45)*. The study of trueness was carried out by analyzing a reference material of olive oil (RM FAPAS T0631, olive oil). The sample preparations of this reference material by both low-temperature cleanup and sweep co-distillation methods were performed in duplicate. The final concentration of each compound was reported.

3.8 The Application of Optimized Condition of Developed Method in Real Cooking Oil

After method was completely validated, the optimized condition was applied with real samples. The pure and used cooking oil samples were analyzed. Several kinds of pure cooking oils such as soybean oil, sunflower oil, canola oil, olive oil, and palm oil were tested. These samples were purchased from super markets and used oils were colledted from local markets. Used oils were the mixed oil, soybean oil that used to fry chicken in several times.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 The Optimization of High Performance Liquid Chromatographic Conditions

PAHs analysis was performed using HPLC with fluorescence detector (Agilent Technologies 1200 series). HPLC parameters were as followed; Guard column: C_{18}, 5 μm, Analytical column: PAH C_{18}, 5 μm, 250 mm x 4.6 mm.id. (Waters), Mobile phase: acetonitrile and water in gradient mode, Flow rate: 1.5 mL/min., Column temperature: 40 ^{o}C, Injection volume: 10 μL. The wavelength for fluorescence detector was set as Table 4.1.

Table 4.1 HPLC-FLD excitation and emission wavelength program.

Time (min)	Excitation (nm)	Emission (nm)	PAHs detected
0.0	280	330	Naph
13.0	280	330	Ace, Fl
33.0	264	410	Phen, Ant, Ft, Pyr, BaA, Chry, BeP, BbF, BkF, BaP
35.5	290	410	DiahA, BghiP
39.0	300	500	123cd

The separation of 16 PAHs; naphthalene (Naph), acenaphthene (Ace), fluorene (Fl), phenanthrene (Phen), anthracene (Ant), fluoranthene (Fl), pyrene (Pyr), benzo(a)anthracene (BaA), benzo(e)pyrene (BeP), chrysene (Chry), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenzo(a,h)anthracene (DiahA), benzo(g,h,i)perylene (BghiP) and indeno(1,2,3-

c,d)pyrene (123cd) was well separated within 40 minutes as shown in Figure 4.1. The gradient program for the mobile phase started with 45% acetonitrile (0 min) then increasing linearly to 90% acetonitrile (35 min) and hold at 90% acetonitrile for 10 min. After 45 min (still 90%) the mobile phase was changed back to the initial composition (45% acetonitrile/55% water) within 10 min and allowed to equilibrate for another 10 min. Total runtime of one analysis was this 55 min.

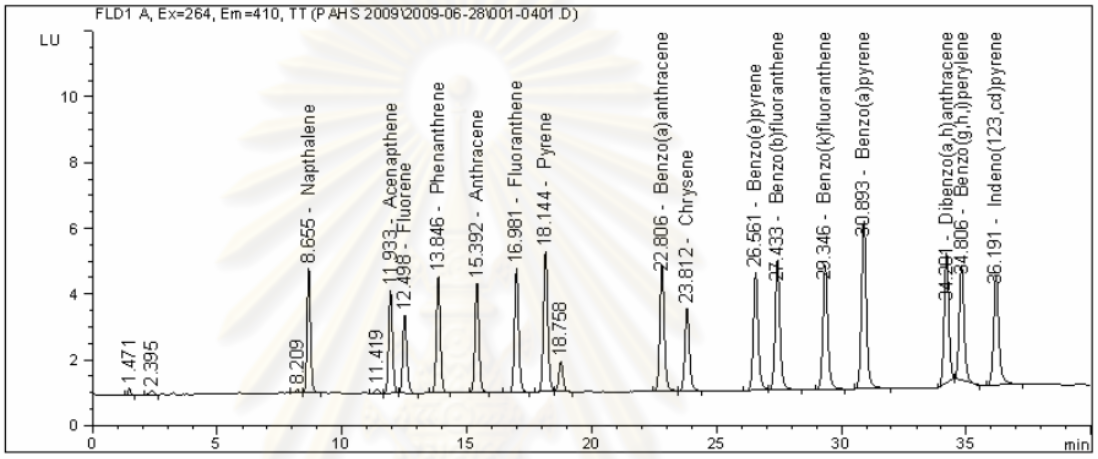


Figure 4.1 The chromatogram of standard mixture solutions of 16 PAHs

4.2 Sample Preparation of Cooking Oil by Low-Temperature Cleanup

Due to fat component of cooking oils, sample preparation was required to get rid of lipid from the samples before determining PAHs by HPLC. Low-temperature cleanup method was applied because this method can precipitate out most of fat in the sample by a simple step. This technique allowed convenient treatment of multiple samples simultaneously. In this study, the method was developed to determine sixteen polycyclic aromatic hydrocarbons in pure and used cooking oils. The extraction procedure was simple and achieved by adding a mixture of organic solvent and subsequently freezing the solution between -18 ^{o}C and -25 ^{o}C. At these temperatures, fat was frozen out and organic solvent can be simply separated and further cleanup by SPE cartridge. The parameters such as freezing time, organic solvent, number of extraction and SPE condition were optimized.

4.2.1 The Optimization of Freezing Time

Freezing time is the time used for precipitation of fat from organic solvent by using low temperature before extraction process. Therefore, percentage of fat remaining in the organic solution after freezing step was observed to optimize the fat removal. Freezing times of 4, 8, 12, 24, 36 hours were investigated and the result was shown in Table 4.2. According to Table 4.2, the minimum time required for leaving samples in the freezer for fat frozen was 12 hours. However, freezing time of 24 and 36 hours result a lower percentage of fat remaining in the extracted solution and less interfering peaks appeared in the chromatograms (Figure 4.2). Though, there was no difference when compared the results from freezing time of 24 and 36 hours (Figure 4.2(b), (c)). Therefore, 24 hours was chosen for freezing time as the shortest time and good efficiency of extraction. All further studied were used 24 hours of freezing time to gain the amelioration of the requisite factor.

Table 4.2 Percentage of fat remaining in the extracted solution from the low-temperature cleanup method at various freezing times.

Freezing time (hrs.)	Results	%fat remaining ± S.D.
4	unfrozen fat	12.20 ± 0.02
8	unfrozen fat	12.05 ± 0.01
12	frozen fat at the bottom of tube	9.71 ± 0.00
24	frozen fat at the bottom of tube	6.83 ± 0.01
36	frozen fat at the bottom of tube	6.77 ± 0.01

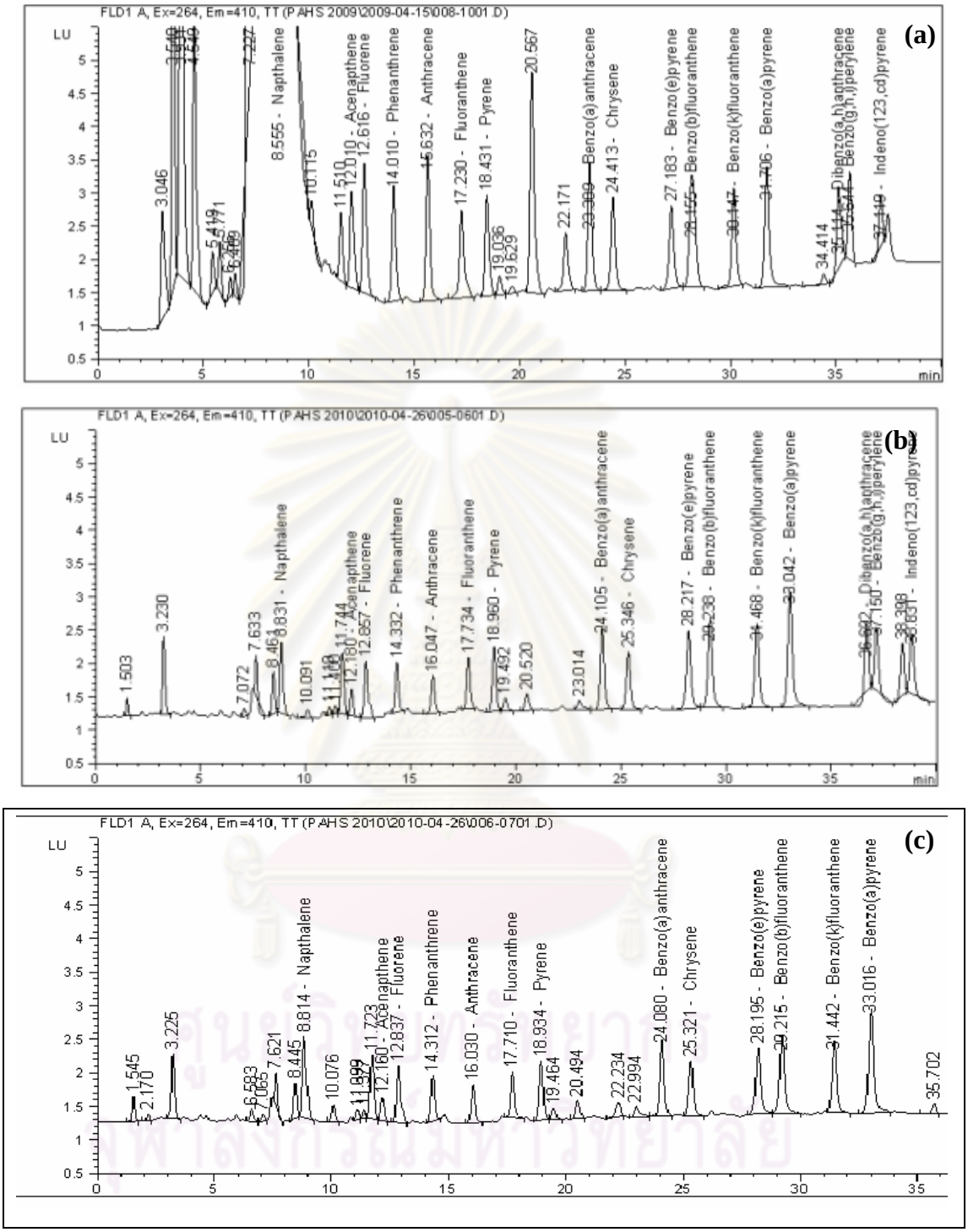


Figure 4.2 The chromatogram of low-temperature cleanup method at different freezing time:

(a) 12 hours

(b) 24 hours

(c) 36 hours

4.2.2 The Optimization of Organic Solvent

In low-temperature cleanup method, selection of organic solvents for extracting PAHs from oil is a critical parameter affecting the extraction efficiency. The polarity of organic solvents must be strong enough to extract both light and heavy PAHs. If the polarity is too high, it may not solubilize the heavy PAHs, whereas if it is too low, oil co-extracted was occured. To get a cleaner final extract with less interference, the use of acetonitrile and a mixture of acetonitrile/acetone as extracting solvents were performed to study the effect of organic solvent polarity. Moreover, the volume of extracting solvent was also studied. Theoretically, acetonitrile is used as a medium-polarity solvent which a wide range of ionic and nonpolar compounds can be extracted. Therefore, it should be easily dissolved PAHs in the sample *(16).* Acetone is classified as polar and non-polar which has an ability to extract nonpolar substances like hydrocarbons *(46).* This solvent might be helpful for extracting PAHs from oils.
According to percentage of fat remaining and percentage of PAHs recovery, a mixture of acetonitrile/acetone at ratio of 80:20 (v/v) and the volume of 8 mL were appropriate as the extracting solvent as shown in Figure 4.3-4.6. Even though, lower amount of oil co-extracted was achieved when using small volume of organic solvent. The low recovery percentage of heavy PAHs were obtained when using 4 mL of all studied organic solvent. For the volume of 8 and 10 mL, pure acetonitrile showed the lower amount of oil co-extracted than acetonitrile/acetone mixtures but the recoveries were unsuitable when compared with those from mixed solvents. Moreover, pure acetonitrile was not satisfactory in cases of evaporation due to its high boiling point *(16).* The improvement of PAHs recovery was greatest with 8 mL of mixed solvent of acetonitrile/acetone in a ratio of 80:20, v/v. At this condition, target analyte recoveries were in the acceptable range (40-120%) *(50).*

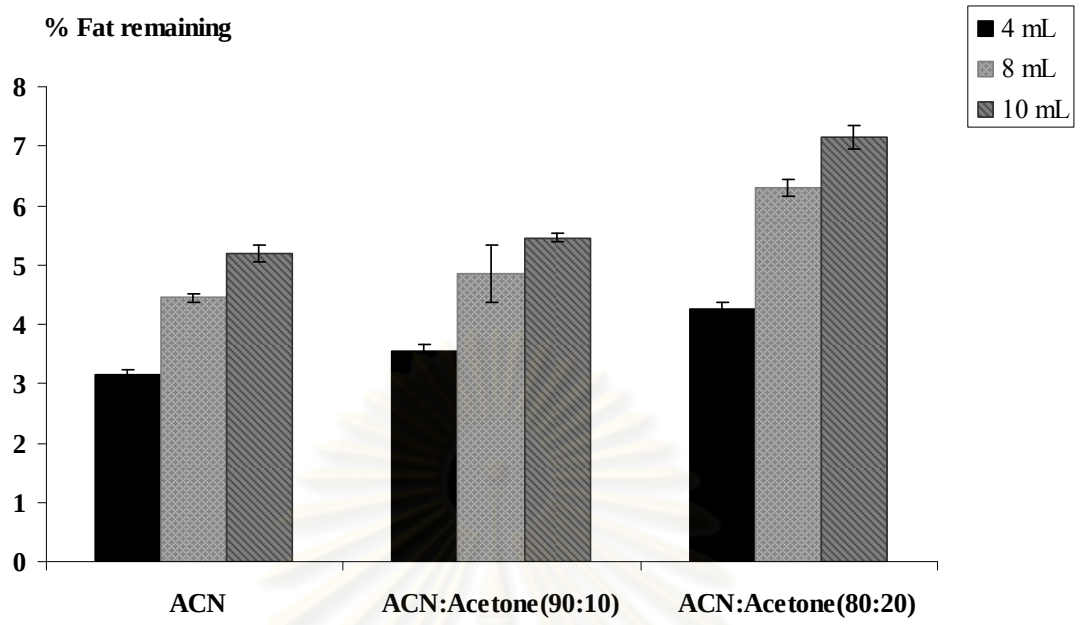


Figure 4.3 The effect of extraction time for fat remaining for 24 hours.

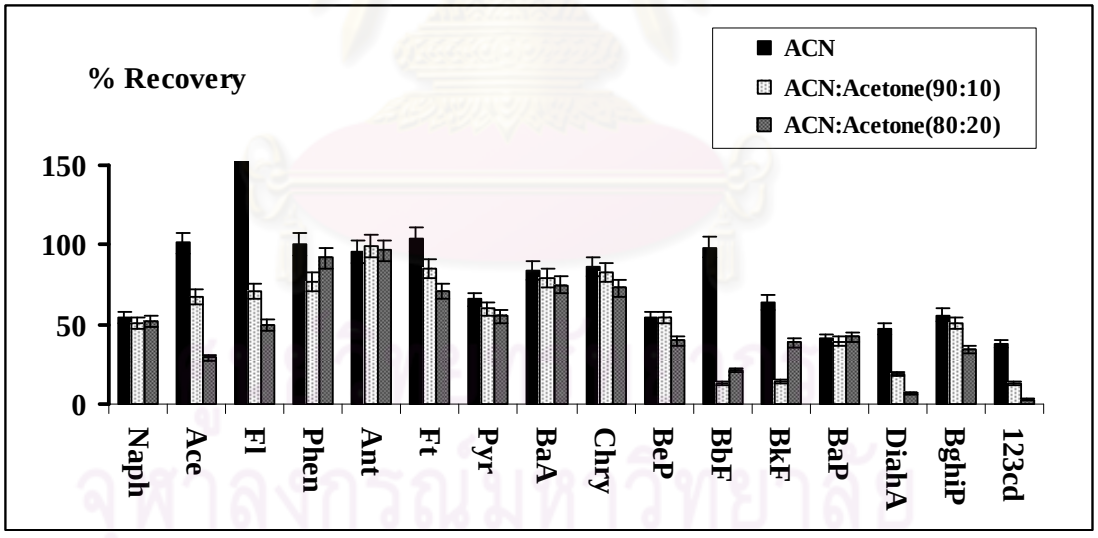


Figure 4.4 Effect of different organic solvent on extraction efficiency of 4 mL of organic solvent.

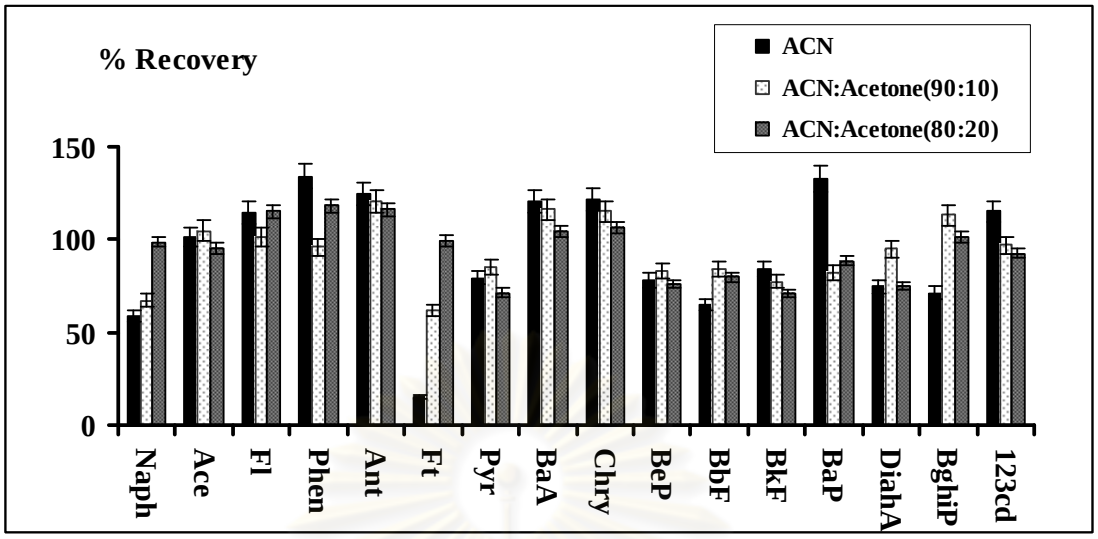


Figure 4.5 Effect of different organic solvent on extraction efficiency of 8 mL of organic solvent.

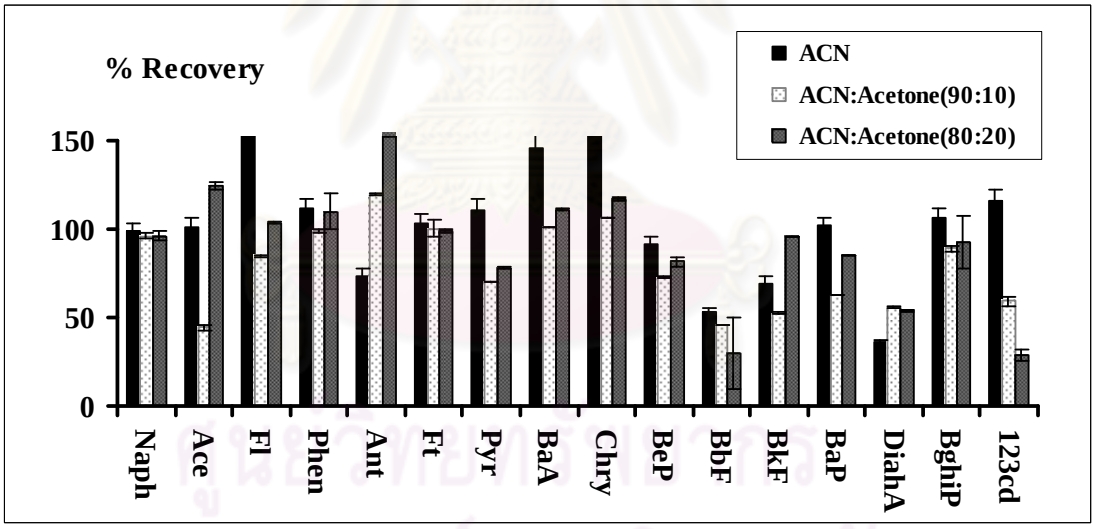


Figure 4.6 Effect of different organic solvent on extraction efficiency of 10 mL of organic solvent.

4.2.3 The Optimization of Number of Extraction

According to preliminary study, 8 mL of acetonitrile/acetone mixture (80:20, v/v) was the optimized extraction condition. However, some of target analyte recoveries were

still low. Therefore, the number of extraction was studied to optimize the percentage of analyte recovery. Table A-4 in Appendix A and Figure 4.7 showed the percentage of target analyte recoveries on different number of extraction. In overall, two times extraction was enough to extract PAHs from the samples as important factors to increase the net amount of extracted analyze were K_D and the ratio of V_{org}/V_{aq}. (K_D = distribution equilibrium, V_{org} = volume of organic solvent, V_{aq} = volume of aqueous solution)

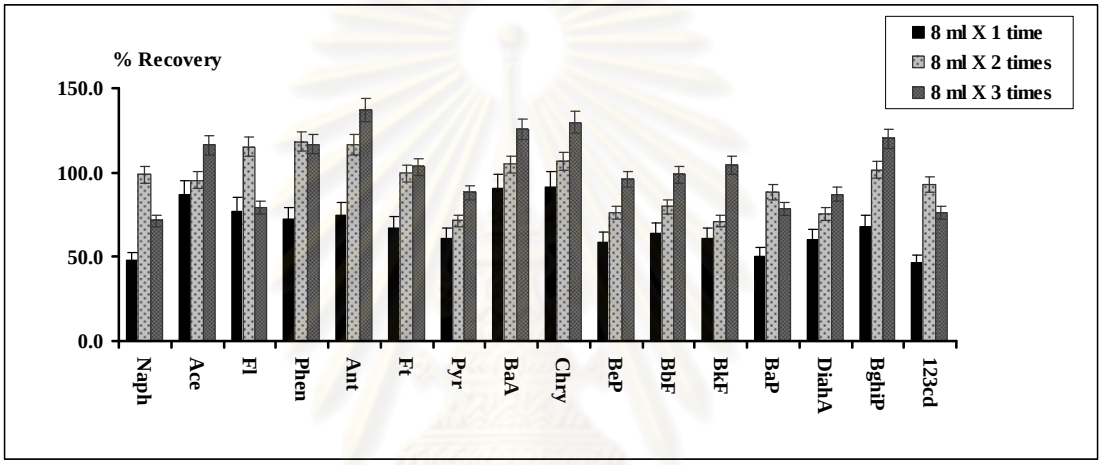


Figure 4.7 Effect of number of extraction time of 8 mL acetonitrile/acetone mixture (80:20, v/v).

