DEVELOPMENT OF EXTRACTION AND SEPARATION TECHNIQUES FOR THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS AND BIOGENIC AMINES IN FOOD

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LIST OF ABBREVIATIONS

5MCh	5-methylchrysene
Ace	Acenaphthene
ACN	Acetonitrile
Acy	Acenaphthylene
AGREE	Analytical GREEness metric profile
ANP-LC	Aqueous normal phase liquid chromatography
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
Ant	Anthracene
BAA	Benz(a)anthracene
BAP	Benzo(a)pyrene
BAs	Biogenic amines
BBF	Benzo(b)fluoranthene
BcF	Benzo(c)fluorene
BghiP	Benzo(g,h,i)perylene
BjF	Benzo(j)fluoranthene
BkF	Benzo(k)fluoranthene
C18 column	Octyldecylsilane column
C18	Carbon-18
C_2Cl_4	Tetrachloroethylene
C4	Carbon-4
CAD	Cadaverine
CCl_4	Tetrachloromethane
$CHCl_3$	Chloroform
CHR	Chrysene
CONTAM	EFSA Panel on Contaminants in the Food Chain
CPP	Cyclopenta(cd)pyrene
DACC	Donor-acceptor complex chromatography

DAD	Diode-array detector
DAO	Diamine oxidase
DAOI	Diamine oxidase inhibitor
DBaeP	Dibenzo(a,e)pyrene
DBahA	Dibenza,hanthracene
DBahP	Dibenzo(a,h)pyrene
DBaiP	Dibenzo(a,i)pyrene
DBalP	Dibenzo(a,l)pyrene
DCM	Dichloromethane
DLLME	Dispersive liquid-liquid extraction
DMC	Dimethylcarbonate
$_d$SPE	Dual-cartridge solid phase extraction
EC	European Commission
EF	Enrichment factor
EFSA	European Food Safety Authority
EI	Electrospray ionization
EtOH	Ethanol
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
Fla	Fluoranthene
FLD	Fluorescence detector
Flu	Fluorene
GAC	Green analytical chemistry
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-HRMS	Gas chromatography-high-resolution mass spectrometry
GC-MS	Gas chromatograph-mass spectrometry
GC-MS/MS	Gas chromatography-triple quadrupole mass spectrometry
GC-SIM	Gas chromatography-selected ion monitoring mode
GC × GC	Comprehensive two-dimensional gas chromatography

GPC	Gel permeation chromatography
HCl	Hydrochloric acid
HILIC	Hydrophilic interaction liquid chromatography
HIS	Histamine
HPAHs	Heavy polycyclic aromatic hydrocarbons
I.D.	Internal diameter
IARC	International Agency for Research on Cancer
IBCF	Isobutyl chloroformate
IP	Indeno(1,2,3-cd)pyrene
IPCS	International Program on Chemical Safety
IS	Internal standard
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LAB	Lactic acid bacteria
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-triple quadrupole mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LPAHs	Light polycyclic aromatic hydrocarbons
LSPTME	Liquid-solid phase transition microextraction
m/z	Mass to charge ratio
MA-LLE	Microwave-assisted LLE
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MeOH	Methanol
$MgSO_4$	Magnesium sulphate
MRM	Multiple reaction monitoring
MS	Mass spectrometry

MS/MS	Triple quadrupole mass spectrometer
Na	Naphthene
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nd	Not detected
$-NH_2$	Amino group
P. Area	Peak area
PAHs	Polycyclic aromatic hydrocarbons
PAO	Polyamine oxidase
PDA	Photodiode array detector
PEA	Phenylethylamine
PFAD	Palm fatty acid distillates
Phe	Phenanthrene
PPs	Penalty points
PUT	Putrescine
py	Pyridine
Pyr	Pyrene
R^2	Correlation coefficient
R. Time	Retention time
RP-HPLC	Reversed-phase HPLC
RSD	Relative standard deviation
S/N	Signal to noise ratio
SALLE	Salting-out assisted liquid-liquid extraction
SALLME	Salting out assisted liquid-liquid microextraction
SCF	Scientific Community on Food
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
SPD	Spermidine
SPE	Solid phase extraction

SRM	Selected reaction monitoring
T_3RF	Tocotrienol-rich fractions
T_3RF15	15% tocotrienol concentrated from PFAD
T_3RF50	50% tocotrienol concentrated from PFAD
TAGs	Triacylglycerides
TCM	Chloroform
TIC	Total ion chromatogram
TRP	Tryptamine
TYR	Tyramine
UHPLC	Ultrahigh performance liquid chromatography
US EPA	The United States Environmental Protection Agency
USD	United States Dollar
UV	Ultraviolet detector
UVA	Ultraviolet A
VOs	Vegetable oils
WHO	World Health Organization