4.2.4 The Optimization of Solid-Phase Extraction (SPE) Cartridges for Cleanup

After the extraction step using low-temperature for fat precipitation, more interfering oil peaks were still occurred close to the retention time of naphthalene, acenaphthene and fluorene. The chromatogram of interference peaks in this problem is proposed in Figure 4.8.

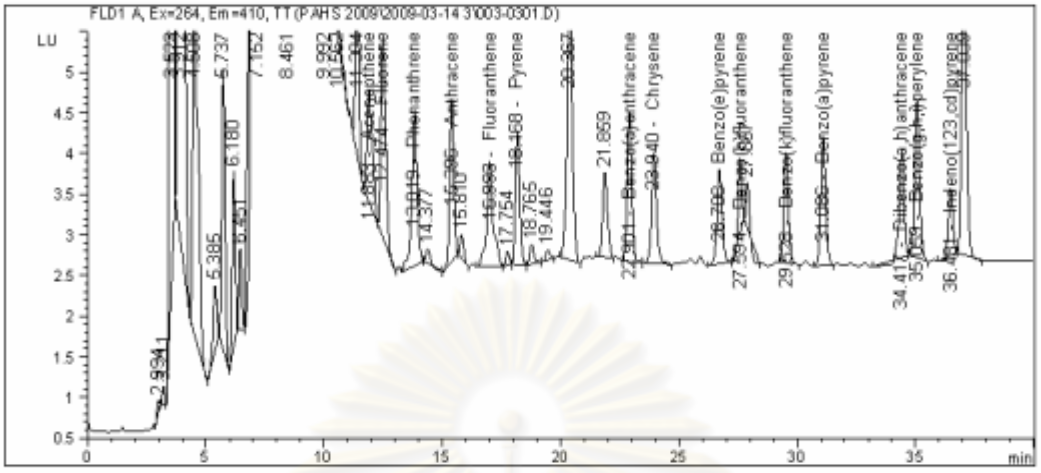


Figure 4.8 The chromatogram of low-temperature extraction without cleanup step

Thus, three types of SPE sorbents which are Sep-Pak alumina N, florisil, and SPE C_{18} cartridge were tested. The selection of SPE sorbent type was based on the compatibility with the analytes and the elimination of interference. From Figure 4.9 florisil obtained the recoveries in the range of 58.63%-105.30% for all 16 PAHs which were similar trend to alumina N. Table A-5 in Appendix A show the extraction results. Nevertheless, the interference peaks in the chromatogram of florisil were more than that of alumina N as shown in Figure 4.10(b), and (c). Physical properties of sorbent have a great influence on the characteristics of the SPE cartridge. Some important parameters of alumina N, florisil, and SPE C_{18} are listed in Table 4.3 *(43).* Florisil and alumina N have larger particle size but smaller surface area than SPE C_{18}. Besides that, C_{18} has a long carbon chain thus it could provide adequate hydrophobic space enough to enhance the interaction with analytes. Since PAHs are weakly or almost nonpolar compounds, it is reasonable to predict that they are retained but the results showed that C_{18} could yield good recoveries for some of 2-3 rings PAHs and poor recoveries for 4-6 rings PAHs. The results are shown in Table A-5 in Appendix A and Figure 4.10(a). While the surface area of sorbent was large, the more analytes will be retained. Alumina N can provide better recoveries for all PAHs and less interference in spite of large particle size. This demonstrated that alumina N has strong adsorption ability for interference and allowed interested analyte (PAHs)

passed through. It is also reasonable to predict that alumina N will has more efficient to reduce interference *(43).*

Table 4.3 Physico-chemical characteristics data of alumina N, florisil, and SPE C_{18}

Sorbent type	Particle size (μm)	Pore size(Å)	Surface Area (m^2/g)
Florisil	50 - 200	60	300
Alumina N	50 - 300	120	155
SPE C_{18}	55 - 105	125	500

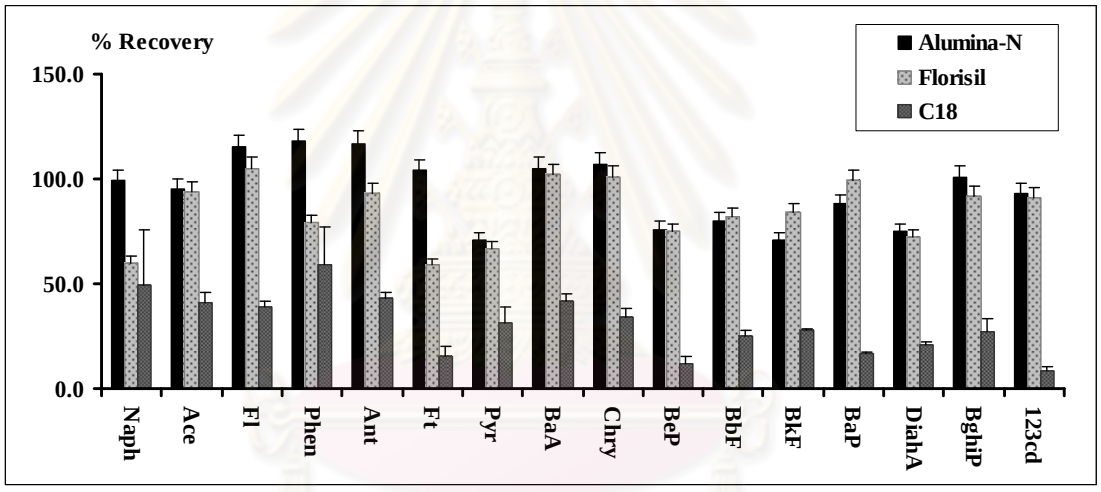


Figure 4.9 Effect of SPE sorbent type (alumina N, florisil, SPE C_{18}) on clean up

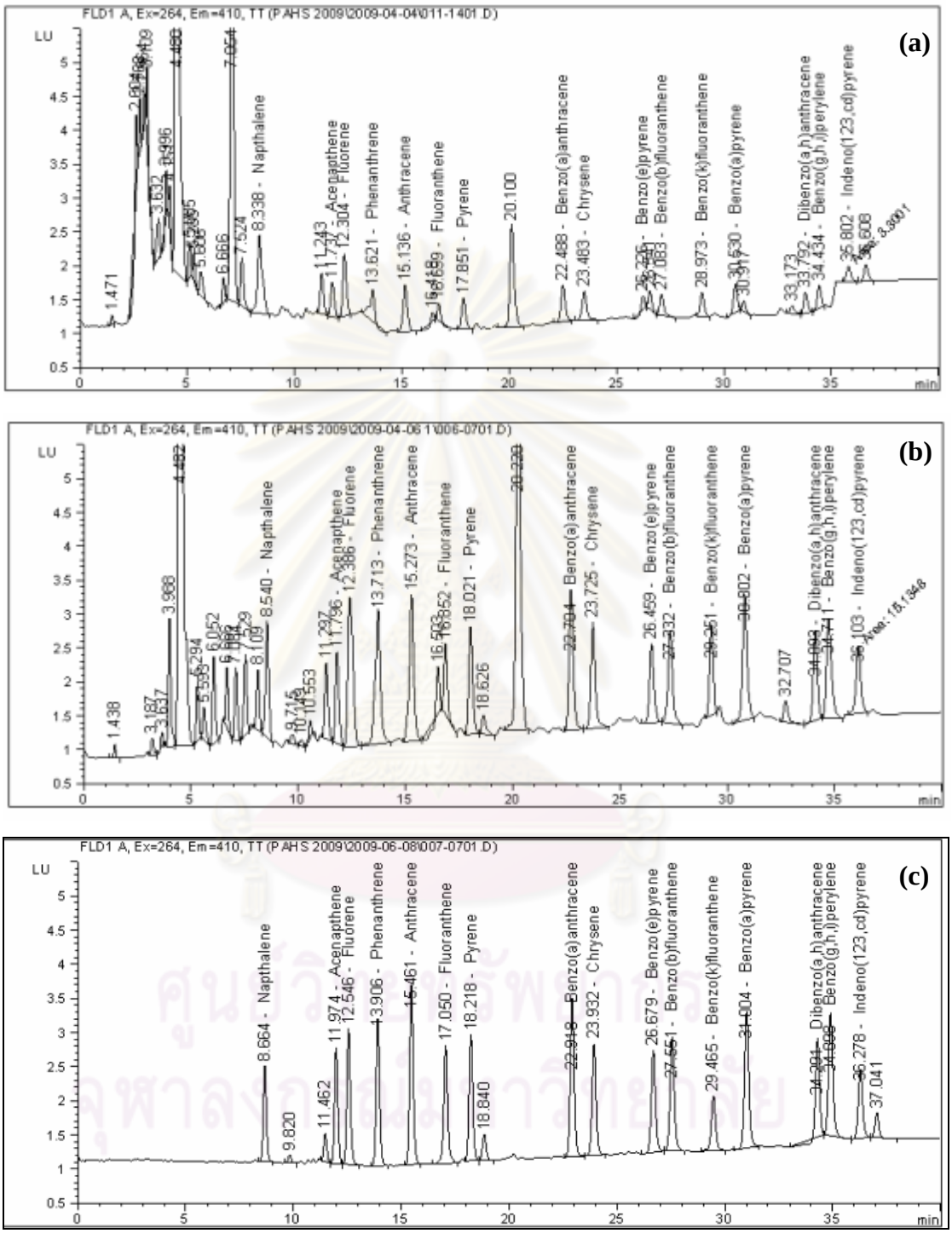


Figure 4.10 The chromatogram of solid-phase extraction when

(a) cleanup with SPE C_{18} cartridge

(b) cleanup with florisil cartridge

(c) cleanup with alumina N cartridge

4.3 Sample Preparation of Cooking Oil by Sweep Co-Distillation

Sweep co-distillation was performed on the UNITREX ™ II system (SGE Incorporated, USA). This technique is a sample preparation that is simple, fast, cost-effective and using only small amount of organic solvent. The technique allows a treatment of multiple samples simultaneously. In this technique, the sample is distillated and then purged by a gas in order to trap the target in the sorbent. Then, the sorbent was eluted by a mixture of organic solvent.

4.3.1 The Optimization of Distillation Temperature and Time

Under experimental conditions, a critical step for extraction of PAHs by sweep co-distillation was the temperature. Since the high temperature may cause the burning of oil matrix in fractionation tubes, distillation temperature were tested at 230 ^{o}C and 235 ^{o}C. Recovery of PAHs at temperature of 230 ± 1 ^{o}C and 235 ± 1 ^{o}C for 30, 45 and 60 minutes were shown in Figure 4.11-4.13. Low recovery of high volatile PAHs such as naphthalene was observed because it is easy volatilize between the extraction process. This may occured from a sample warming step before injecting the sample into the sweep co-distillation system because of the fact that oil sample was viscous and difficult to inject into the system and during the evaporation step. In the effect of distillation time, 60 minutes gave the most satisfactory recovery of PAHs in cooking oils at two temperatures because the longer time of distillation will enhance the removal of PAHs from fractionation tube. Therefore, optimum extraction was accomplished when distilled at 235 ± 1 ^{o}C for 60 minutes. Only 8 PAHs which are Ace, Fl, Phen, Ant, Ft, Pyr, BaA, Chry were received an acceptable recovery which ranging from 59.83 to 110.45%. Recovery(%) of the other PAHs which are benzo(e)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and indeno (1,2,3-c,d) pyrene was lower than 40% because of a poor volatility (BeP b.p. 311 ^{o}C, BbF b.p. 480 ^{o}C, BkF b.p. 480 ^{o}C, BaP b.p. 495 ^{o}C, , DiahA b.p. 524 ^{o}C, BghiP b.p. 550 ^{o}C and 123cd b.p. 530 ^{o}C). Hence, only their qualitative evaluation was possible.

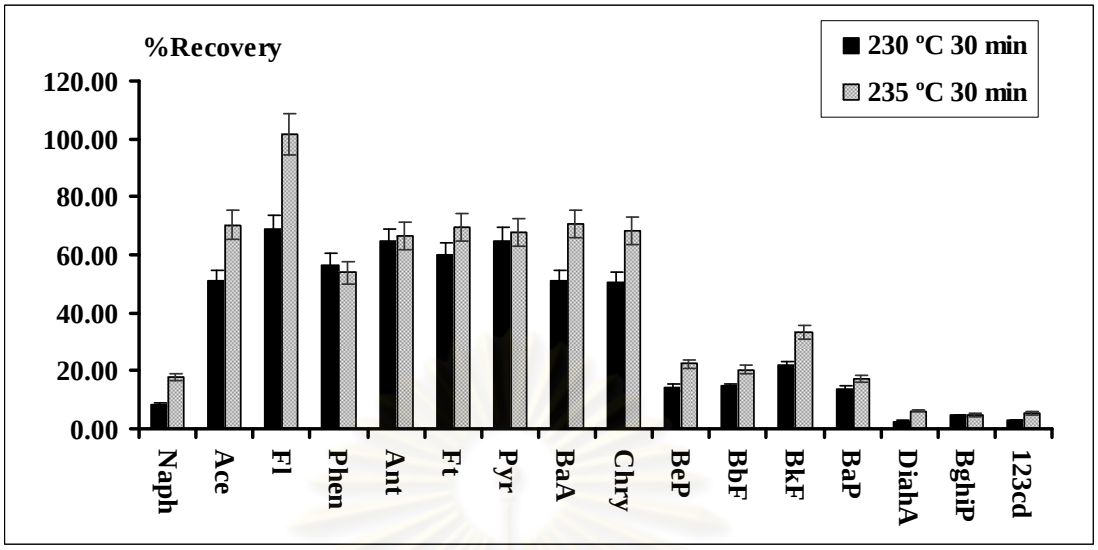


Figure 4.11 % Recovery of 16 PAHs at 230 ± 1 ^{o}C and 235 ± 1 ^{o}C for 30 minutes of sweep co-distillation.

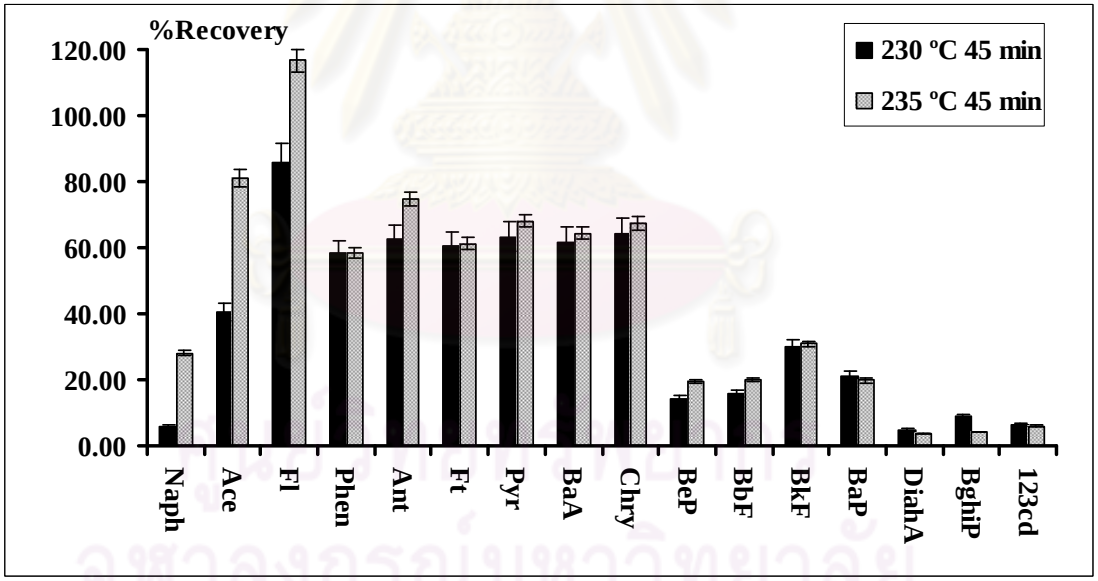


Figure 4.12 % Recovery of 16 PAHs at 230 ± 1 ^{o}C and 235 ± 1 ^{o}C for 45 minutes of sweep co-distillation.

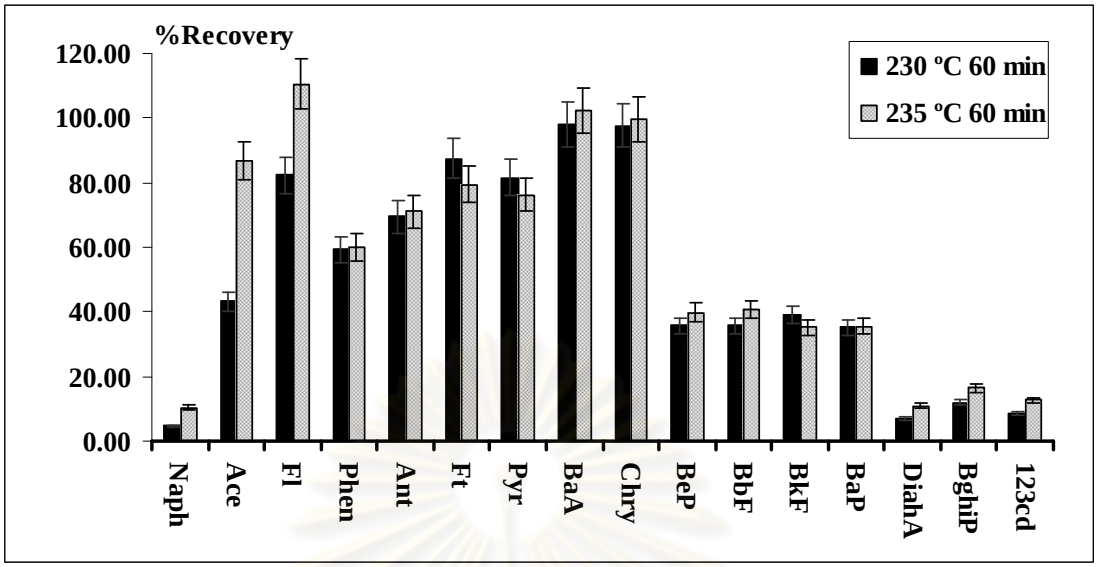


Figure 4.13 % Recovery of 16 PAHs at 230 ± 1 ^{o}C and 235 ± 1 ^{o}C for 60 minutes of sweep co-distillation**.**

4.4 Method Validation

4.4.1 Standard Calibration Curve

Based on the EU regulation for a limit of 2 μg/kg PAHs in oils and fats *(4)*, a mixture of 16 PAHs in methanol solution were investigated in a range of 0.25-6.0 ng/mL with three replicates. All calibration curves were displayed in Appendix B. The results of the coefficient of determination (R^2) and regression data were summarized in Table 4.4. The calibration curves were all fit for the purpose. The corresponding coefficient of determination (R^2) in Table 4.4 were greater than 0.9900.

Table 4.4 Slope, y-intercept, and coefficient of determination from standard calibration curve of 16 PAHs.

No.	Compounds	Slope	y-Intercept	R^2
1.	Naphthalene	0.7045	0.7830	0.9990
2.	Acenaphthene	1.6474	0.1280	0.9996
3.	Fluorene	3.7444	0.0038	0.9983
4.	Phenanthrene	0.3855	0.1685	0.9994
5.	Anthracene	3.7851	0.0297	0.9995
6.	Fluoranthene	0.1748	0.0309	0.9995
7.	Pyrene	0.9931	0.1315	0.9995
8.	Benzo(a)anthracene	2.4373	0.0276	0.9996
9.	Chrysene	1.5713	0.0656	0.9996
10.	Benzo(e)pyrene	0.5999	0.0108	0.9995
11.	Benzo(b)fluoranthene	1.3276	-0.0340	0.9997
12.	Benzo(k)fluoranthene	5.1632	-0.0945	0.9991
13.	Benzo(a)pyrene	7.4941	-0.5221	0.9994
14.	Dibenzo(a,h)anthracene	1.8074	0.0401	0.9997
15.	Benzo(g,h,i)perylene	0.4884	0.0164	0.9994
16.	Indeno(1,2,3-c,d)pyrene	0.3302	-0.0487	0.9995

4.4.2 Linearity and Working Range

The study of linearity and working range was carried out at 6 concentration levels as present in Table 3.4. Enrichment capability of the method was obtained from the extraction of these six spiked levels with optimized low-temperature cleanup and sweep co-distillation conditions. Each concentration was achieved in three replicates. The linearity and working range were plotted as peak area versus analytes concentration. The results of linearity and working range for low-temperature cleanup were displayed in Appendix C and Appendix D for sweep co-distillation method. In addition, the linear regression data for linearity and working range studies of all PAHs were shown in Table 4.5.

The linearity of each PAH was evaluated in term of coefficient of determination (R^2). The coefficient of determination for low-temperature cleanup were obtained between 0.9739-0.9980. Some of PAHs showed poor linearity ($R^2 < 0.99$) such as fluorene, anthracene, benzo(e)pyrene, benzo(g,h,i)perylene and indeno(1,2,3-c,d)pyrene. However, their coefficient of determination (R^2) were acceptable as the value were greater than 0.97. For sweep co-distillation method, only eight PAHs could be quantitatively analyzed (acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, and chrysene). The coefficient of determination (R^2) were ranged from 0.9776 to 0.9987. Furthermore, the sensitivity of each analyte that best shows the detect response is indicated by slope values. The compounds with the high slope value is the greater of the detector response and higher sensitivity. In the study, fluoranthene, anthracene, benzo(k)fluoranthene, and benzo(a)pyrene for low-temperature cleanup and only fluorene for sweep co-distillation method have the highest sensitivity, while benzo(g,h,i)perylene and indeno(1,2,3-c,d)pyrene for low-temperature cleanup and fluoranthene for sweep co-distillation method have the lowest sensitivity.

Table 4.5 Slope, y-intercept, and coefficient of determination from linearity and working range of 16 PAHs in optimized methods low-temperature cleanup and sweep co-distillation.

No.	Compounds	Low-temperature cleanup method			Sweep co-distillation method		
		Slope	y-Intercept	R^2	Slope	y-Intercept	R^2
1.	Naph	0.5696	0.6244	0.9903	-	-	-
2.	Ace	1.7955	-0.2472	0.9974	1.5213	-0.8416	0.9776
3.	Fl	4.0303	-0.4230	0.9739	3.3317	-0.4548	0.9872
4.	Phen	0.4161	-0.1096	0.9908	0.2640	-0.4660	0.9869
5.	Ant	4.6365	-0.3675	0.9848	2.7309	0.2707	0.9880
6.	Ft	0.1579	-0.4519	0.9904	0.1496	0.2322	0.9984
7.	Pyr	0.7994	0.2914	0.9980	0.7512	0.3801	0.9987
8.	BaA	2.8280	-0.7491	0.9968	2.3008	-0.2858	0.9949
9.	Chry	1.8983	-0.3396	0.9938	1.4009	0.2740	0.9909
10.	BeP	0.4648	-0.3663	0.9788	-	-	-
11.	BbF	1.1150	-0.7388	0.9962	-	-	-
12.	BkF	4.6288	-0.9979	0.9961	-	-	-
13.	BaP	4.9982	-0.0265	0.9900	-	-	-
14.	DiahA	1.5179	-1.1624	0.9959	-	-	-
15.	BghiP	0.5371	-1.8388	0.9844	-	-	-
16.	123cd	0.2067	-0.2677	0.9882	-	-	-

4.4.3 Limit of Detection (LODs) and Limit of Quantifications (LOQs)

The method limits of detections (LODs) were calculated from chromatographic signal (peak height) at three times higher than the baseline noise (S/N=3). The lowest spiked concentration of each PAH under optimized condition for low-temperature cleanup and sweep co-distillation method was done in ten replicates. In the same way, the method limits of quantification were also calculated from chromatographic signal

(peak height) at 10 times higher than the baseline noise (S/N=10). Ten replicates of LOQs were studied. The detection limits of each PAH were shown in Table 4.6.

For low-temperature cleanup method, the highly sensitive compound showed a low detection limit i.e naphthalene, acenaphthene, fluorene, anthracene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene while the low sensitive compound had a high detection limit i.e. phenanthrene, fluoranthene benzo(e)pyrene, benzo(g,h,i)perylene, and indeno(1,2,3-c,d)pyrene. For sweep co-distillation method, it could be quantitively detected only eight PAHs as acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, and chrysene. Moreover, the LODs and LOQs values were about two times higher than low-temperature cleanup method. As the EU regulations for PAHs in fat and oil samples represented by benzo(a)pyrene as a marker at the level of 2 ng/g, only sample preparation of oil samples by low-temperature cleanup method could applied to perform the legal requirements. The LOQs values in low-temperature cleanup method was 0.25 ng/g for benzo(a)pyrene, indicated that the level of LOQs was eight-fold lower than EU regulation. From the results, it can be concluded that the simultaneous extraction of 16 PAHs in cooking oil samples should be done by low-temperature cleanup method. If determination only 8 PAHs, sweep co-distillation method could reached the legal requirements such as German Society for Fat Science proposed a value of 25 μg/kg for sum of both, light and heavy PAHs *(17)*.

Table 4.6 The limit of detections and limit of quantifications of 16 PAHs (n=10).

No.	Compounds	Low-temperature cleanup method		Sweep co-distillation method	
		LOD ± S.D. (ng/g)	LOQ ± S.D. (ng/g)	LOD ± S.D. (ng/g)	LOQ ± S.D. (ng/g)
1.	Naph	0.63 ± 0.69	1.25 ± 0.15	-	-
2.	Ace	0.25 ± 0.07	0.50 ± 0.08	0.50 ± 0.40	2.00 ± 0.10
3.	Fl	0.13 ± 0.77	0.25 ± 0.05	0.25 ± 0.27	1.00 ± 0.06
4.	Phen	1.25 ± 0.20	2.50 ± 0.28	2.50 ± 0.80	10.00 ± 0.61
5.	Ant	0.13 ± 0.39	0.25 ± 0.02	0.25 ± 0.30	1.00 ±0.31
6.	Ft	3.13 ± 0.16	6.25 ± 0.15	6.25 ± 0.27	25.00 ± 0.89
7.	Pyr	0.63 ± 0.19	1.25 ± 0.15	1.25 ± 0.37	5.00 ± 0.21
8.	BaA	0.25 ± 0.17	0.50 ± 0.04	0.50 ± 0.44	2.00 ± 0.11
9.	Chry	0.25 ± 0.21	0.50 ± 0.03	0.50 ± 0.40	2.00 ± 0.08
10.	BeP	1.00 ± 0.28	2.00 ± 0.16	-	-
11.	BbF	0.50 ± 0.36	1.00 ± 0.03	-	-
12.	BkF	0.13 ± 0.20	0.25 ± 0.02	-	-
13.	BaP	0.13 ± 0.84	0.25 ± 0.03	-	-
14.	DiahA	0.38 ± 0.19	0.75 ± 0.02	-	-
15.	BghiP	1.25 ± 0.35	2.50 ± 0.09	-	-
16.	123cd	1.88 ± 0.60	3.75 ± 0.03	-	-

4.4.4 Accuracy

The method accuracy is the degree of how to closeness of measurements of a quantity to the true value of analytes in sample. Generally, the method accuracy was reported in term of recovery *(42)*. The recovery is calculated from the analytical signal as the ratio between found and expected expressed in %. In this study, the accuracy of the method was based on studied at 5 concentration levels as shown in Table 3.4 (level 2 to level 6) with the mean of all values is reported. The standard deviation are calculated from the ten replicate analysis and the average of all results is reported in Table 4.7 for low-temperature cleanup and Table 4.8 for sweep co-distillation method.

Table 4.7 The result of the accuracy study at concentration level of 0.5, 1.0, 2.0, 4.0, and 6.0 ng/g of benzo(a)pyrene in low-temperature cleanup method

Compounds	% Mean recovery ± S.D. (n=10)				
	0.5 ng/g	1.0 ng/g	2.0 ng/g	4.0 ng/g	6.0 ng/g
Naphthalene	100.04 ± 0.28	99.34 ± 0.31	103.00 ± 1.24	75.93 ± 2.89	67.32 ± 1.30
Acenaphthene	102.80 ± 0.09	88.60 ± 0.08	113.80 ± 0.17	92.93 ± 0.26	102.56 ± 0.22
Fluorene	87.40 ± 0.10	61.80 ± 0.07	113.25 ± 0.07	94.23 ± 0.22	118.47 ± 0.41
Phenanthrene	69.56 ± 0.84	107.62 ± 0.55	86.99 ± 3.01	99.08 ± 3.34	97.40 ± 5.43
Anthracene	88.60 ± 0.06	104.50 ± 0.07	109.45 ± 0.18	107.78 ± 0.57	95.50 ± 0.40
Fluoranthene	86.64 ± 1.46	85.24 ± 2.41	88.33 ± 3.03	80.75 ± 10.04	91.03 ± 6.18
Pyrene	85.24 ± 0.31	70.88 ± 0.22	86.11 ± 0.63	75.92 ± 1.85	76.85 ± 0.31
Benzo(a)anthracene	106.10 ± 0.10	101.45 ± 0.07	112.95 ± 0.12	96.19 ± 0.27	110.33 ± 0.18
Chrysene	91.00 ± 0.14	99.25 ± 0.14	111.63 ± 0.31	95.94 ± 0.47	113.61 ± 0.15
Benzo(e)pyrene	86.18 ± 0.54	73.04 ± 0.17	68.90 ± 1.95	67.85 ± 3.55	71.21 ± 3.37
Benzo(b)fluoranthene	92.25 ± 0.26	73.47 ± 0.14	84.03 ± 0.79	72.35 ± 1.60	79.19 ± 0.66
Benzo(k)fluoranthene	93.00 ± 0.04	73.30 ± 0.05	93.06 ± 0.09	76.25 ± 0.32	83.32 ± 0.09
Benzo(a)pyrene	105.80 ± 0.06	107.40 ± 0.07	89.25 ± 0.12	64.69 ± 0.55	74.00 ± 0.22
Dibenzo(a,h)anthracene	102.00 ± 0.17	64.39 ± 0.04	81.54 ± 0.51	70.07 ± 1.57	74.32 ± 0.14
Benzo(g,h,i)perylene	80.54 ± 0.73	71.59 ± 0.36	99.99 ± 4.04	74.12 ± 6.79	95.44 ± 1.17
Indeno(1,2,3-c,d)pyrene	89.36 ± 0.83	55.21 ± 0.38	45.88 ± 0.99	54.30 ± 2.56	58.42 ± 4.67

Table 4.8 The result of the accuracy study at concentration level of 0.5, 1.0, 2.0, 4.0, and 6.0 ng/g of benzo(a)pyrene in sweep co-distillation method

Compounds	% Mean recovery ± S.D. (n=10)				
	0.5 ng/g	1.0 ng/g	2.0 ng/g	4.0 ng/g	6.0 ng/g
Acenaphthene	88.62 ± 0.40	76.46 ± 0.10	52.49 ± 0.12	71.13 ± 2.70	95.40 ± 4.08
Fluorene	89.97 ± 0.27	59.73 ± 0.06	101.38 ± 0.74	115.09 ± 2.55	119.47 ± 1.85
Phenanthrene	101.18 ± 0.80	96.87 ± 0.61	60.72 ± 1.00	47.48 ± 1.78	61.40 ± 0.80
Anthracene	92.71 ± 0.30	91.14 ± 0.31	78.53 ± 1.32	58.39 ± 2.08	71.89 ± 0.84
Fluoranthene	64.96 ± 0.27	75.23 ± 0.89	85.64 ± 0.61	66.64 ± 2.89	75.17 ± 2.89
Pyrene	85.15 ± 0.37	64.47 ± 0.21	85.44 ± 0.57	65.23 ± 2.55	79.65 ± 0.70
Benzo(a)anthracene	71.63 ± 0.44	96.88 ± 0.11	103.23 ± 0.69	82.39 ± 3.75	95.33 ± 1.74
Chrysene	91.91 ± 0.40	113.42 ± 0.08	110.14 ± 0.62	86.68 ± 2.39	98.66 ± 1.29

According to Table 4.7, the recovery reported were 45.88-118.47% for the PAHs standard concentration of 0.5, 1.0, 2.0, 4.0 and 6.0 ng/g for benzo(a)pyrene, respectively. For sweep co-distillation method, the standard spiking levels were similar to low-temperature cleanup and the recovery ranged from 47.48-119.47%. The recovery result of sweep co-distillation method was calculated only for acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene and chrysene. In this research, these recovery values were accepted by the AOAC Peer-verified methods that recommended the acceptable recovery values of the method at ppb concentration level to be 40-120 %. The results indicated that the developed extraction method provided good accuracy for the analysis of 16 PAHs for low-temperature cleanup and only 8 PAHs for sweep co-distillation in cooking oil.

4.4.5 Precision

In this work, precision was determined in a same concentration spiked level as accuracy study under optimized conditions of low-temperature cleanup and sweep co-distillation methods. Generally, the percent of relative standard deviations (%R.S.D.) were presented. Moreover, repeatability was evaluated because the analytical results were derived from identical test portions in the same laboratory, using the same equipment, finished within a short period of time, and represented in term of RSDr. On the other hand, reproducibility of the method can be estimated on the basis of results obtained when the method has been used to analyze identical test portions in different laboratories, using different equipment and represented in term of RSD_R. Both repeatability and reproducibility are generally depended on analyte concentration. Additionally, the precision values can be evaluated with the modified Horwitz equation which calculated as Eq.1.

$$\text{HORRAT} = \frac{\text{Experimental RSDr}}{\text{Predicted RSDr}} \qquad \text{(Eq.1)}$$

Where

$$\text{Predicted RSDr} = 0.67 \times 2^{(1-0.5 \log C)} = 0.67 \times 2C^{-0.1505} \qquad \text{where C}$$

C = mass fraction or concentration of analyte in the sample

Experimental RSDr = the relative standard deviation calculated from results generated under repeatability conditions (within- laboratory)

Table 4.9 Expected % RSDr values that reflect the mass fraction amount of analyte

Concentration	Mass fraction, C	Expected % RSDr
100%	1.0	2
1%	0.01	4
0.01%	0.0001	8
1 ppm	0.000001	16
10 ppb	0.00000001	32
1 ppb	0.000000001	45

The Horwitz ratio (HORRAT) should be smaller than two *(45).* The calculated HORRAT value of both low-temperature cleanup and sweep co-distillation method were shown in Table 4.10 to 4.14. According to these results, the %R.S.D. were in the rang of 1.07 to 24.43 for low-temperature cleaup and 2.56 to 28.71 for sweep co-distillation at the PAHs standard concentration of 0.5, 1.0, 2.0, 4.0 and 6.0 ng/g for benzo(a)pyrene, respectively. The precision of both methods were acceptable because the HORRAT values were smaller than acceptable value and overall R.S.D. values were also satisfactory.

Table 4.10 The result of the HORRAT values of all analytes at concentration spiked level 0.5 ng/g of benzo(a)pyrene (n=10).

Compounds	Concentration (ng/g)	Low-temperature cleanup		Sweep co-distillation	
		Experimental RSDr	HORRAT	Experimental RSDr	HORRAT
Naph	2.50	11.10	0.43	-	-
Ace	1.00	8.69	0.29	10.21	0.35
Fl	0.50	22.99	0.69	5.29	0.16
Phen	5.00	24.26	1.04	15.86	0.68
Ant	0.50	14.55	0.44	9.35	0.29
Ft	12.50	13.44	0.66	27.81	1.37
Pyr	2.50	14.39	0.55	11.80	0.46
BaA	1.00	9.65	0.32	7.27	0.25
Chry	1.00	14.92	0.50	3.59	0.12
BeP	4.00	15.54	0.64	-	-
BbF	2.00	14.17	0.53	-	-
BkF	0.50	8.19	0.30	-	-
BaP	0.50	10.71	0.32	-	-
DiahA	1.50	10.94	0.39	-	-
BghiP	5.00	18.14	0.77	-	-
123cd	7.50	12.40	0.56	-	-

Table 4.11 The result of the HORRAT values of all analytes at concentration spiked level 1.0 ng/g of benzo(a)pyrene (n=10).

Compounds	Concentration (ng/g)	Low-temperature cleanup		Sweep co-distillation	
		RSDr	HORRAT	RSDr	HORRAT
Naph	5.00	6.23	0.27	-	-
Ace	2.00	4.48	0.17	4.70	0.18
Fl	1.00	11.91	0.40	4.29	0.15
Phen	10.00	5.11	0.24	18.56	0.89
Ant	1.00	6.46	0.22	7.26	0.25
Ft	25.00	12.29	0.61	28.71	1.57
Pyr	5.00	6.35	0.27	8.11	0.35
BaA	2.00	3.57	0.13	2.77	0.10
Chry	2.00	7.17	0.27	2.56	0.10
BeP	8.00	2.96	0.13	-	-
BbF	4.00	4.90	0.20	-	-
BkF	1.00	7.25	0.24	-	-
BaP	1.00	6.75	0.23	-	-
DiahA	3.00	2.01	0.10	-	-
BghiP	10.00	5.04	0.24	-	-
123cd	15.00	4.64	0.23	-	-

Table 4.12 The result of the HORRAT values of all analytes at concentration spiked level 2.0 ng/g of benzo(a)pyrene (n=10).

Compounds	Concentration (ng/g)	Low-temperature cleanup		Sweep co-distillation	
		RSDr	HORRAT	RSDr	HORRAT
Naph	10.00	12.17	0.58	-	-
Ace	4.00	3.79	0.16	3.57	0.15
Fl	2.00	3.02	0.11	12.87	0.48
Phen	20.00	17.30	0.91	22.89	1.21
Ant	2.00	8.43	0.31	23.94	0.90
Ft	50.00	6.86	0.41	8.73	0.53
Pyr	10.00	7.29	0.34	7.57	0.36
BaA	4.00	2.73	0.11	7.90	0.33
Chry	4.00	6.90	0.28	10.07	0.30
BeP	16.00	17.58	0.89	-	-
BbF	8.00	11.87	0.54	-	-
BkF	2.00	4.79	0.18	-	-
BaP	2.00	6.51	0.24	-	-
DiahA	6.00	10.54	0.44	-	-
BghiP	20.00	19.88	1.04	-	-
123cd	30.00	7.18	0.40	-	-

Table 4.13 The result of the HORRAT values of all analytes at concentration spiked level 4.0 ng/g of benzo(a)pyrene (n=10).

Compounds	Concentration (ng/g)	Low-temperature cleanup		Sweep co-distillation	
		RSDr	HORRAT	RSDr	HORRAT
Naph	20.00	19.06	1.32	-	-
Ace	8.00	3.44	0.16	26.07	1.21
Fl	4.00	5.84	0.24	19.45	0.81
Phen	40.00	8.43	0.49	21.49	1.26
Ant	4.00	13.30	0.55	21.53	0.90
Ft	100.00	12.43	0.83	22.37	1.51
Pyr	20.00	12.17	0.64	19.10	1.01
BaA	8.00	3.55	0.16	22.84	1.06
Chry	8.00	6.16	0.28	21.63	1.00
BeP	32.00	17.29	0.99	-	-
BbF	16.00	14.40	0.73	-	-
BkF	4.00	10.96	0.45	-	-
BaP	4.00	22.71	0.99	-	-
DiahA	12.00	19.51	0.95	-	-
BghiP	40.00	24.43	1.42	-	-
123cd	60.00	7.85	0.49	-	-

Table 4.14 The result of the HORRAT values of all analytes at concentration spiked level 6.0 ng/g of benzo(a)pyrene (n=10).

Compounds	Concentration (ng/g)	Low-temperature cleanup		Sweep co-distillation	
		RSDr	HORRAT	RSDr	HORRAT
Naph	30.00	6.42	0.36	-	-
Ace	12.00	1.82	0.09	23.69	1.16
Fl	6.00	5.65	0.25	9.98	0.44
Phen	60.00	9.14	0.56	6.05	0.36
Ant	6.00	7.03	0.31	5.62	0.25
Ft	150.00	4.53	0.32	27.51	1.97
Pyr	30.00	1.33	0.07	3.45	0.19
BaA	12.00	1.34	0.06	7.31	0.36
Chry	12.00	1.11	0.05	8.14	0.40
BeP	48.00	9.95	0.59	-	-
BbF	24.00	3.58	0.19	-	-
BkF	6.00	1.76	0.08	-	-
BaP	6.00	4.87	0.21	-	-
DiahA	18.00	1.07	0.06	-	-
BghiP	60.00	2.03	0.12	-	-
123cd	90.00	9.08	0.60	-	-

4.4.6 Method Trueness

To confirm the suitability of method for intended use, a validation process was carried out by applying the optimized extraction procedure to the reference material RM FAPAS T0631, olive oil (which were 8 mL of mixed acetonitrile/acetone at ratio 80:20 (v/v), cleanup with alumina N cartridge for low-temperature cleanup, and at temperature 235 ^{o}C for 60 minutes for sweep co-distillation method). The RM FAPAS T0631 has been certified for 5 of the 16 PAHs. The material was extracted in duplicates for PAHs using both methods (low-temperature cleanup and sweep co-distillation), and analyzed by HPLC. The results obtained were shown in Table 4.15

Table 4.15 Analysis of RM FAPAS T0631 by low-temperature cleanup (Method 1) and sweep co-distillation (Method 2)

Compounds	Reference material FAPAS T0631 (μg/kg)			
	Assigned value	Satisfactory Range	Measured by Method 1	Measured by Method 2
Benzo(a)anthracene	0.68	0.38-0.97	0.71	0.94
Benzo(b)fluoranthene	2.61	1.46-3.76	2.33	NT
Benzo(a)pyrene	0.79	0.44-1.14	0.47	NT
Benzo(g,h,i) perylene	2.66	1.49-3.83	3.38	NT
Indeno (1,2,3,-c,d)pyrene	1.24	0.69-1.78	ND	NT

Where

NT = Not test

ND = Not detected

From Table 4.15, the results from lwo-temperature method were in good agreement with satisfactory range of reference values except indeno(1,2,3-c,d)pyrene because the assigned value of reference was lower than the limit of quantification of this method. On the contrary, the determination of PAHs in reference material by sweep co-

distillation for sample preparation prior to HPLC analysis was tested, only one of them, benzo(a)anthracene because the optimal condition for sweep-co ditillation was not sensitive enough for the large PAHs.

4.5 The Application of Optimized Method in Cooking Oil Samples

This successful method development using low-temperature cleanup was used to extract PAHs from refined and used cooking oil sample. The refined oils were purchased from supermarket in Bangkok and used cooking oils were collected from nine local markets in Bangkok. The example of HPLC chromatogram from these experiments were shown and illustrated in Table 4.16-4.17 and Figure 4.14. From Table 4.16, it can be seen that a range of PAHs for refined oils very concentrations were found in the palm oils. Generally it was the light PAHs (up to four rings) and some of heavy PAHs as five ring compounds that were found in the soybean oils. This may be due to the fact that palm oil and soybean oil preparation requires special treatment like drying which may generate PAHs. Furthermore, PAHs in edible oils could arise from atmospheric deposition to plants or through contamination of extraction solvents use. It has been shown that specific refining steps like deodorization may drastically reduce the content of these contaminants and should be an integral part of the edible oil refining process so that the risk of PAHs contamination can be minimized *(8)*. For sunflower, canola, and olive oils were found the light PAHs more than heavy PAHs. The largest contribution to this arising from the compounds phenanthrene, fluoranthene, pyrene. Naphthalene also dominated in all samples. On the other hand, nine used oil samples have been shown to contain a high range of total PAHs, compared with the corresponding refined oils. However, the chromatogram for both refined and used oil samples show less interference peak.

Table 4.16 Concentrations (ng/g) of PAHs in different type of refined oil.