LIST OF UNITS AND SYMBOLS

%	percent
<	less than
>	greater than
≤	less than or equal to
°C	degree Celsius
°C/min	degree Celsius per minute
µg/kg	micrograms per kilogram
µg/L	micrograms per litre
µg/mL	microgram per millilitres
µL	microlitre
µm	micrometre
amu/second	atomic mass unit per second
cm/s	centimetres per second
D	Debye
eV	electron volts
g/cm^3	grams per cubic centimetre
Hz	Hertz
kg	kilograms
m	metre
mg	milligrams per litre
mg/kg	milligrams per kilogram
mg/L	milligrams per litre
min	minute
mL	millilitre
mL/min	millitres per minute

mm	millimetre
ms	millisecond
ng/g	nanograms per gram
ng/mL	nanograms per millilitres
pH	potential of hydrogen
pK_a	Acid dissociation constant at the logarithmic scale
ppb	parts per billion
rpm	revolutions per minute
v/v	volume per volume
v/v/v	volume per volume per volume
cm	centimetre
mm	millimetre
µm	micrometre

PEMBANGUNAN TEKNIK PENGEKSTRAKAN DAN PEMISAHAN UNTUK PENENTUAN HIDROKARBON POLISIKLIK AROMATIK DAN AMINA BIOGENIK DALAM MAKANAN

ABSTRAK

Hidrokarbon polisiklik aromatik (PAHs) dan amina biogenik (BAs) merupakan pencemar organik berpotensi yang terkandung dalam pelbagai produk makanan. Analisis kuantitatif terhadap pencemar-pencemar ini dalam makanan adalah mencabar disebabkan oleh variasi fisikokimia yang tinggi dan kompleksiti matriks makanan. Tesis ini menerangkan pembangunan kaedah untuk analisis kuantitatif PAHs dan BAs dalam produk makanan terpilih. Fasa pertama penyelidikan bertujuan untuk membangunkan dan mengesahkan kaedah penentuan PAHs (BAA, BBF, CHR dan BAP) dalam pecahan kaya tokotrienol (T_3RF) dan sulingan asid lemak kelapa sawit (PFAD) menggunakan pengekstrakan fasa pepejal dwi-kartrij ($_d$SPE) diikuti oleh kromatografi gas-spektrometri jisim (GC-MS). Pengekstrakan dan penulenan PAH dari sampel dilakukan menggunakan prosedur $_d$SPE yang dioptimumkan. Beberapa parameter pengekstrakan dan kromatografi dioptimumkan secara sistematik untuk mencapai perolehan maksimum dan gangguan minimum. Kaedah ini menunjukkan lineariti yang baik ($R^2 > 0.99$) dalam julat 1.5 - 25 µg/L dan kepersisan yang cemerlang dengan %RSD ≤ 6.85% untuk kawasan puncak bagi PAHs yang disasarkan. Had pengesanan terendah (LOD) ditentukan sebagai 0.48 - 1.35 µg/L, manakala perolehan semula PAH dari sampel T_3RF dan PFAD didapati memuaskan dalam julat 97.5% hingga 102%. Kaedah yang dibangunkan telah berjaya digunakan untuk analisis PAH dalam pelbagai sampel T_3RF dan PFAD. Keputusan ini menunjukkan kebolehgunaan dan kebolehpercayaan kaedah $_d$SPE-GC-MS yang dibangunkan untuk penentuan PAH