Compounds	Range of positive values (ng/g)				
	Soybean oil	Sunflower oil	Canola oil	Olive oil	Palm oil
Naph	7.0-12.5	5.9-15.2	3.1-11.9	7.4-11.2	4.9-9.5
Ace	ND	ND	ND	< 0.5	< 0.5
Fl	0.6-0.7	0.5	0.4-0.5	ND	0.3-3.7
Phen	12.2-13.4	11.5-15.5	5.0-6.6	11.1	2.8-96.5
Ant	0.3-0.6	0.3-0.6	0.3-0.4	ND	< 0.25-0.9
Ft	3.5-13.8	3.4-11.0	ND	8.7-13.8	11.5-73.6
Pyr	3.5-5.7	2.8-3.1	< 1.25	ND	28.9-29.4
BaA	0.6-2.4	ND	ND	ND	0.6-6.0
Chry	0.6-2.4	0.9	ND	0.7	0.9-24.1
BeP	< 2.0-2.7	ND	ND	ND	3.2-4.8
BbF	< 1.0-1.1	ND	ND	ND	< 1.00-2.1
BkF	< 0.25-0.6	ND	1.5	ND	0.3-1.3
BaP	0.3-1.0	ND	ND	0.5-1.1	0.9-3.2
DiahA	ND	2.9	ND	0.9	< 0.75-15.4
BghiP	ND	ND	ND	ND	2.5-4.8
123cd	ND	ND	ND	ND	10.5-11.4

ND = Not detected

Soybean oil = four samples

Sunflower oil = three samples

Canola oil = two samples

Olive oil = five samples

Palm oil = five samples

Table 4.17 Concentrations (ng/g) of PAHs in different used cooking oil analyzed (9 samples)

Compounds	Range of positive values (ng/g)
Naphthalene	2.80-11.5
Acenaphthene	ND
Fluorene	0.60-2.70
Phenanthrene	5.40-11.50
Anthracene	0.60-1.20
Fluoranthene	9.40-17.20
Pyrene	<1.25-3.20
Benzo(a)anthracene	1.30-4.40
Chrysene	1.00-4.30
Benzo(e)pyrene	2.90-5.50
Benzo(b)fluoranthene	<1.00
Benzo(k)fluoranthene	<0.25-0.70
Benzo(a)pyrene	0.50-2.20
Dibenzo(a,h)anthracene	1.00-5.10
Benzo(g,h,i)perylene	3.10-6.80
Indeno(1,2,3-c,d)pyrene	17.70-54.00

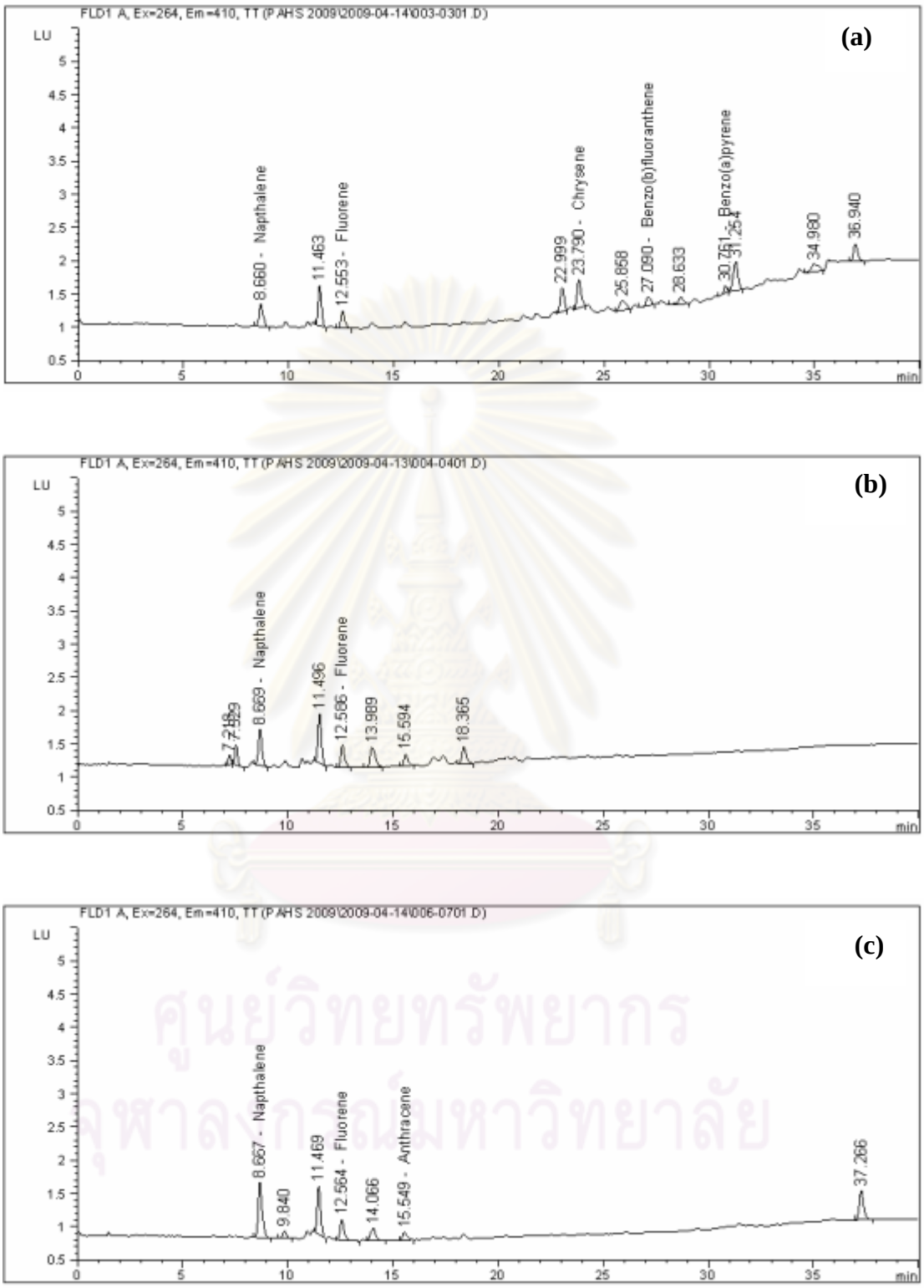


Figure 4.14 The chromatogram of cooking oil samples

(a) Palm oil
(b) Sunflower oil
(c) Canola oil
(d) Soybean oil
(e) Olive oil
(f) Used oil

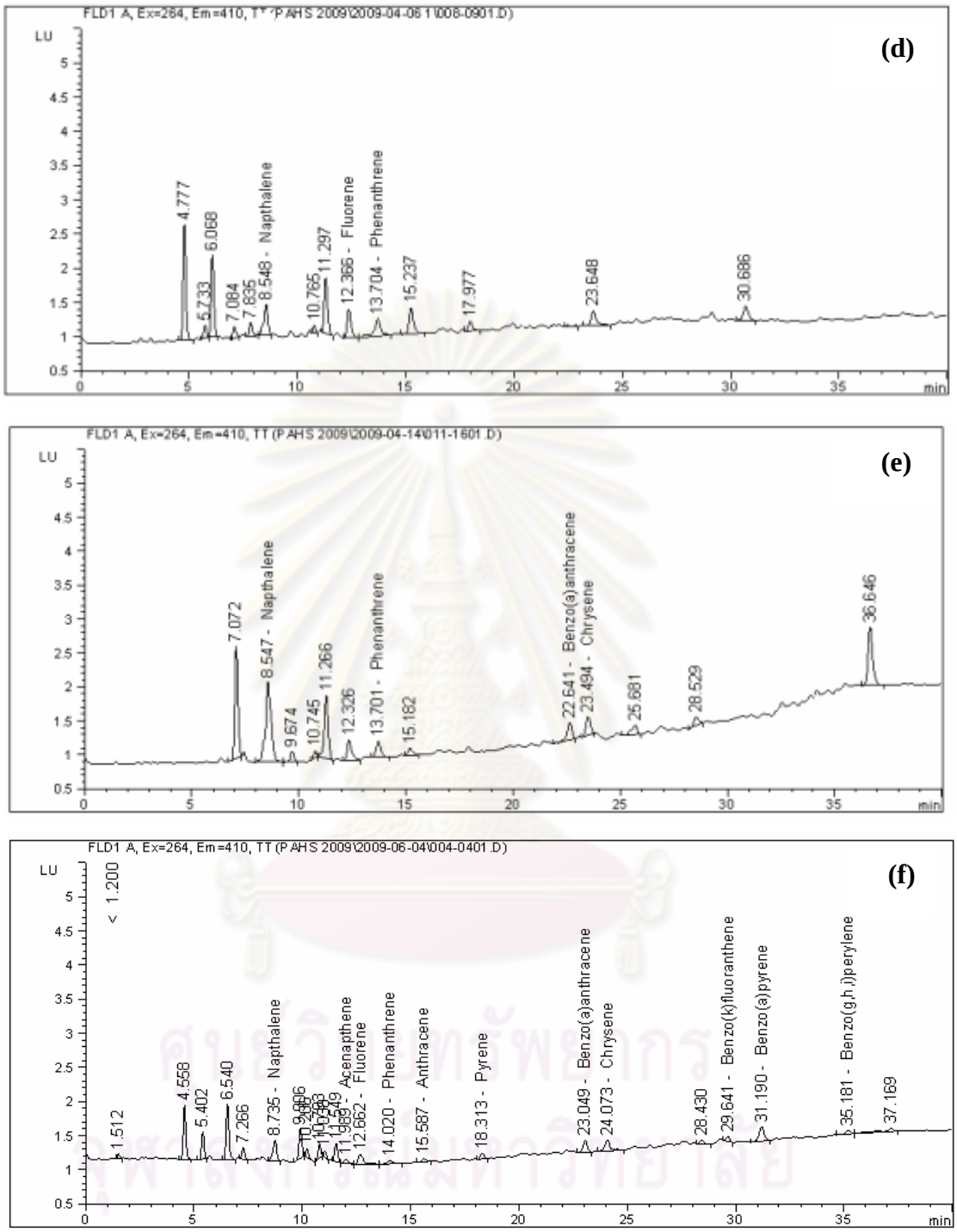


Figure 4.14 (continue) The chromatogram of cooking oil samples

(d) Palm oil **(d)** Soybean oil

(e) Sunflower oil **(e)** Olive oil

(f) Canola oil **(f)** Used oil

CHAPTER V

CONCLUSION AND SUGGESTION FOR FURTHER STUDY

Polycyclic aromatic hydrocarbons (PAHs) are well-known contaminants in environment and food processing. The European Union has set a limit for PAHs in foods for direct human consumption or use as an ingredient in foods (excluding cocoa butter until 01/04/07) of 2 μg/kg by using benzo(a)pyrene as marker. However, Thailand has no regulation about the level of PAHs in food yet. This work was aimed to develop sample preparation method for determination of PAHs in cooking oils using low-temperature cleanup and sweep co-distillation technique prior to analysis by HPLC. Both techniques provide a simple, cheap, rapid, efficient and suitable routine analysis methods for PAHs contaminated in cooking oil samples.

A new method for analysis of 16 PAHs (naphthalene, acenapthlene, fluorene, phenanthrene, anthracene, fluoranthene, benzo(a)anthracene, chrysene, benzo(e)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, Indeno(1,2,3-c,d)pyrene, and pyrene) in oil sample was developed. Low-temperature cleanup could used to determined all of the 16 analytes but sweep co-distillation method was utilized to analyze only for light PAHs (up to four aromatic ring) as acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, and chrysene except naphthalene due to its high volatility (Naph b.p. 218 ^{o}C).

Low-temperature cleanup method was demonstrated low-cost method and convenient treatment of multiple samples simultaneously. Moreover, a small amount of organic solvent was employed, resulting in an environmentally friendly technique. Most lipids in the extract were easily eliminated in form of frozen fat precipitation. During freezing step, about 94% of the lipids in the cooking oils were easily removed without any significant losses of PAHs analytes. For cleanup step, the study of different SPE cartridge (alumina N, florisil and SPE C_{18}) was evaluated by using the optimized

condition for each cartridge. The result of alumina N showed the satisfactory recovery and high efficient in elimination of lipid-interferences from the extracts. Thus, the low-temperature cleanup method was developed for the determination of 16 PAHs in cooking oils with simple configuration as summarized in Figure 5.1.

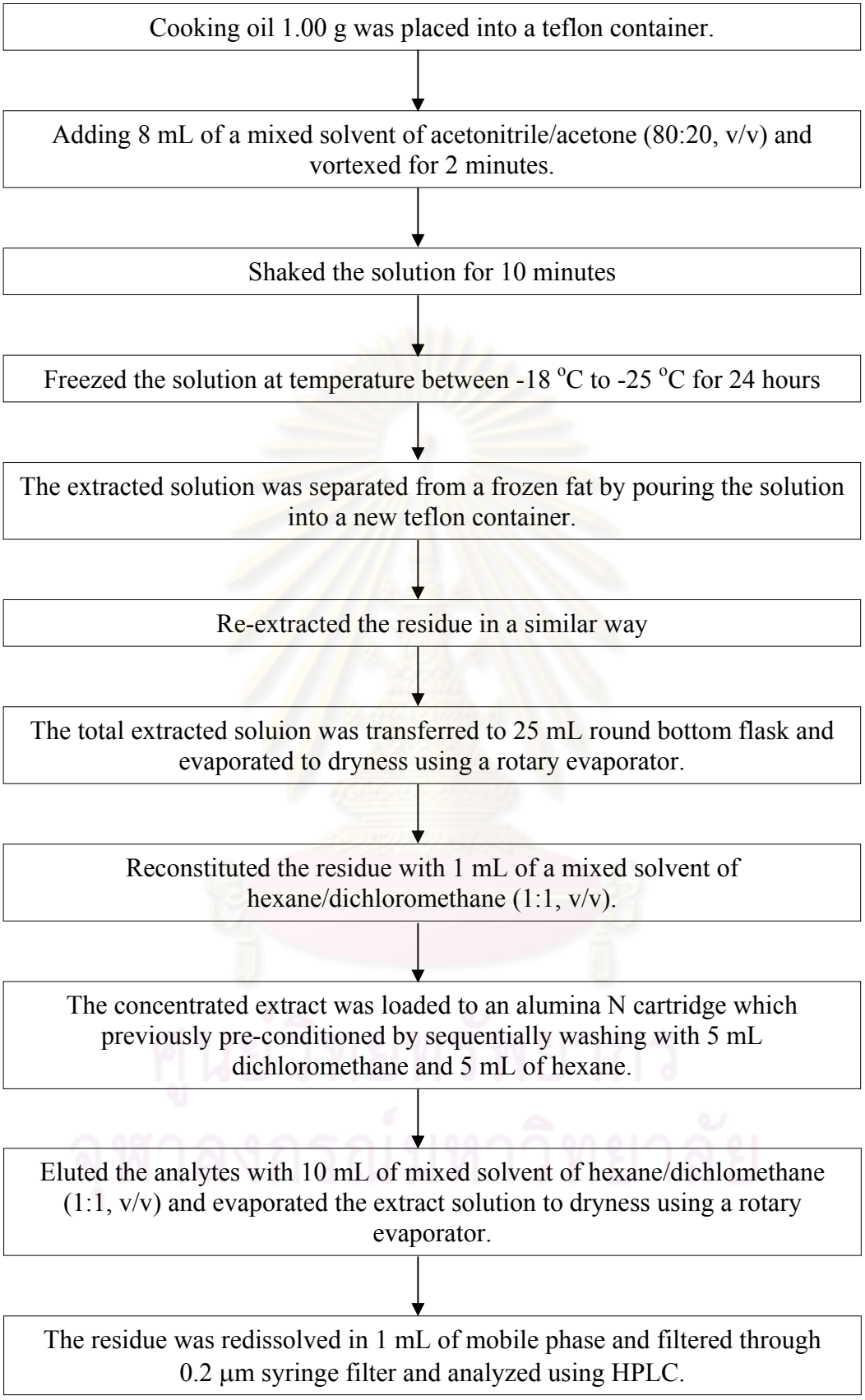


Figure 5.1 Schematic diagram of low-temperature cleanup procedure with optimized condition**.**

After the utilization of low-temperature cleanup in the determination of 16 PAHs, this optimized condition was validated to observe the performance of method before applying to real sample application. The summary of low-temperature cleanup method validation was reported in Table 5.1.

Table 5.1 Method performance of low-temperature cleanup method for determination of 16 PAHs in cooking oils

Compounds	Linear range (ng/mL)	R^2	LODs ng/g	LOQs ng/g	%Recovery[a]
Naph	1.25 – 30.00	0.9990	0.63 ± 0.69	1.25 ± 0.15	103.00 ± 1.24
Ace	0.50 – 12.00	0.9996	0.25 ± 0.07	0.50 ± 0.08	113.80 ± 0.17
Fl	0.25 – 6.00	0.9983	0.13 ± 0.77	0.25 ± 0.05	113.25 ± 0.07
Phen	2.50 – 60.00	0.9994	1.25 ± 0.20	2.50 ± 0.28	86.99 ± 3.01
Ant	0.25 – 6.00	0.9995	0.13 ± 0.39	0.25 ± 0.02	109.45 ± 0.18
Ft	6.25 – 150.00	0.9995	3.13 ± 0.16	6.25 ± 0.15	88.33 ± 3.03
Pyr	1.25 – 30.00	0.9995	0.63 ± 0.19	1.25 ± 0.15	86.11 ± 0.63
BaA	0.50 – 12.00	0.9996	0.25 ± 0.17	0.50 ± 0.04	112.95 ± 0.12
Chry	0.50 – 12.00	0.9996	0.25 ± 0.21	0.50 ± 0.03	111.63 ± 0.31
BeP	2.00 – 48.00	0.9995	1.00 ± 0.28	2.00 ± 0.16	68.90 ± 1.95
BbF	1.00 – 24.00	0.9997	0.50 ± 0.36	1.00 ± 0.03	84.03 ± 0.79
BkF	0.25 – 6.00	0.9991	0.13 ± 0.20	0.25 ± 0.02	93.06 ± 0.09
BaP	0.25 – 6.00	0.9994	0.13 ± 0.84	0.25 ± 0.03	89.25 ± 0.12
DiahA	0.75 – 18.00	0.9997	0.38 ± 0.19	0.75 ± 0.02	81.54 ± 0.51
BghiP	2.50 – 60.00	0.9994	1.25 ± 0.35	2.50 ± 0.09	99.99 ± 4.04
123cd	3.75 – 90.00	0.9995	1.88 ± 0.60	3.75 ± 0.03	45.88 ± 0.99

[a] Recovery at concentration level of 2 ng/kg benzo(a)pyrene

The standard calibration curve of 16 analytes showed the values of coefficient of determination (R^2) over 0.99 representing a good linear dynamic range of the method. The LODs were ranged 0.13 to 3.13 ng/g. Comparison of LODs of this work with other methods was illustrated in Table 5.2.

Table 5.2 Comparison of limits of detection of this work and other publications in determination of PAHs in refined oils.

Method	LODs (ng/g)	Sample preparation
Diletti *(10)*	0.10-0.40	Liquid-liquid extraction
Barranco *(14)*	0.09-0.20	Donor-acceptor complex chromatography (DACC)
Bogusz*(18)*	0.30-1.60	Solid-phase column extraction
Veyrand *(47)*	0.008-0.15	Pressurized liquid extraction
Ballesteros *(49)*	0.05-0.07	Liquid-liquid extraction
This work	0.13-3.13	Low-temperature cleanup

The LODs of this presented method were comparable to other works. This technique has a potentially excellent due to a satisfactory matrix cleanup, low amounts of organic solvent, and shorter analysis times. Moreover, the method recoveries representing method accuracy were ranged from 45.88 to 113.80 % at 2.0 ng/g spiking concentration level of benzo(a)pyrene. The precision was reported as relative standard deviation (%R.S.D.) and ranged from 2.73 to 19.88 %. When evaluating the method precision by Horwitz equation as HORRAT value, the results were within the acceptable value (lower than expected value)

In case of sweep co-distillation method, different temperatures and distillation times were studied to determine optimum condition. The results showed the satisfactory extraction at temperature 235 ^{o}C and 60 minutes of distillation time. However, eight target analytes out of sixteen analytes were quantitatively determined. Simple configuration for sweep co-distillation was illustrated in Figure 5.2.

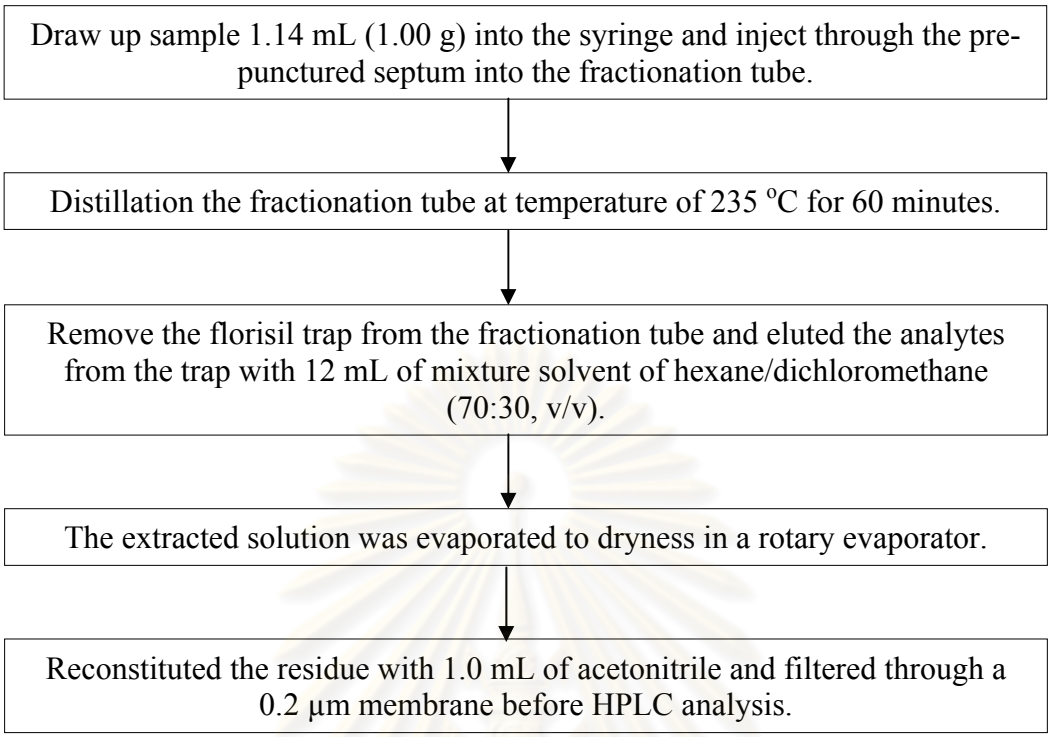


Figure 5.2 Schematic diagram of sweep co-distillation procedure with optimized condition.

After the application of sweep co-distillation in the determination of 8 analytes, this optimized condition was validated to observe the performance of method. The summary of sweep co-distillation method validation was reported in Table 5.3.

Table 5.3 Method performance of sweep co-distillation method for determination of 8 PAHs in cooking oils.

Compounds	Linear range (ng/mL)	R^2	LODs (ng/g)	LOQs (ng/g)	% Recovery[a]
Ace	0.50 – 12.00	0.9996	0.50 ± 0.40	2.00 ± 0.10	52.49 ± 0.12
Fl	0.25 – 6.00	0.9983	0.25 ± 0.27	1.00 ± 0.06	101.38 ± 0.74
Phen	2.50 – 60.00	0.9994	2.50 ± 0.80	10.00 ± 0.61	60.72 ± 1.00
Ant	0.25 – 6.00	0.9995	0.25 ± 0.30	1.00 ± 0.31	78.53 ± 1.32
Ft	6.25 – 150.00	0.9995	6.25 ± 0.27	25.0 ± 0.89	85.64 ± 0.61
Pyr	1.25 – 30.00	0.9995	1.25 ± 0.37	5.00 ± 0.21	85.44 ± 0.57
BaA	0.50 – 12.00	0.9996	0.50 ± 0.44	2.00 ± 0.11	103.22 ± 0.69
Chry	0.50 – 12.00	0.9996	0.50 ± 0.40	2.00 ± 0.08	110.14 ± 0.62

[a] Recovery at concentration level of 2 ng/kg benzo(a)pyrene

The LODs of this method were ranging from 0.25 to 6.25 ng/g. It was two times higher than that from low-temperature cleanup method. However, sweep co-distillation method is an alternative technique to extract eight PAHs in cooking oil because it is easy to operate, cheap and employ small amount of solvent. On the other hand, the limitation of this method is still applicable and can determine light PAHs (i.e. Ace, Fl, Phen, Ant, Ft, Pyr, BaA, and Chry).

Suggestion of Further Study

The enrichment ability of the sweep co-distillation method can be improved by increasing temperature but the burning of oil matrix in fractionation tubes at high temperature should be awared. The analysis of light PAHs could be possible to test by this method.

Analytical problems for low-temperature cleanup method were associated with fat components which is oil co-extraction. Besides, the other matrix such as pigments and other soluble components can remain in solvent as co-extracts. So the selected organic solvents can be further investigated for another system. The containers may also

affect the fat precipitation and separation because they tended to adhere with the walls resulting from the dependence of volume with temperature. Finally, the separation process of the solvent from the lipid must be carried out rapidly to remove the sample from the freezer because they are quickly turned into a liquid state.

According to this preliminary study, the further studies of low-temperature cleanup should be considered about the investigation of mixed solvent ratio. The matrix effect such as lipids, various pigments and other soluble components can remain in solvent as co-extracts. Therefore, the interferences should be typically focused in analysis because of the trace level of analytes.

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APPENDICES

APPENDIX A

Table A-1 Effect of different organic solvent on extraction efficiency of 4 mL of organic solvent

No.	Compounds	% Recovery ± S.D. (n=2)		
		CH_3CN	90:10 CH_3CN/Acetone	80:20 CH_3CN/Acetone
1.	Naph	54.56 ± 1.67	51.00 ± 0.35	51.60 ± 0.85
2.	Ace	101.00 ± 1.59	66.95 ± 1.66	28.95 ± 0.80
3.	Fl	181.70 ± 1.18	70.70 ± 0.71	49.80 ± 0.00
4.	Phen	100.96 ± 5.19	76.83 ± 5.64	91.87 ± 2.92
5.	Ant	95.70 ± 0.77	98.90 ± 0.70	6.30 ± 0.16
6.	Ft	103.40 ± 3.54	85.00 ± 12.13	70.82 ± 4.21
7.	Pyr	65.62 ± 0.80	59.76 ± 2.23	54.94 ± 1.18
8.	BaA	84.10 ± 0.24	79.05 ± 1.18	74.60 ± 0.33
9.	Chry	85.90 ± 0.04	82.85 ± 1.32	72.70 ± 0.65
10.	BeP	54.01 ± 0.11	54.59 ± 3.15	39.85 ± 1.56
11.	BbF	98.55 ± 0.47	12.95 ± 0.35	21.00 ± 2.16
12.	BkF	63.60 ± 0.13	14.20 ± 0.25	38.60 ± 1.47
13.	BaP	41.00 ± 0.21	39.50 ± 0.28	42.20 ± 0.08
14.	DiahA	47.00 ± 0.51	19.17 ± 0.28	6.83 ± 2.38
15.	BghiP	56.04 ± 1.22	50.96 ± 4.67	34.05 ± 0.63
16.	123cd	37.67 ± 1.73	12.79 ± 0.18	2.93 ± 0.79

Table A-2 Effect of different organic solvent on extraction efficiency of 8 mL of organic solvent

No.	Compounds	% Recovery ± S.D. (n=2)		
		CH_3CN	90:10 CH_3CN/Acetone	80:20 CH_3CN/Acetone
1.	Naph	58.89 ± 1.44	67.16 ± 3.11	98.76 ± 0.44
2.	Ace	101.00 ± 0.31	104.75 ± 1.07	95.45 ± 0.45
3.	Fl	114.60 ± 0.03	101.40 ± 0.58	115.30 ± 0.42
4.	Phen	133.91 ± 1.68	95.94 ± 0.16	118.40 ± 5.11
5.	Ant	124.80 ± 0.44	120.40 ± 0.04	116.50 ± 0.39
6.	Ft	15.38 ± 4.44	61.74 ± 4.11	99.68 ± 0.57
7.	Pyr	79.54 ± 1.05	84.92 ± 0.85	71.34 ± 0.88
8.	BaA	120.75 ± 0.59	116.20 ± 0.49	104.70 ± 0.44
9.	Chry	121.35 ± 0.63	115.05 ± 0.42	106.75 ± 0.64
10.	BeP	77.93 ± 1.46	82.96 ± 4.82	76.04 ± 0.86
11.	BbF	64.95 ± 0.96	84.08 ± 1.46	79.63 ± 0.46
12.	BkF	84.40 ± 0.21	77.40 ± 0.25	71.00 ± 0.11
13.	BaP	132.80 ± 0.91	81.90 ± 0.13	88.30 ± 0.15
14.	DiahA	74.73 ± 1.32	94.77 ± 0.50	75.17 ± 0.25
15.	BghiP	71.44 ± 5.28	113.29 ± 2.13	101.40 ± 0.16
16.	123cd	115.15 ± 7.18	96.93 ± 1.38	92.73 ± 1.95

Table A-3 Effect of different organic solvent on extraction efficiency of 10 mL of organic solvent

No.	Compounds	% Recovery ± S.D. (n=2)		
		CH_3CN	90:10 CH_3CN/Acetone	80:20 CH_3CN/Acetone
1.	Naph	98.67 ± 1.53	96.00 ± 1.85	96.00 ± 2.83
2.	Ace	101.53 ± 6.25	44.30 ± 1.60	124.45 ± 1.75
3.	Fl	186.33 ± 1.15	84.70 ± 0.18	103.40 ± 0.38
4.	Phen	111.23 ± 1.70	99.19 ± 0.94	110.07 ± 9.88
5.	Ant	73.60 ± 0.52	119.50 ± 0.86	154.30 ± 2.20
6.	Ft	103.67 ± 22.20	100.36 ± 4.78	98.84 ± 1.46
7.	Pyr	111.03 ± 1.34	70.00 ± 0.09	78.16 ± 0.41
8.	BaA	146.13 ± 0.49	100.60 ± 0.04	110.95 ± 0.54
9.	Chry	159.70 ± 0.35	106.15 ± 0.18	116.90 ± 0.69
10.	BeP	91.09 ± 4.48	72.84 ± 0.04	81.61 ± 2.38
11.	BbF	53.03 ± 13.77	45.55 ± 0.26	29.60 ± 20.12
12.	BkF	69.60 ± 2.21	52.50 ± 0.16	96.10 ± 0.05
13.	BaP	101.60 ± 0.25	62.30 ± 0.05	84.80 ± 0.03
14.	DiahA	35.73 ± 2.31	55.70 ± 0.33	53.90 ± 0.42
15.	BghiP	106.87 ± 4.50	88.83 ± 1.20	92.27± 15.04
16.	123cd	116.07 ± 12.33	59.20 ± 2.30	28.71 ± 2.93

Table A-4 Effect of number of extraction time of acetonitrile/acetone mixture (80:20, v/v)

No.	Compounds	% Recovery ± S.D. (n=2)		
		1 Time	2 Times	3 Times
1.	Naph	47.62 ± 2.13	98.76 ± 0.44	71.24 ± 1.26
2.	Ace	86.65 ± 0.36	95.45 ± 0.45	116.30 ± 0.40
3.	Fl	77.20 ± 0.07	115.30 ± 0.42	79.10 ± 0.15
4.	Phen	72.33 ± 0.01	118.40 ± 5.11	116.79 ± 3.90
5.	Ant	74.80 ± 0.03	116.50 ± 0.39	136.70 ± 0.59
6.	Ft	66.98 ± 9.83	99.68 ± 0.57	103.31 ± 1.58
7.	Pyr	61.00 ± 0.14	71.34 ± 0.88	87.96 ± 0.21
8.	BaA	90.30 ± 0.11	104.70 ± 0.44	125.65 ± 0.21
9.	Chry	91.60 ± 0.03	106.75 ± 0.64	129.65 ± 0.29
10.	BeP	58.65 ± 0.08	76.04 ± 0.86	95.90 ± 0.86
11.	BbF	64.00 ± 0.06	79.63 ± 0.46	98.65 ± 0.18
12.	BkF	60.70 ± 0.56	71.00 ± 0.11	104.50 ± 0.09
13.	BaP	50.50 ± 0.01	88.30 ± 0.15	78.20 ± 0.04
14.	DiahA	60.43 ± 0.19	75.17 ± 0.25	87.00 ± 0.16
15.	BghiP	67.71 ± 0.28	101.40 ± 0.16	120.00 ± 0.49
16.	123cd	46.19 ± 0.74	92.73 ± 1.95	76.46 ± 0.59

Table A-5 Effect of SPE sorbent types (alumina N, florisil, and SPE C_{18}) for 8 mL on clean up

No.	Compounds	% Recovery ± S.D. (n=2)		
		SPE C_{18}	Florisil	Alumina N
1.	Naph	49.22 ± 26.91	60.42 ± 4.07	98.76 ± 0.44
2.	Ace	40.55 ± 4.60	94.25 ± 0.07	95.45 ± 0.45
3.	Fl	38.70 ± 2.92	105.30 ± 0.19	115.30 ± 0.42
4.	Phen	58.84 ± 2.67	78.64 ± 1.60	118.40 ± 5.11
5.	Ant	14.64 ± 4.82	58.63 ± 1.95	116.50 ± 0.39
6.	Ft	31.22 ± 7.66	66.84 ± 0.12	99.68 ± 0.57
7.	Pyr	42.35 ± 3.06	102.30 ± 0.16	71.34 ± 0.88
8.	BaA	34.05 ± 4.05	101.45 ± 0.03	104.70 ± 0.44
9.	Chry	11.84 ± 2.99	75.38 ± 1.00	106.75 ± 0.64
10.	BeP	24.55 ± 2.62	81.83 ± 0.23	76.04 ± 0.86
11.	BbF	28.20 ± 0.52	83.90 ± 0.10	79.63 ± 0.46
12.	BkF	17.20 ± 0.57	99.30 ± 0.12	71.00 ± 0.11
13.	BaP	20.70 ± 1.00	72.23 ± 0.90	88.30 ± 0.15
14.	DiahA	26.91 ± 6.65	91.99 ± 0.02	75.17 ± 0.25
15.	BghiP	8.31 ± 2.63	90.73 ± 8.00	101.40 ± 0.16
16.	123cd	115.15 ± 7.18	96.93 ± 1.38	92.73 ± 1.95

Table A-6 Effect of distillation temperature and time for sweep co-distillation method

Compounds	% Recovery ± S.D. (n=2)					
	230 °C			235 °C		
	30 mins	45 mins	60 mins	30 mins	45 mins	60 mins
Naph	8.50 ± 6.82	5.72 ± 0.85	4.64 ± 0.85	17.84 ± 0.86	28.00 ± 0.16	10.28 ± 0.11
Ace	51.33 ± 1.59	40.55 ± 0.96	43.20 ± 0.96	70.28 ± 0.60	81.00 ± 0.27	86.74 ± 2.85
Fl	69.05 ± 2.19	85.73 ± 0.71	82.35 ± 0.71	101.47 ± 1.75	116.75 ± 1.20	110.45 ± 1.48
Phen	56.49 ± 1.82	58.21 ± 0.01	59.20 ± 0.01	53.88 ± 0.42	58.48 ± 0.14	59.83 ± 2.42
Ant	64.55 ± 2.90	62.56 ± 3.61	69.40 ± 3.61	66.41 ± 1.69	74.70 ± 1.13	71.05 ± 0.07
Ft	60.09 ± 0.16	60.74 ± 0.94	87.42 ± 0.94	69.33 ± 2.85	61.26 ± 0.83	79.53 ± 0.18
Pyr	64.85 ± 2.64	63.36 ± 0.73	81.57 ± 0.78	67.65 ± 0.52	68.15 ± 1.99	76.33 ± 1.77
BaA	51.30 ± 1.41	61.75 ± 0.65	97.93 ± 0.65	70.61 ± 0.79	64.39 ± 1.15	102.25 ± 3.75
Chry	50.75 ± 1.34	64.46 ± 1.91	97.75 ± 1.91	68.43 ± 3.50	67.20 ± 1.27	99.59 ± 0.62
BeP	14.37 ± 0.47	14.46 ± 0.78	35.73 ± 0.78	22.36 ± 2.88	19.31 ± 0.57	39.90 ± 0.15
BbF	14.70 ± 0.11	15.69 ± 0.69	35.79 ± 0.69	20.43 ± 0.54	19.89 ± 0.80	40.78 ± 1.09
BkF	21.80 ± 0.14	30.06 ± 0.09	39.00 ± 0.09	33.27 ± 3.72	30.85 ± 0.49	35.11 ± 2.11
BaP	13.75 ± 1.48	21.10 ± 1.28	35.20 ± 1.28	17.20 ± 2.31	19.78 ± 0.59	35.61 ± 0.84
DiahA	2.53 ± 0.71	4.72 ± 0.73	7.00 ± 0.78	6.10 ± 0.10	3.67 ± 3.21	10.97 ± 2.17
BghiP	5.42 ± 0.54	8.80 ± 1.43	13.02 ± 1.43	4.76 ± 1.14	4.18 ± 2.16	16.40 ± 2.25
123cd	2.97 ± 0.70	6.40 ± 0.71	8.69 ± 0.71	5.36 ± 0.50	5.95 ± 0.07	12.63 ± 1.80