dalam matriks T_3RF dan PFAD, menjadikannya berguna untuk kawalan kualiti dan penilaian keselamatan dalam pengeluaran pecahan ini untuk pelbagai aplikasi. Dalam penyelidikan seterusnya, satu kajian telah dijalankan untuk mewujudkan kaedah penentuan BA dalam sos soya dengan menggunakan teknik pengekstrakan mikro cecair-cecair penyebar (DLLME) digandingkan dengan GC-FID. DLLME digunakan sebagai teknik penyediaan sampel untuk mengekstrak dan memekatkan BA daripada matriks sos soya. Pelbagai parameter termasuk jenis dan isipadu pelarut pengekstrak dan penyebar, pH, jisim garam, dan masa pengekstrakan telah dioptimumkan untuk meningkatkan kecekapan pengekstrakan. BA yang diekstrak telah diterbitkan menggunakan isobutil kloroformat (IBCF) sebelum dianalisis dengan GC-FID. Kaedah yang dibangunkan menunjukkan lineariti yang cemerlang ($R^2 > 0.995$) dalam julat 0.5 - 25 mg/L dengan LOD yang ditentukan sebagai 0.14 - 0.47 mg/L, menunjukkan keupayaan kaedah ini untuk mengesan pada kepekatan yang rendah. Selain itu, ketepatan kaedah menghasilkan perolehan semula yang baik dalam julat 88.59% hingga 118.67%. Kepersisan untuk kawasan puncak adalah dari 0.88% hingga 9.22%. Kaedah ini telah berjaya digunakan untuk analisis BA dalam pelbagai sampel sos soya dari pasaran Asia. Kaedah DLLME dan GC-FID yang dicadangkan ini memainkan peranan yang penting untuk penentuan serentak BAs dalam sos soya, dengan itu menyumbang kepada kawalan kualiti dan memastikan keselamatan produk sos soya.

DEVELOPMENT OF EXTRACTION AND SEPARATION TECHNIQUES FOR THE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS AND BIOGENIC AMINES IN FOOD

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) and biogenic amines (BAs) are potential contaminants in various food products. Quantitative analysis of these contaminants in food is challenging due to their high physicochemical variability and complexity. This thesis describes the method developments for quantitative analysis of PAHs and BAs in selected food products. The first phase of the investigation aimed to develop and validate a method for the determination of PAHs (benz(a)anthracene, benzo(a)pyrene, chrysene and benzo(b)fluoranthene), in tocotrienol-rich fractions (T_3RF) and palm fatty acid distillates (PFAD) using dual-cartridge solid phase extraction ($_d$SPE) followed by gas chromatography-mass spectrometry (GC-MS) analysis. The extraction and purification of PAHs were performed using an optimized $_d$SPE procedure. Several extraction and chromatographic parameters were optimized to achieve maximum recovery and minimal interference. The method showed good linearity ($R^2 > 0.99$) over the range of 1.5 – 25 µg/L and excellent precision with %RSD of ≤ 6.85% for peak areas. The limits of detection (LOD) were determined to be 0.48 – 1.35 µg/L, while obtaining good recoveries (97.5% to 102%). The developed method was successfully applied to the analysis of PAHs in T_3RF and PFAD samples. The results demonstrate the applicability and reliability of the developed $_d$SPE-GC-MS method for the determination of PAHs in complex T_3RF and PFAD matrices, making it useful for quality control and safety assessments. In the following investigation, a method for the determination of BAs (putrescine, cadaverine,

phenylethylamine, histamine, tryptamine, tyramine and spermidine) in soy sauce utilizing dispersive liquid-liquid microextraction (DLLME) coupled with gas chromatography-flame ionization detector (GC-FID) was developed. DLLME was employed as a sample preparation technique to extract and concentrate the BAs from soy sauce. Various parameters including the type and volume of extractive and dispersive solvents, pH, mass of salt, and extraction time were optimized to enhance the extraction efficiency. The extracted BAs were derivatized using isobutyl chloroformate (IBCF) prior to GC-FID analysis. The developed method exhibited excellent linearity ($R^2 > 0.995$) within the range of 0.5 - 25 mg/L with LOD determined to be 0.14 – 0.47 mg/L, demonstrating the method's capability to detect BAs at low concentrations. Additionally, the accuracy of the method produced good recoveries (88.59% to 118.67%). The precision ranges from 0.88% to 9.22% for peak areas. The method was successfully applied to the analysis of BAs in soy sauce. This proposed DLLME and GC-FID method is useful for the simultaneous determination of BAs in soy sauce, thereby contributing to quality control and ensuring the safety of soy sauce.

CHAPTER ONE

INTRODUCTION

1.1 Research background

The food market is one of the biggest markets globally, amounting to 9.43 trillion US dollars (USD) in 2023 with a projected growth rate of 6.21% per annum for the next 4 years. The rising awareness for "Clean Label" products, increased intake of plant-based food, and growing demand for regional food has greatly influenced the food market, causing the market to adapt quickly towards its global sustainability [1]. With due consideration, it is without a doubt that there will be increasing concerns regarding the safety of various food products traded in the market. Among the wide variety of toxic chemical compounds that may be present in food, polycyclic aromatic hydrocarbons (PAHs) and biogenic amines (BAs) are commonly studied due to their presence in a wide range of food products, especially in agricultural goods [2, 3].

PAHs are organic hydrocarbons possessing two or more fused benzene rings. Selected PAHs such as benzo(a)pyrene (BAP), benz(a)anthracene (BAA) and dibenz(a,h)anthracene (DBahA) are known carcinogens, essentially causing cancer by damaging the genome or by the obstruction of cell metabolism [4]. In the broad spectrum of PAHs present in food matrices, the PAH4 (BAP, BAA, BBF and CHR) as listed by the European Union (EU) is of great interest among researchers [5]. These PAHs are documented by the International Agency for Research on Cancer (IARC) as either class 1 carcinogen (compounds with proven carcinogenic effects on humans), or class 2B (compounds possibly carcinogenic to humans) [6]. Amid the diverse range of sample matrices known to possess a variety of PAHs, edible oil samples are especially prone to PAHs contaminations. The multiple modes of plant crops

contamination include exposure of seeds to volcanic eruptions [7], forest fires [8] and exhaust gases as well as during manufacturing and processing [9, 10]. The detection and quantitation of PAHs in edible oil samples are mainly conducted using chromatographic techniques such as liquid chromatography (LC) and gas chromatography (GC) with different detection modalities [11].

BAs on the other hand are naturally occurring organic molecules with the amino ($-NH_2$) group present in their structure. BAs such as tyramine (TYR) and histamine (HIS) are known causes of hypotension, hypertension, and allergic reactions, upon excessive consumption [12]. Additionally, some BAs such as putrescine (PUT) and cadaverine (CAD) have the potential to form carcinogenic nitrosamines when reacted with nitrites [13]. In recent times, the demand for soy products such as soy sauce has been growing [14]. Studies have shown that BAs are found to be present in soy sauce samples with varying concentrations even though the fermentation process is overall similar [15]. For example, high amounts of TYR and HIS are found in Japanese soy sauces, while PUT and CAD are predominant in Thai soy sauces [16, 17]. Regardless, the lack of an international standard for the regulation of BAs in food samples such as soy sauce presents a cause of concern for consumers worldwide, motivating the need for relevant data for the establishment of a universal regulation.

In this study, a combination of dual-cartridge solid phase extraction ($_d$SPE) and gas chromatograph-mass spectrometry (GC-MS) approaches were evaluated for the quantitative analysis of PAH4 in tocotrienol rich fraction (T_3RF) and palm fatty acid distillates (PFAD). A review of the literature reveals that the detection of PAHs in edible oil samples such as soybean oil [18-20], coconut oil [18, 21], sesame oil [20, 22, 23], sunflower oil [20, 24] and olive oil [22, 23], has been successfully executed. To the best of my knowledge, this is the first study that examines the applicability of

$_d$SPE and GC-MS for the quantitation of PAHs in T_3RF and PFAD samples. On the contrary, literature suggests that LC techniques are the most frequently used tools for the analysis of BAs in soy sauce samples [25-27]. Therefore, there is limited research that examines the usage of a gas chromatographic technique for the detection of BAs in soy sauce samples. The current study proposes a dispersive liquid-liquid microextraction (DLLME) technique for the extraction and pre-concentration of BAs in soy sauce samples followed by gas chromatography-flame ionization detector (GC-FID) analysis.

1.2 Problem Statement

Vegetable oils are the most consumed edible oil, typically composed of more than 95% triacylglyceride with 5% minor components such as glycolipids, phospholipids, free fatty acids, and others [28]. Within the extensive array of vegetable oil, palm oil is the leading edible oil by production volume due to its high yields and low production costs [29]. Palm oil naturally contains various bioactive constituents (e.g., carotenoids, vitamin E (tocopherols and tocotrienols) and glycolipids) with health-promoting benefits [30]. Among those components, the pursuit towards tocotrienols as a dietary supplement is expanding. Crude palm oil, is one of the most abundant natural sources of tocotrienols, containing up to 800 mg of tocotrienols per kg of oil [31]. Despite the vast increase in interest towards tocotrienols, the literature regarding the detection of PAHs in T_3RF and PFAD is scarce, therefore motivating the need for the development of a sensitive and reliable method to monitor these contaminants in T_3RF.

On the other hand, soy sauce samples are consumed mainly by the Asian community for more than two millennia, with a market size of ~USD 40.63 billion in

2019 and poised to hit USD 56.67 billion in the year 2027 [14]. The detection of BAs in soy sauce is mainly conducted using LC approaches. Chromatographically, GC is known to possess a greater resolving power compared to LC [32]. Currently, there is a lack of literature reporting the use of GC for the quantitative measurement of BAs in food. Moreover, the legal limits for BAs in soy sauce are not well defined by international governing bodies making it essential for researchers to provide relevant data to aid in the realization of a standardized BAs limit in food with an emphasis on soy sauce products.