APPENDIX B

APPENDIX B Calibration curve

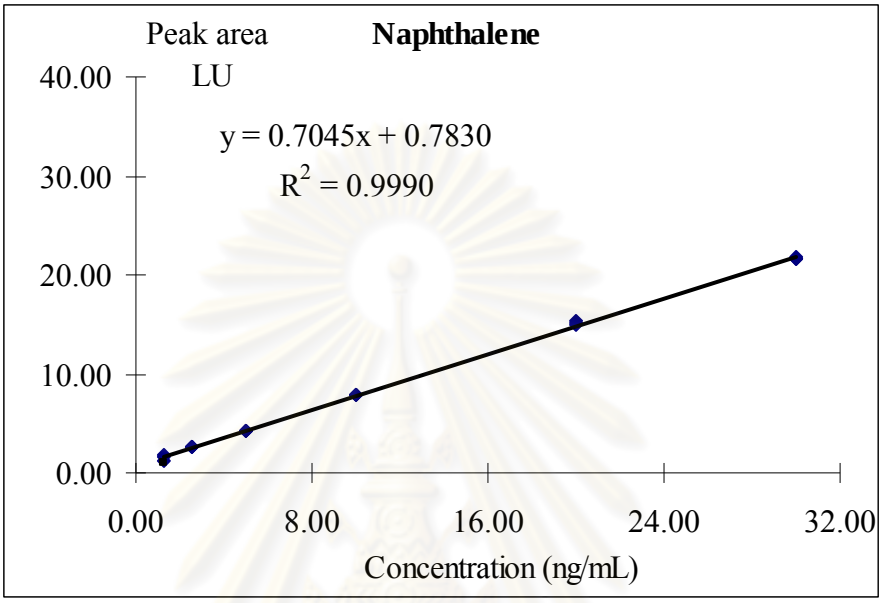

Figure B-1 Standard calibration curve of naphthalene

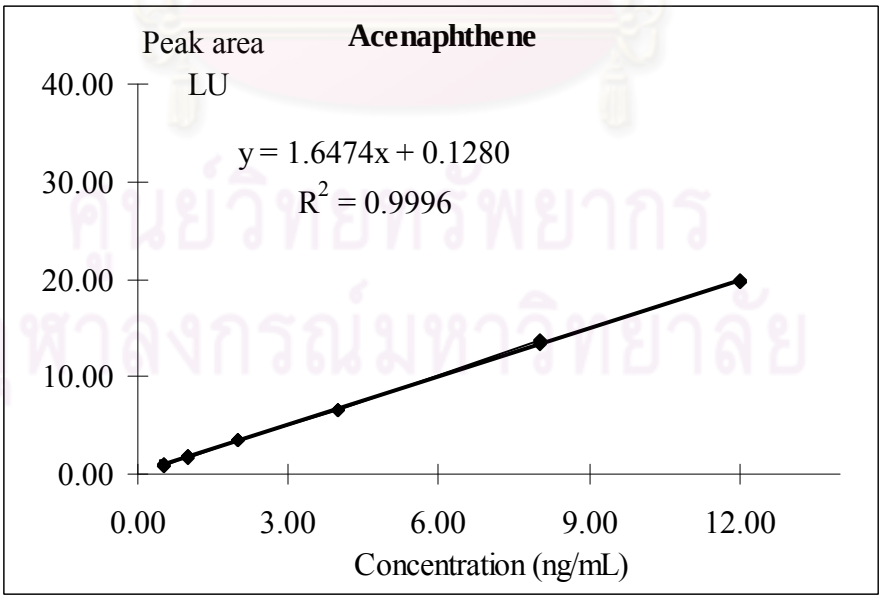

Figure B-2 Standard calibration curve of acenaphthene

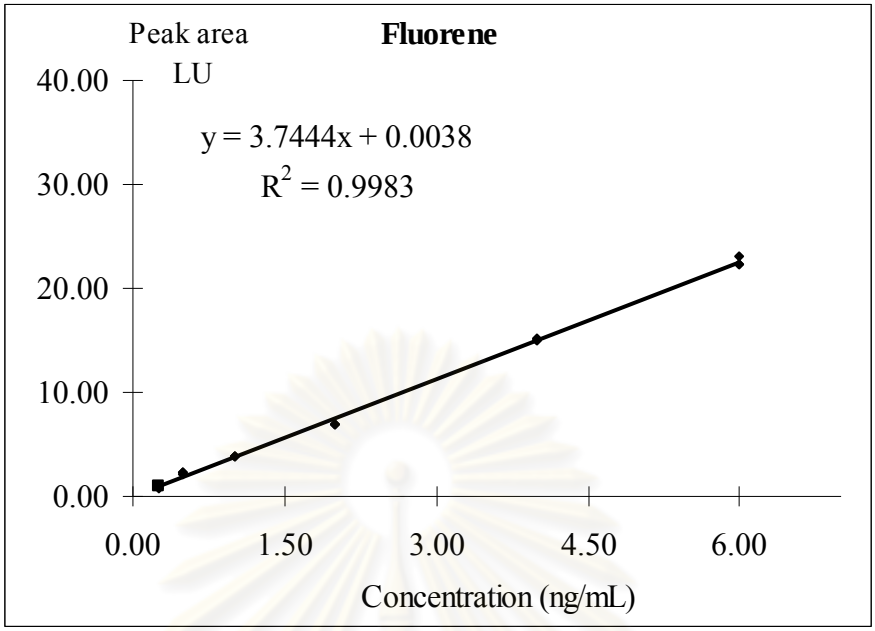

Figure B-3 Standard calibration curve of fluorene

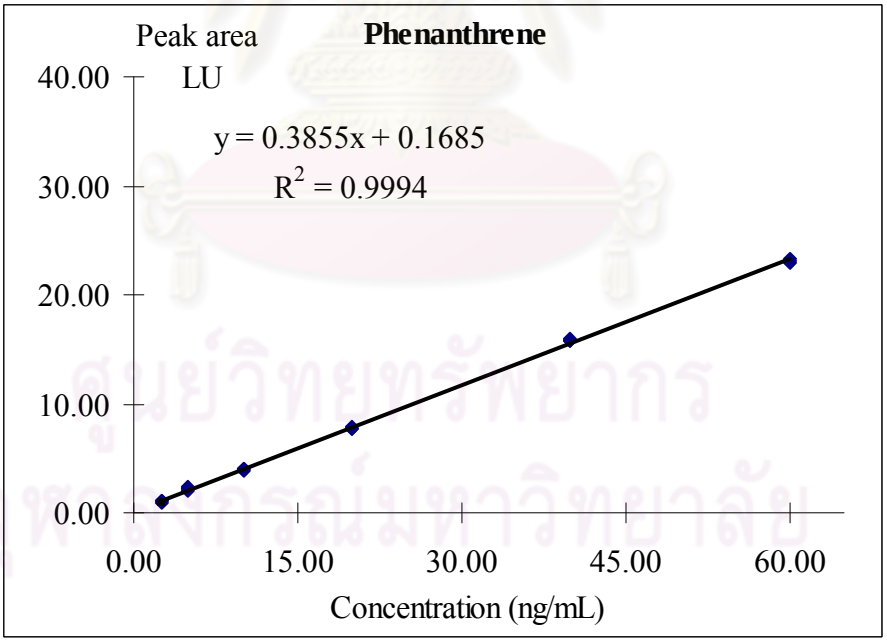

Figure B-4 Standard calibration curve of phenanthrene

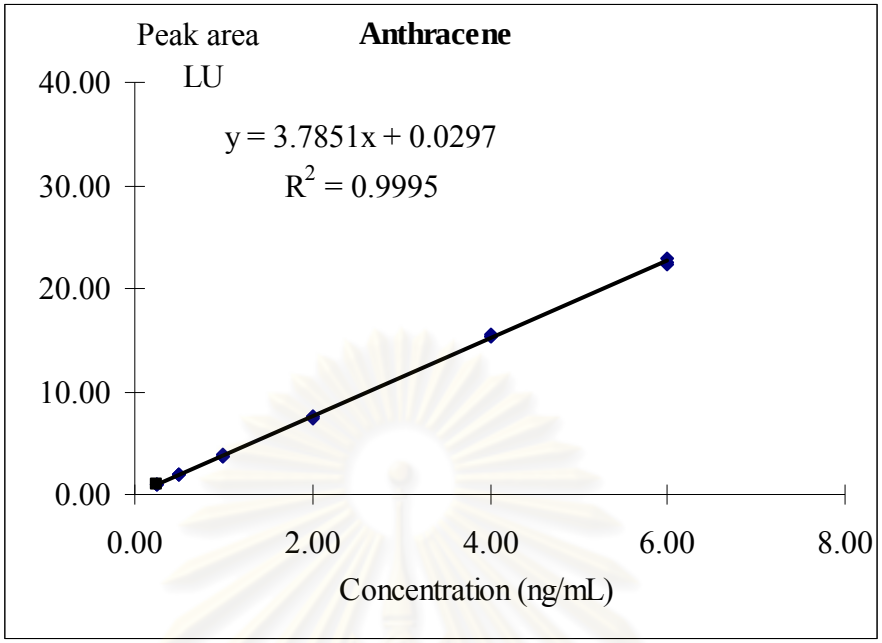

Figure B-5 Standard calibration curve of anthracene

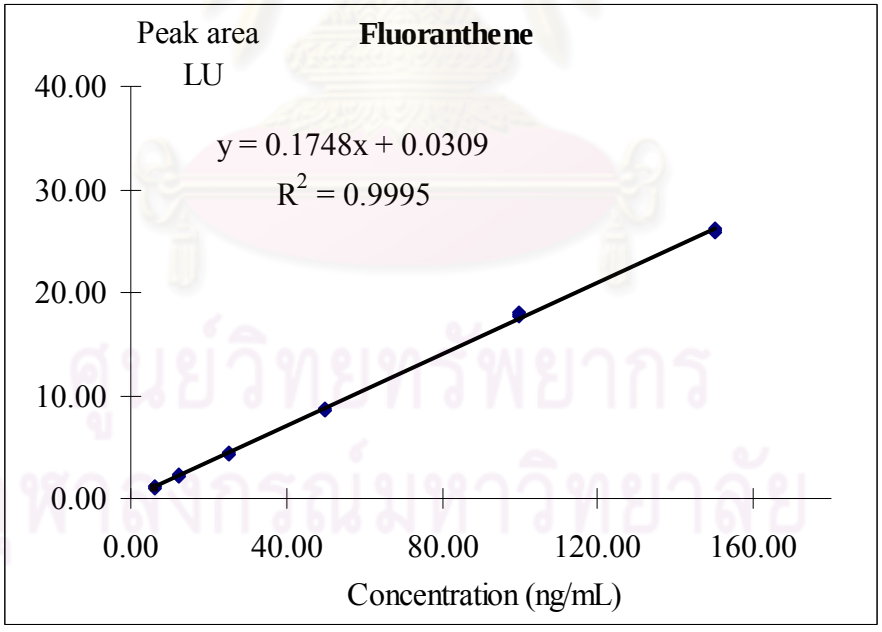

Figure B-6 Standard calibration curve of fluoranthene

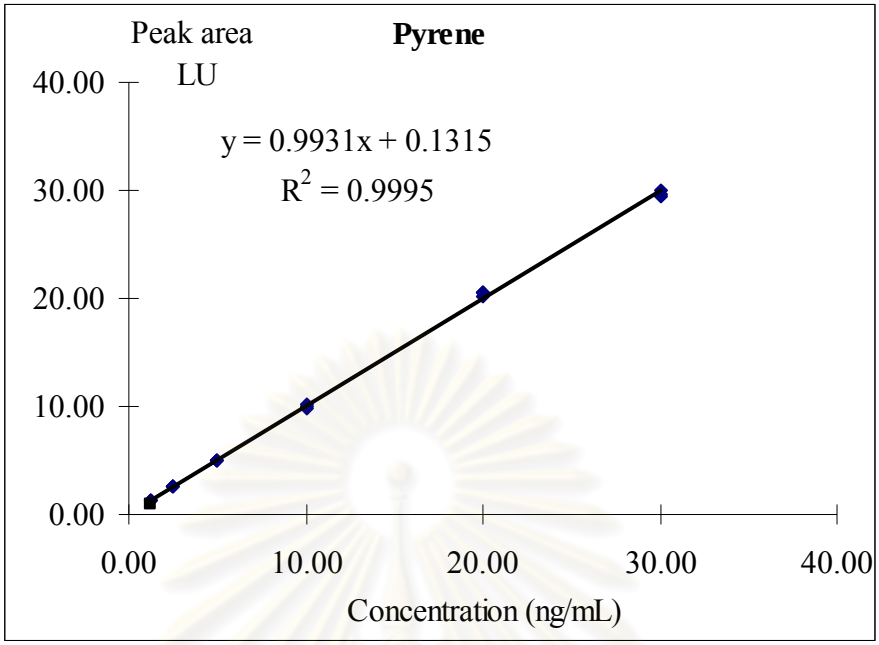

Figure B-7 Standard calibration curve of pyrene

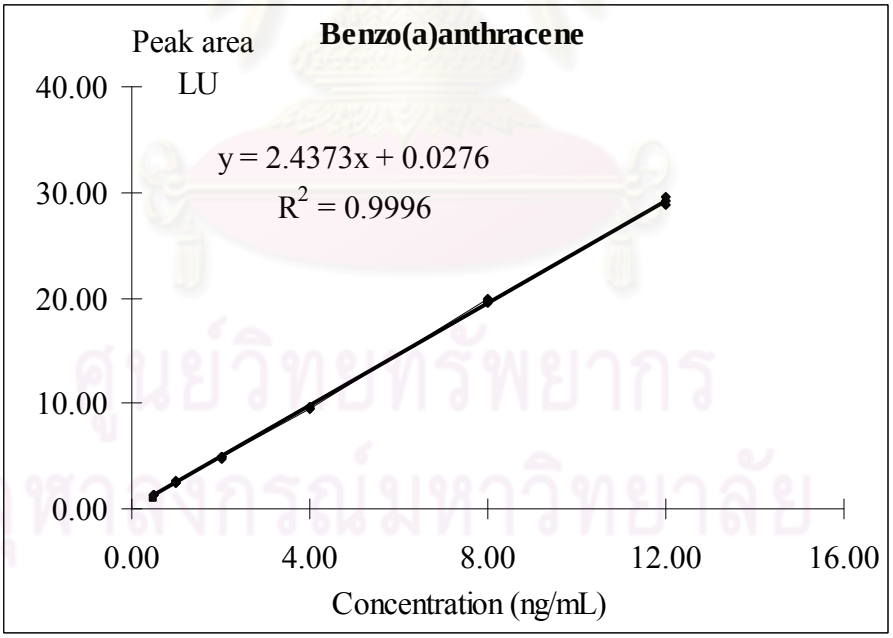

Figure B-8 Standard calibration curve of benzo(a)anthracene

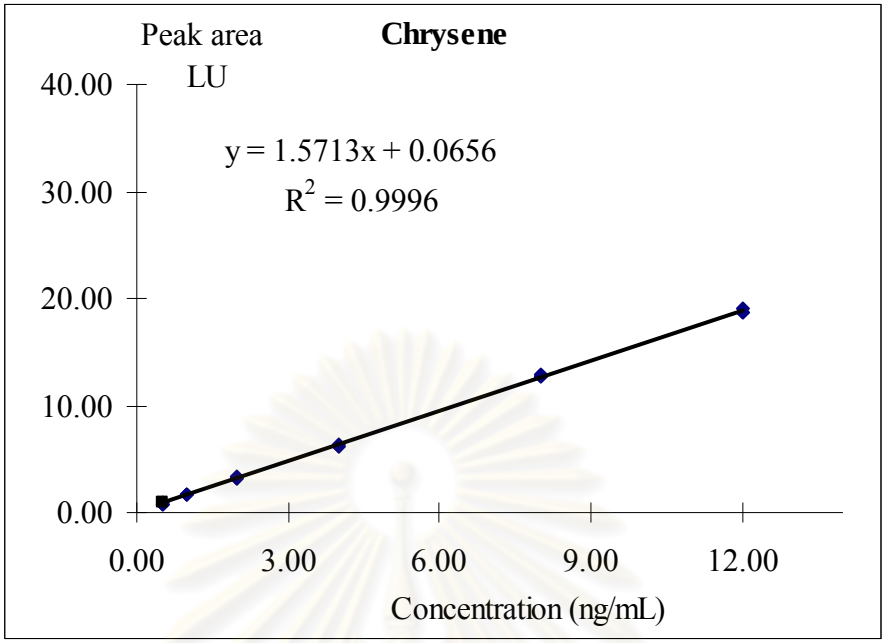

Figure B-9 Standard calibration curve of chrysene

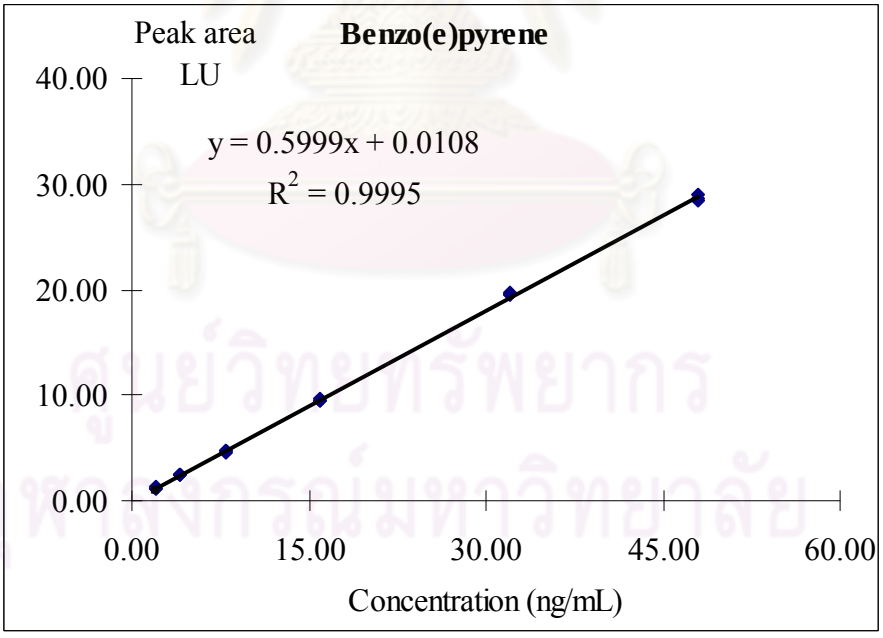

Figure B-10 Standard calibration curve of benzo(e)pyrene

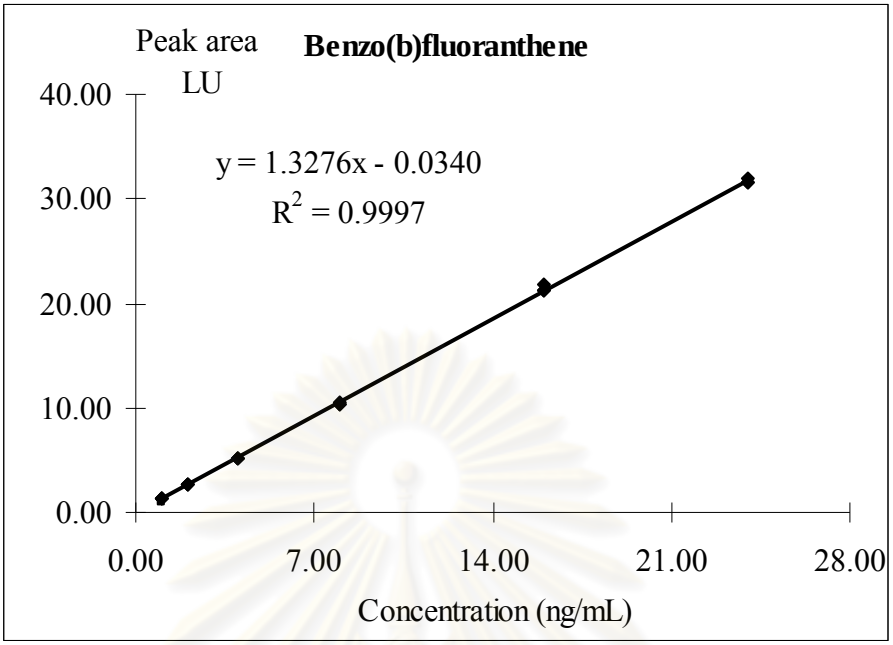

Figure B-11 Standard calibration curve of benzo(b)fluoranthene

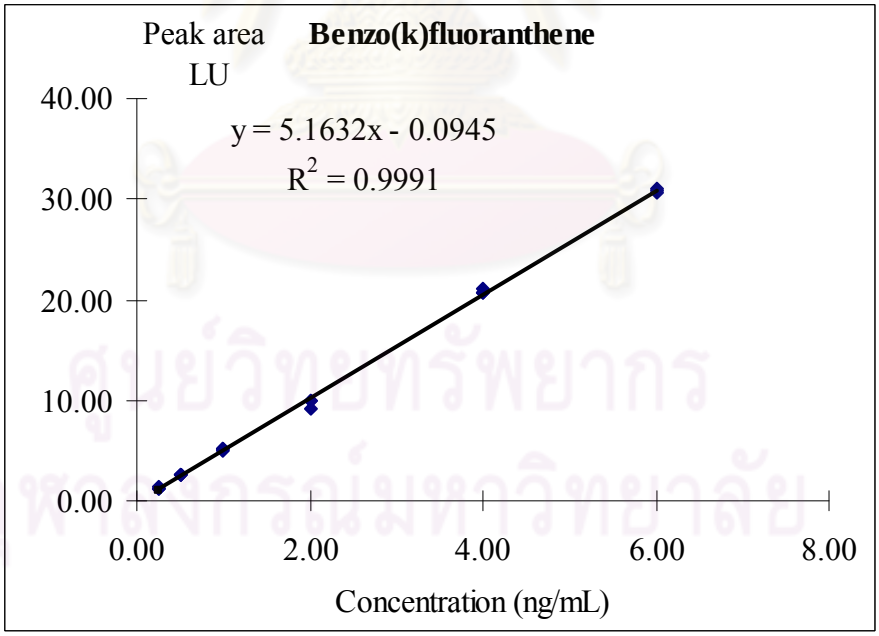

Figure B-12 Standard calibration curve of benzo(k)fluoranthene

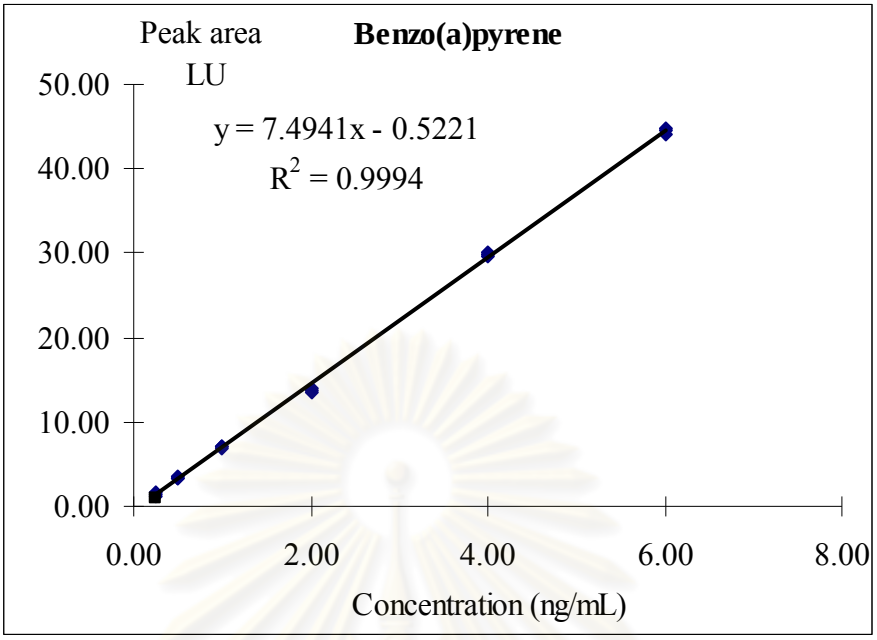

Figure B-13 Standard calibration curve of benzo(a)pyrene

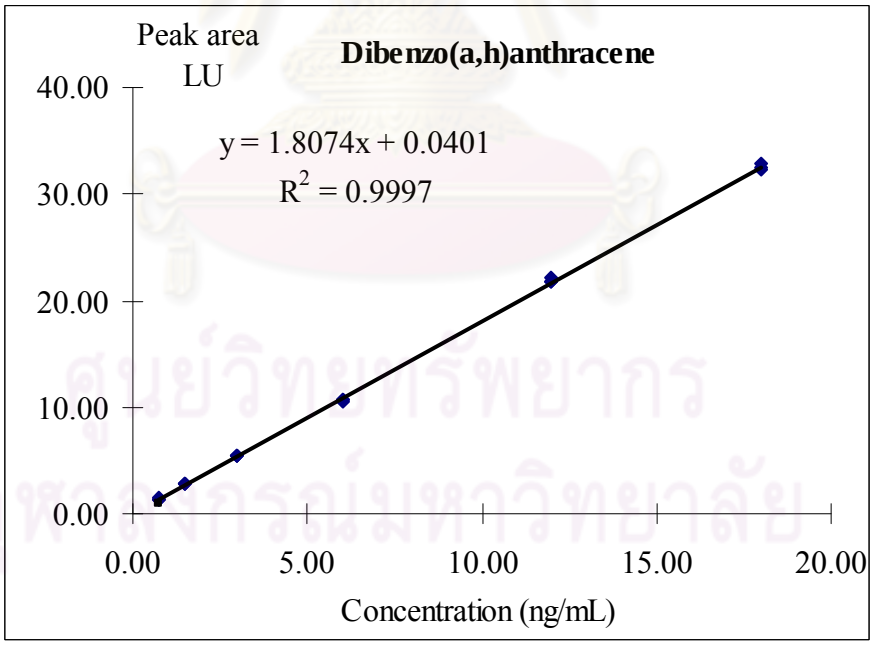

Figure B-14 Standard calibration curve of dibenzo(a,h)anthracene

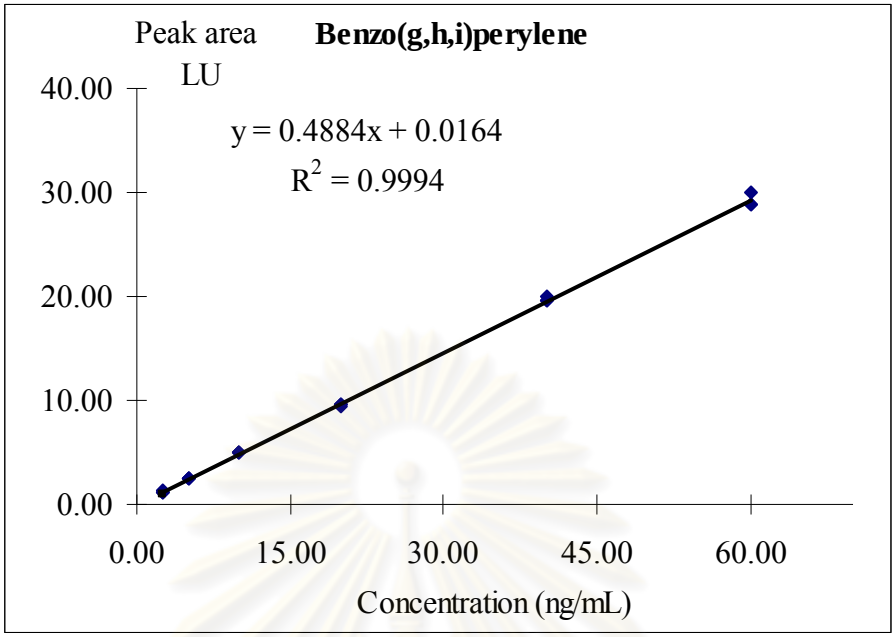

Figure B-15 Standard calibration curve of benzo(g,h,i)perylene

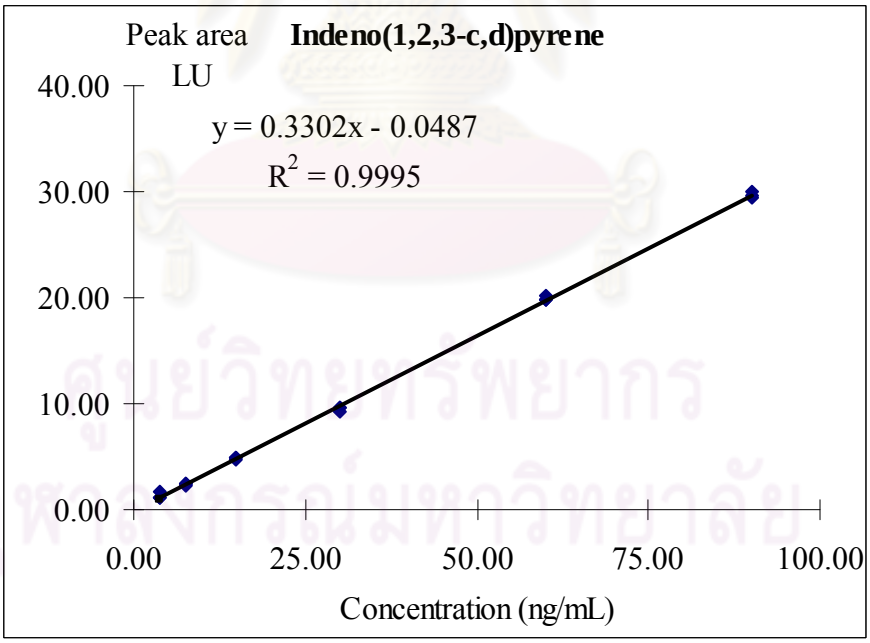

Figure B-16 Standard calibration curve of indeno(1,2,3-c,d)pyrene

APPENDIX C

APPENDIX C Linearity and working range under the optimum low-temperature cleanup condition

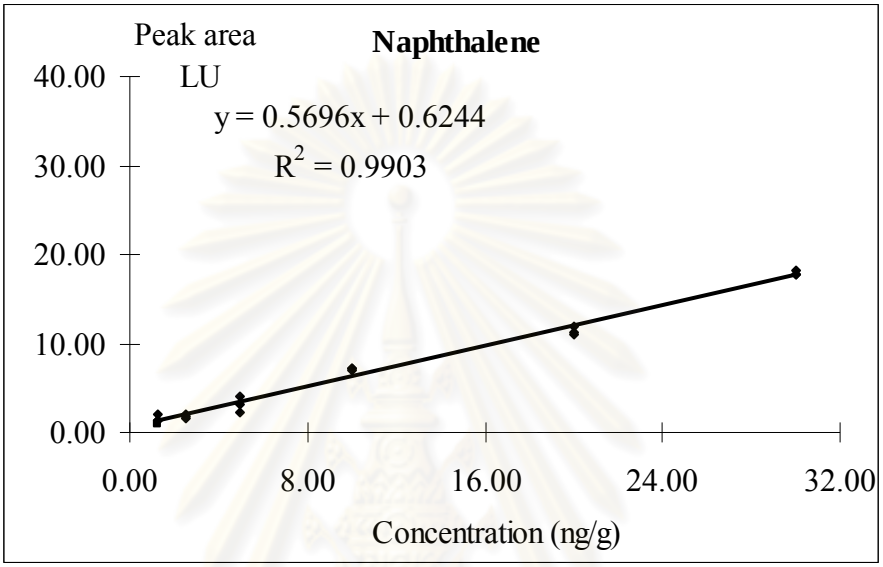

Figure C-1 Linearity and working range of naphthalene under the optimum low-temperature cleanup condition

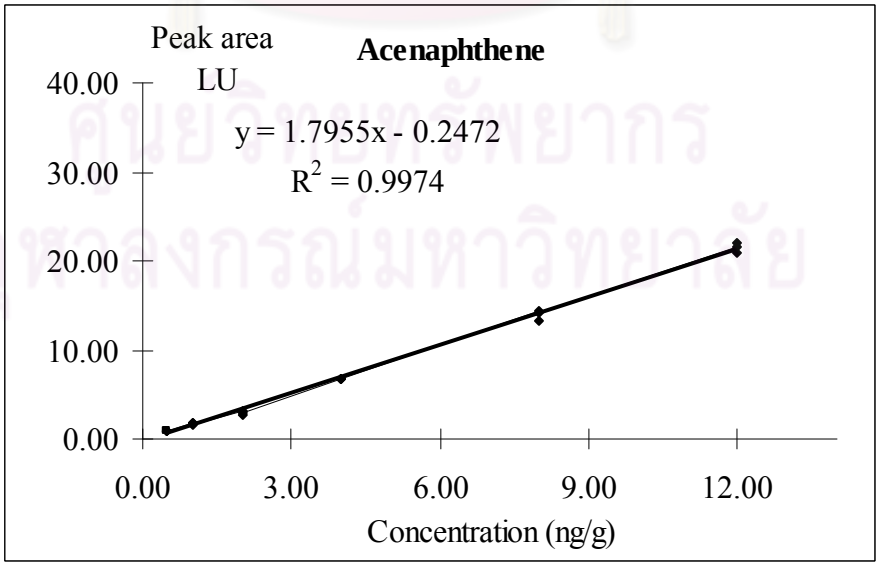

Figure C-2 Linearity and working range of acenaphthene under the optimum low-temperature cleanup condition

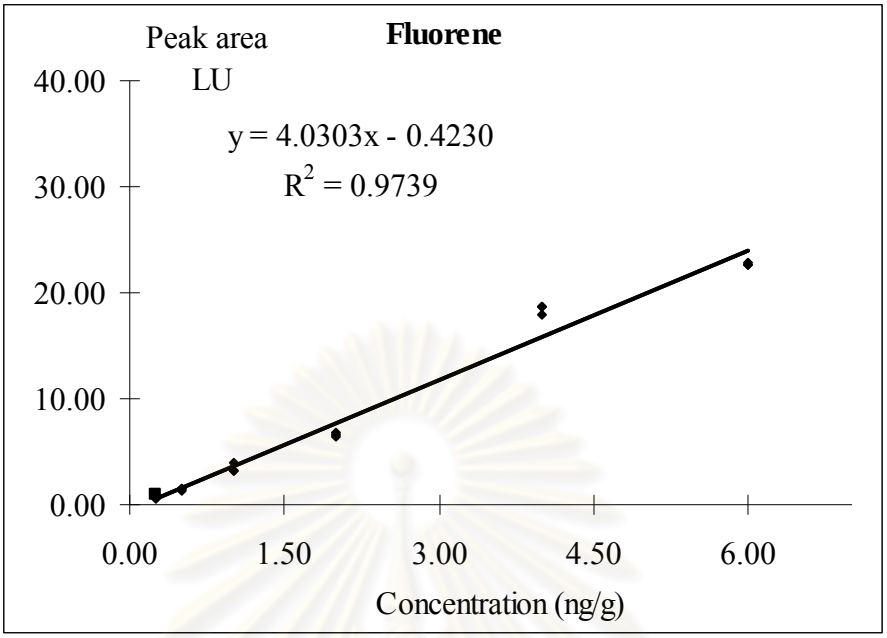

Figure C-3 Linearity and working range of fluorene under the optimum low-temperature cleanup condition

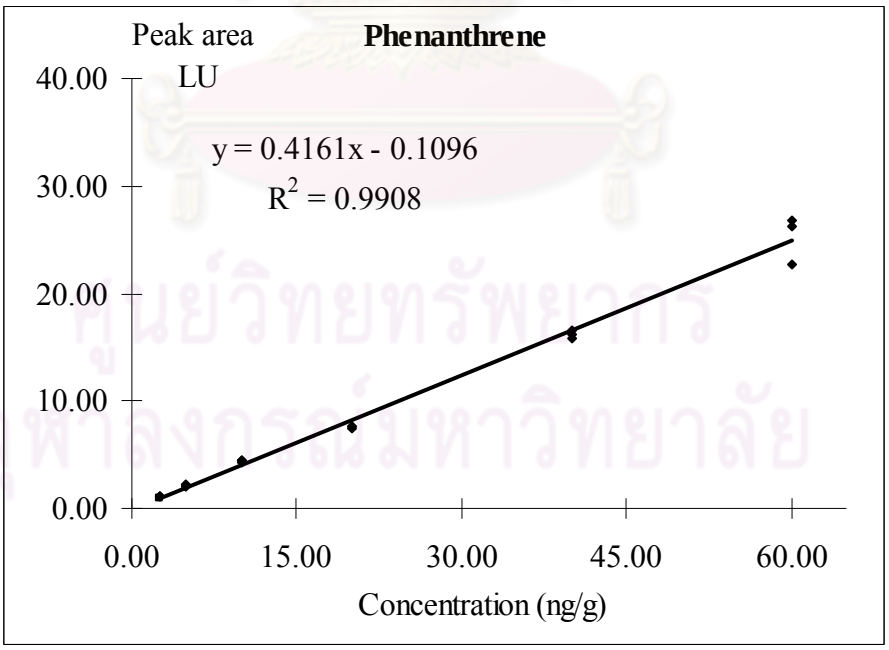

Figure C-4 Linearity and working range of phenanthrene under the optimum low-temperature cleanup condition

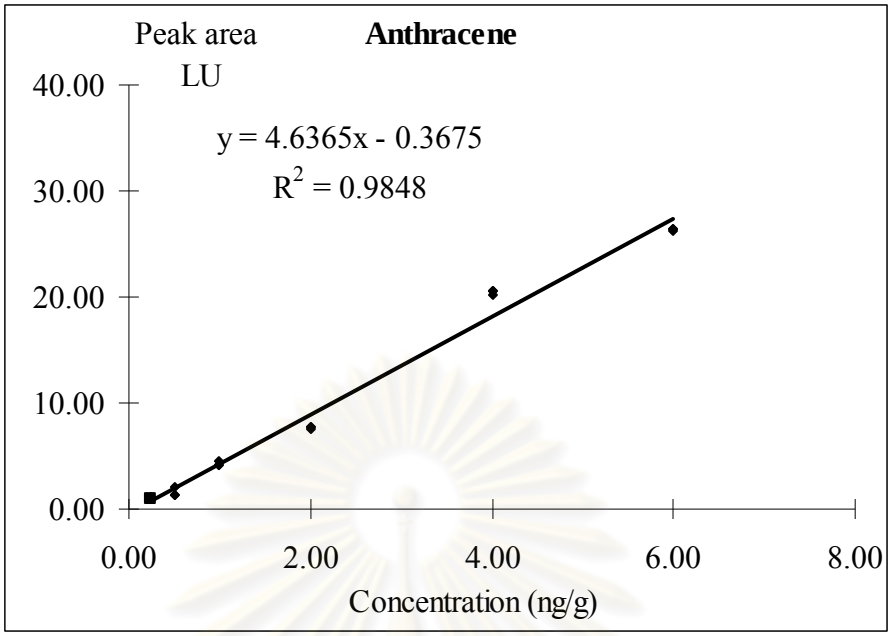

Figure C-5 Linearity and working range of anthracene under the optimum low-temperature cleanup condition

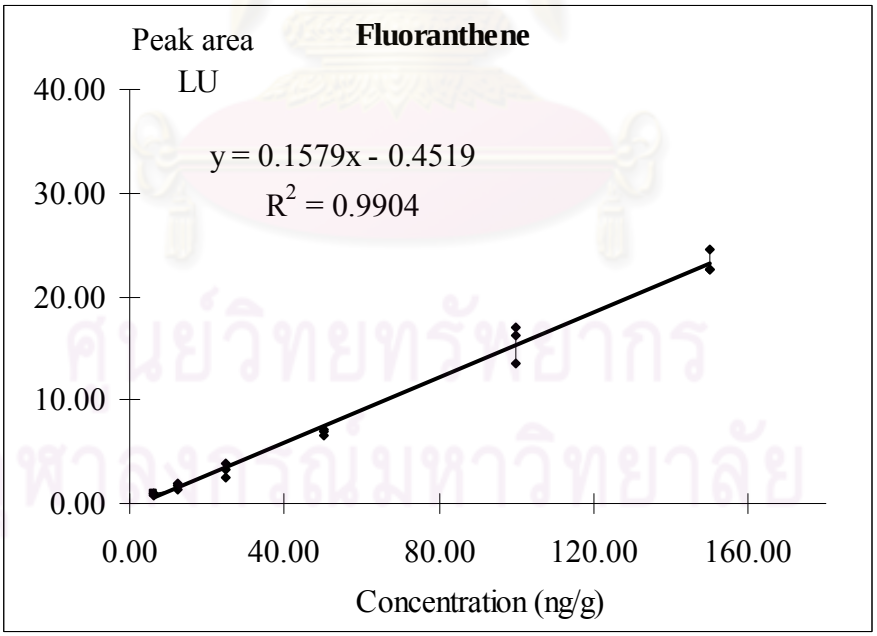

Figure C-6 Linearity and working range of fluoranthene under the optimum low-temperature cleanup condition

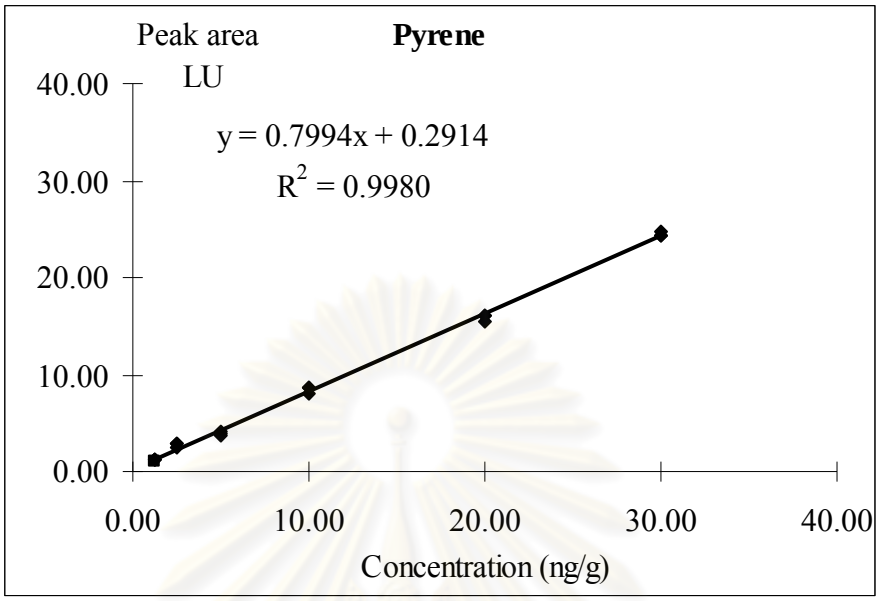

Figure C-7 Linearity and working range of pyrene under the optimum low-temperature cleanup condition

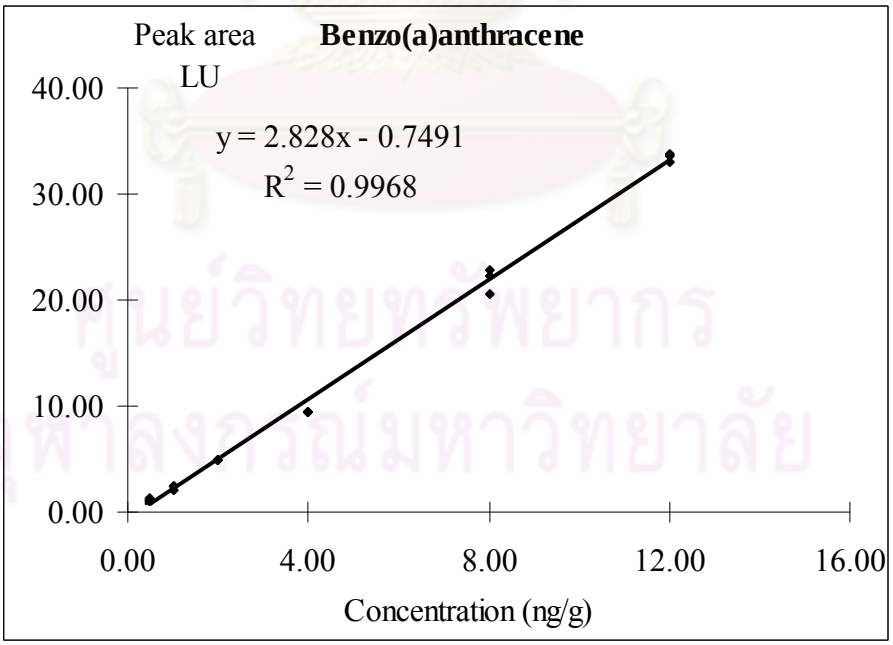

Figure C-8 Linearity and working range of benzo(a)anthracene under the optimum low-temperature cleanup condition

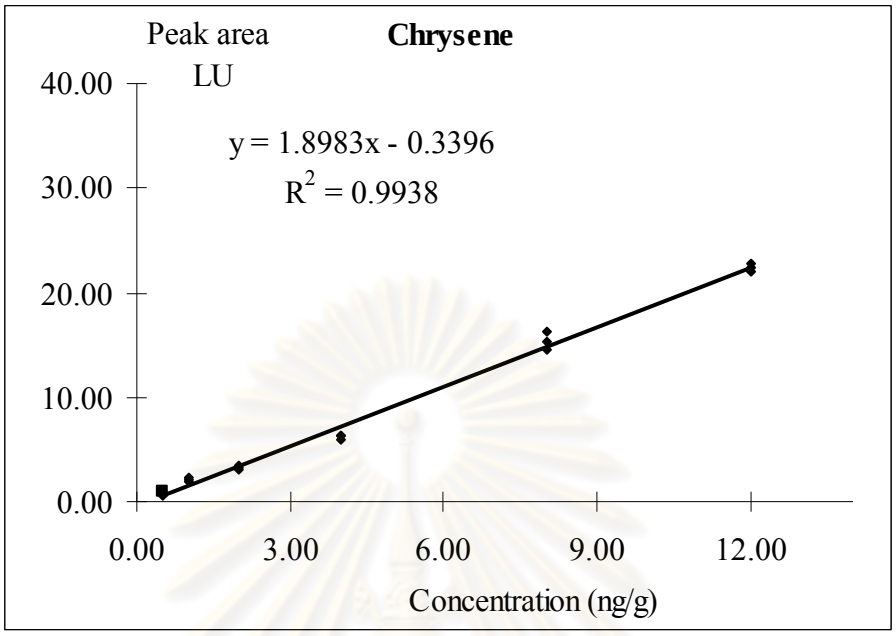

Figure C-9 Linearity and working range of chrysene under the optimum low-temperature cleanup condition

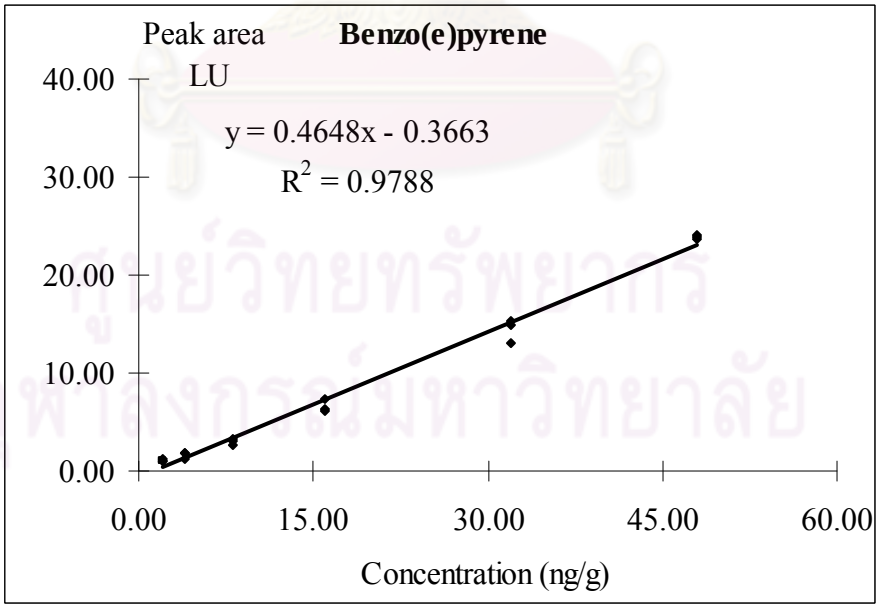

Figure C-10 Linearity and working range of benzo(e)pyrene under the optimum low-temperature cleanup condition

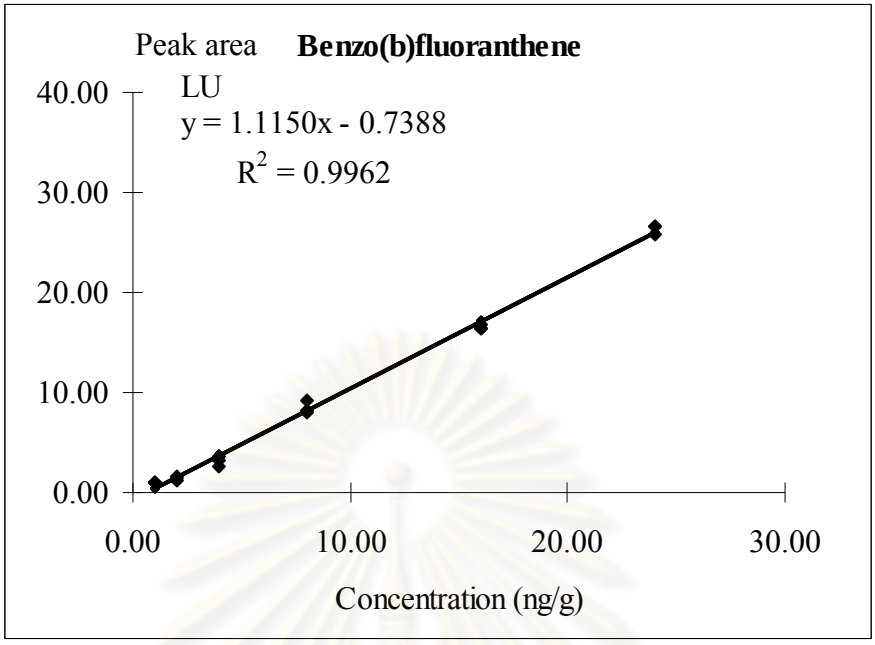

Figure C-11 Linearity and working range of benzo(b)fluoranthene under the optimum low-temperature cleanup condition

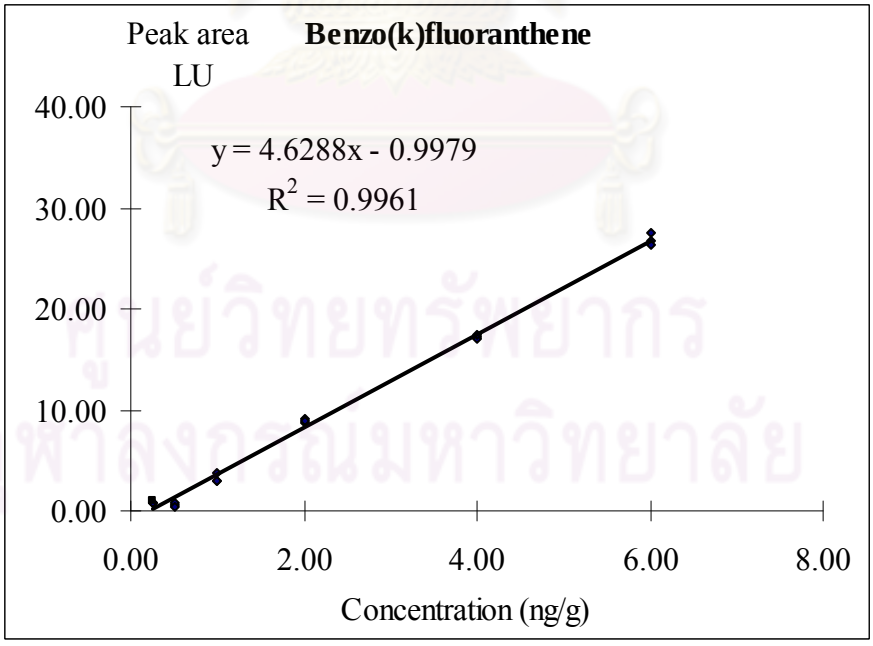

Figure C-12 Linearity and working range of benzo(k)fluoranthene under the optimum low-temperature cleanup condition

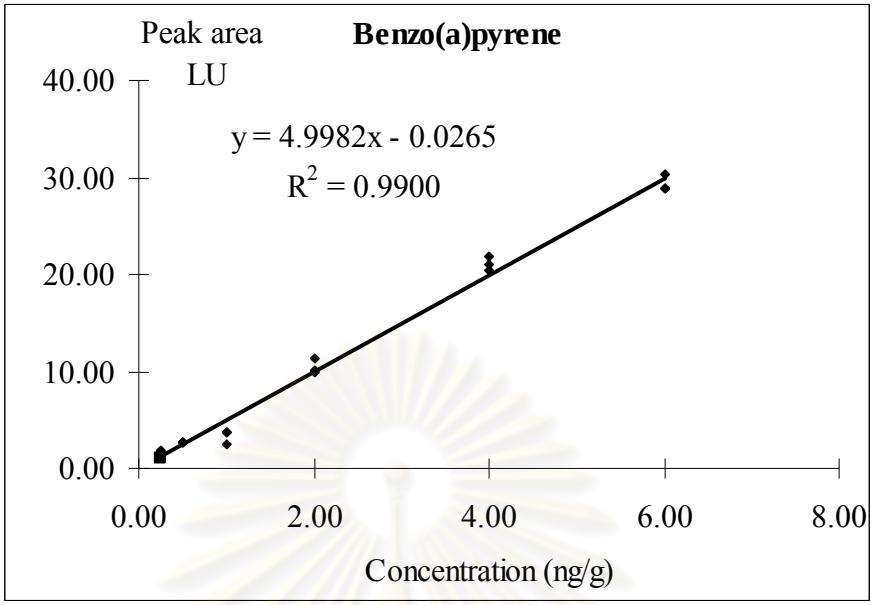

Figure C-13 Linearity and working range of benzo(a)pyrene under the optimum low-temperature cleanup condition

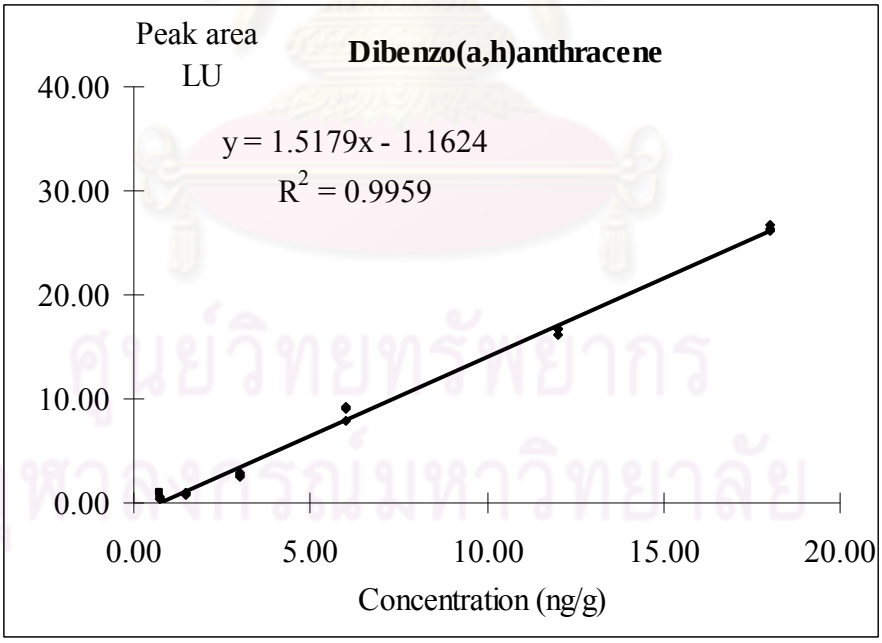

Figure C-14 Linearity and working range of dibenzo(a,h)anthracene under the optimum low-temperature cleanup condition

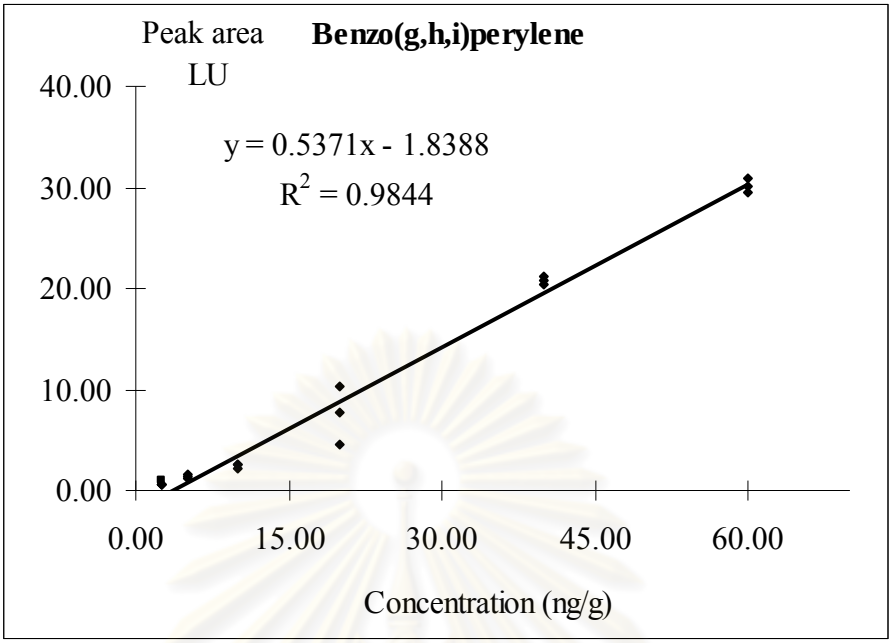

Figure C-15 Linearity and working range of benzo(g,h,i)perylene under the optimum low-temperature cleanup condition

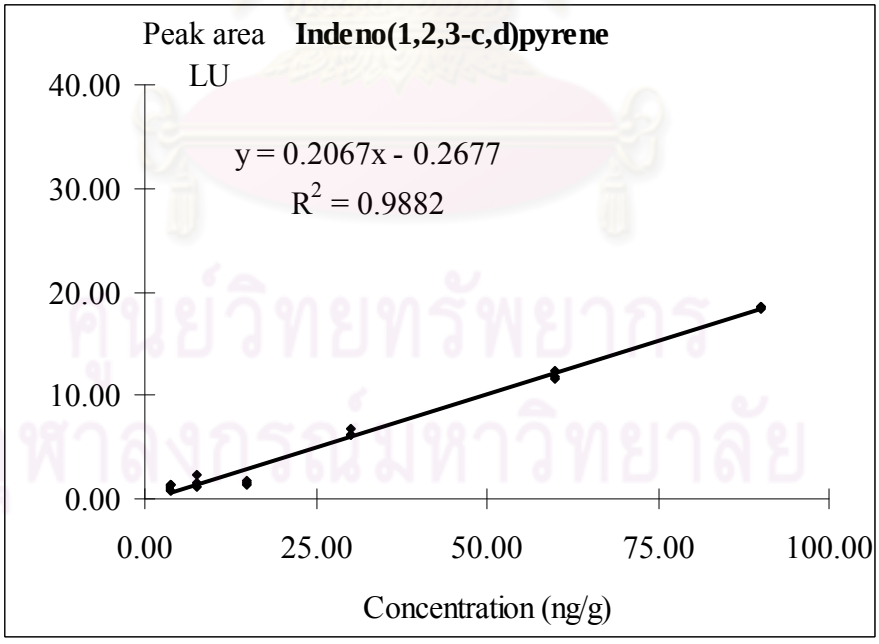

Figure C-16 Linearity and working range of indeno(1,2,3-c,d)pyrene under the optimum low-temperature cleanup condition

APPENDIX D

APPENDIX D Linearity and working range of naphthalene under the optimum sweep co-distillation condition

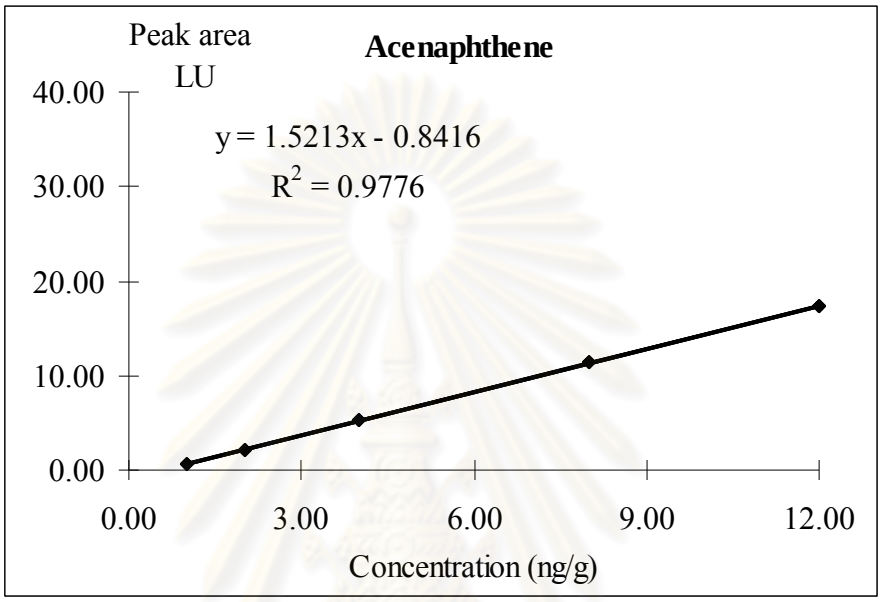

Figure D-1 Linearity and working range of acenaphthene under the optimum sweep co-distillation condition

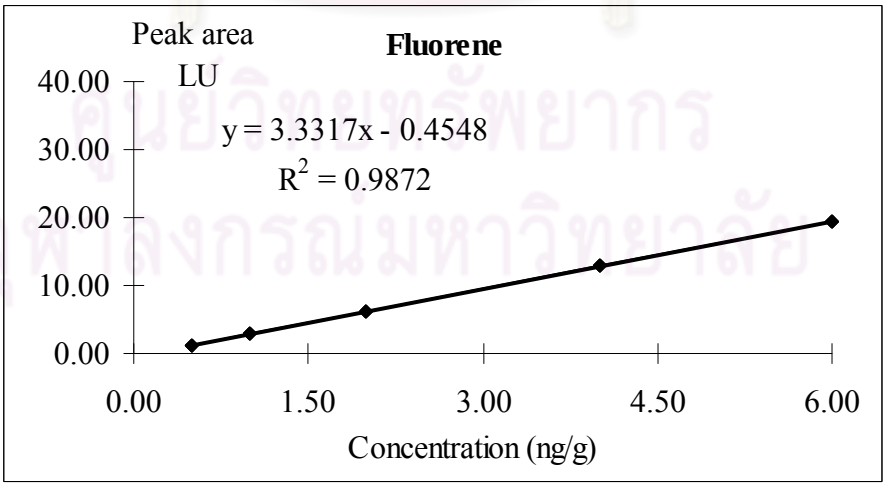

Figure D-2 Linearity and working range of fluorene under the optimum sweep co-distillation condition

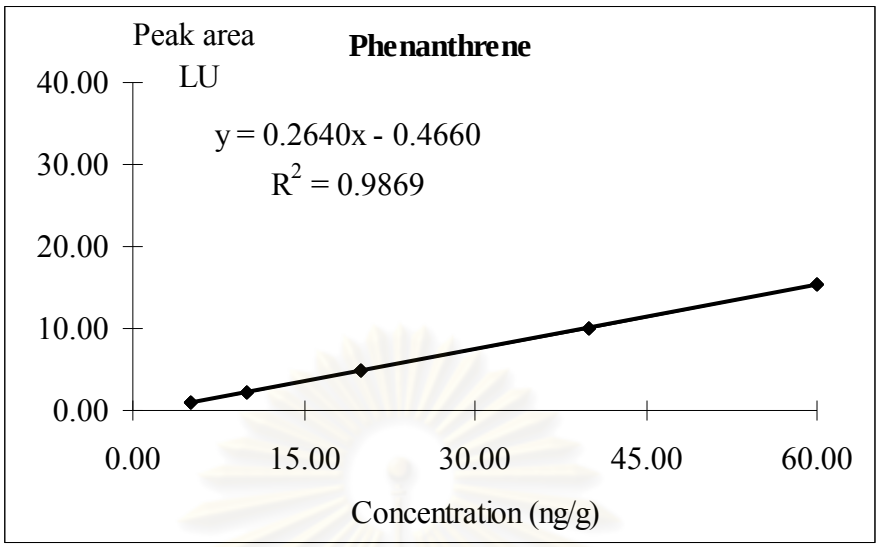

Figure D-3 Linearity and working range of phenanthrene under the optimum sweep co-distillation condition

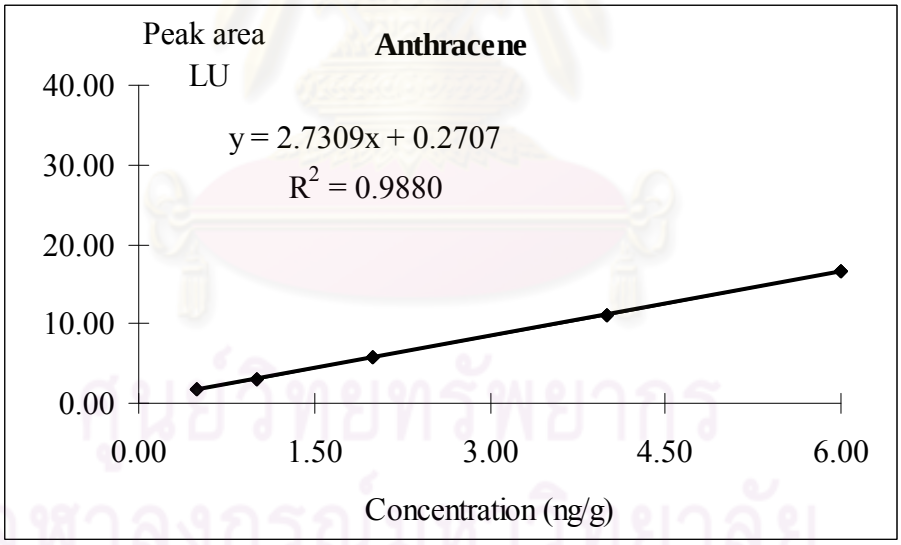

Figure D-4 Linearity and working range of anthracene under the optimum sweep co-distillation condition

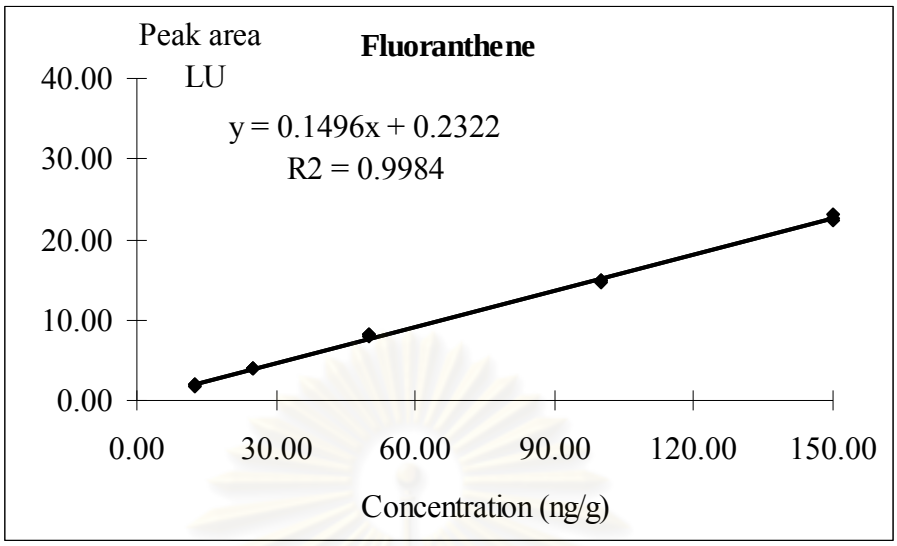

Figure D-5 Linearity and working range of fluoranthene under the optimum sweep co-distillation condition

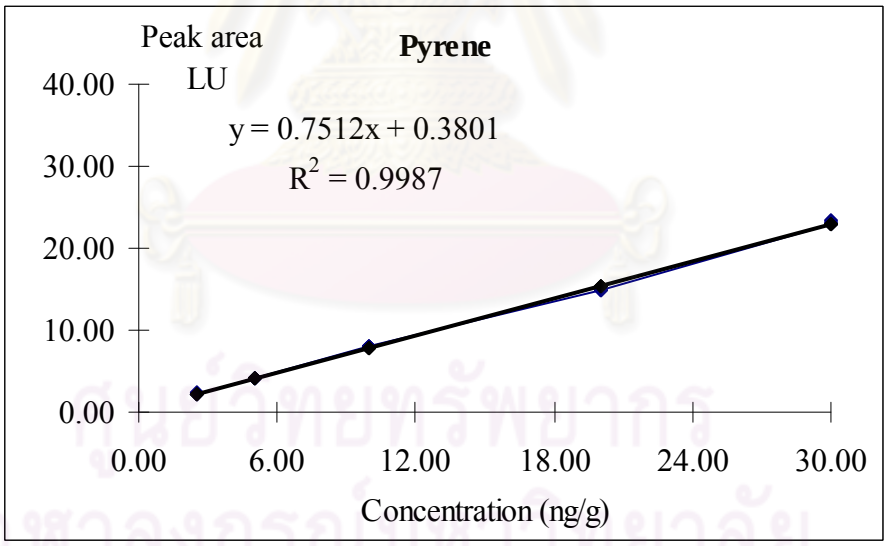

Figure D-6 Linearity and working range of pyrene under the optimum sweep co-distillation condition

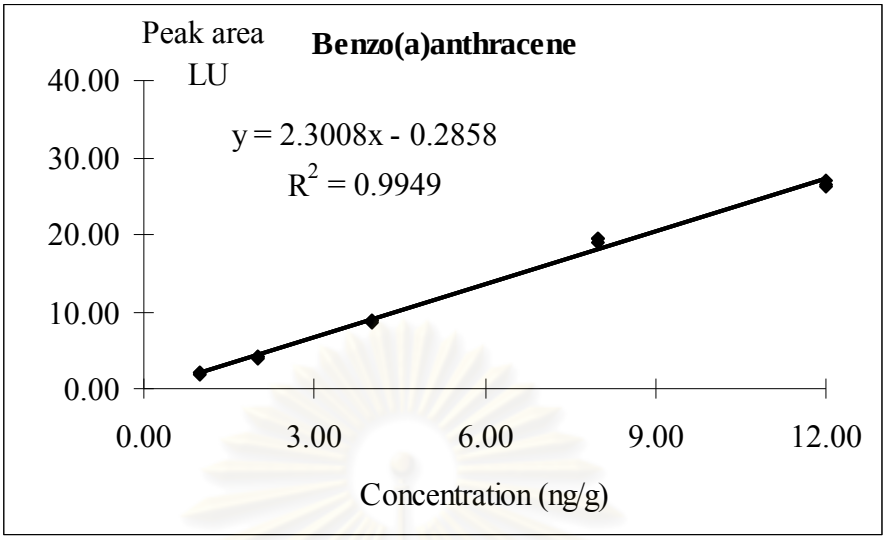

Figure D-7 Linearity and working range of benzo(a)anthracene under the optimum sweep co-distillation condition

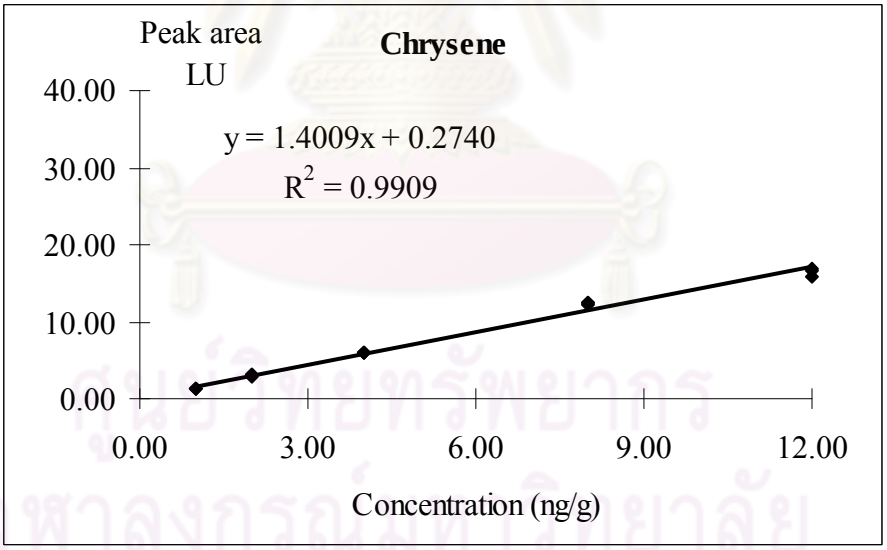

Figure D-8 Linearity and working range of chrysene under the optimum sweep co-distillation condition

VITA

Mrs Thongsuk Payanan was born on July 10, 1967, in Samutsakorn, Thailand. She had graduated a Bachelor's degree of Science in Chemistry from Ramkumheang University in 1993. Afterwards, she continued her academic education for Master degree at Department of Chemistry, Faculty of Science, Chulalongkorn University. She had completed the program in April 2009 and received her Master's degree of Science in Analytical Chemistry in July 2010. At present, she has been working in Bureau of Quality and Safety of Food, Department of Medical Sciences, Ministry of Publich Health since 1976.