1.3 Objectives

Therefore, the main objectives of this research are to:

i. develop dual-cartridge solid phase extraction ($_d$SPE) and gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) method for the simultaneous determination of selected PAHs (BAA, BBF, CHR and BAP) in palm fatty acid distillates (PFAD) and tocotrienol rich fractions (T_3RF).
ii. develop a dispersive liquid-liquid microextraction (DLLME) and gas chromatography flame ionization detector (GC-FID) approach for the simultaneous extraction and determination of BAs (PUT, CAD, PEA, TRP, TYR, HIS and SPD) in soy sauce.
iii. validate and apply the developed methods for the analysis of PAHs and BAs in PFAD, T_3RF and soy sauce respectively.

1.4 Scope of work

In the current study, focus is made on the analytical extraction, separation and detection of two classes of compounds namely: PAHs (BAA, BBF, CHR, and BAP) and BAs (phenylethylamine (PEA), PUT, CAD, tryptamine (TRP), HIS, TYR, and spermidine (SPD)). Chapter 1 describes an overview of the study. Chapter 2 presents the literature review regarding the occurrence, toxicity and analytical methods for the detection of PAH4 in PFAD and T_3RF and BAs in soy sauce respectively. Chapter 3 investigates the applicability of SPE and GC-MS techniques for the simultaneous determination of 4 EU-regulated PAHs: BAP, BAA, BBF and CHR in T_3RF and PFAD. Dual cartridge solid-phase extraction ($_d$SPE) was investigated as the sample clean-up procedure and chromatographic parameters affecting the separation of the targeted PAHs were optimized and the validated method was used for the determination of the targeted PAHs in T_3RF and PFAD samples. Chapter 4 demonstrates the development of DLLME and GC-FID approach for the simultaneous determination of seven BAs (PUT, PEA, CAD, HIS, TRP, TYR and SPD) in soy sauce samples. The extraction and chromatographic parameters were investigated and discussed in this chapter. The prospect of using the validated DLLME and GC-FID methods to quantify the targeted BAs in soy sauce samples of different origins was described. Finally, chapter 5 concludes the research presented in this thesis and provides recommendations for future studies.

CHAPTER TWO

LITERATURE REVIEW

2.1 Polycyclic aromatic hydrocarbons

PAHs are a large group of aromatic organic hydrocarbons comprising two or more fused aromatic (benzene) rings or pentacyclic rings which are arranged in either linear, angular or cluster formations. These compounds are classified as heavy or light according to the number of condensed aromatic rings. Lightweight PAHs (LPAHs) have two to four aromatic rings while heavy PAHs (HPAHs) have four or more aromatic rings [33]. LPAHs are semi-volatile compounds and are generally considered to exhibit relatively low toxicity, while HPAHs are mostly associated with airborne particles and are more stable and toxic than LPAHs [34]. Due to their lipophilic nature and increased molecular stability, a few known HPAHs such as BAP, BAA, and DBahA are found to be carcinogenic, mutagenic and teratogenic thus posing a threat to human life [4]. The toxicity of PAHs is made possible due to various achievable linkages and configurations of the carbon and hydrogen ring systems that have brought forth an extensive diversity of physical, chemical and toxicological variations [33, 35].

2.1.1 Occurrence of PAHs in food and vegetable oil matrices

The presence of toxic and carcinogenic PAHs, in particular HPAHs, is a matter of concern that requires continuous monitoring, especially in food samples. Several epidemiological studies have shown that a large proportion of human cancers are attributable, at least in part, to dietary factors, including dietary exposure to PAHs [36]. Environmental factors play a critical role in the exposure of PAHs to humans such as industrial emissions during the processing of raw materials (aluminium, coke,

petrochemicals, rubber tires, etc.), emissions from open burning of agricultural crops, cigarette smoking (first hand and second hand) as well as leeching from industrial effluents into water and soil [37]. However, for non-occupational settings, dietary exposure to PAHs as high as 70% as a result of dietary sources such as barbecuing food over charcoal or wood can be attributed to a non-smoking individual. Charred meat and fish have been shown to contain PAHs with concentration levels of 10-20 μg/kg which is significantly more dominant than their uncooked equivalents [38].

Of the multitude of agricultural goods, vegetable oils (VOs) have seen an increase in interest on the global stage for the past decade considering the boost in global demand by virtue of increased global wealth, rising awareness regarding health-related issues by trans fatty acids and health benefits of VOs [39]. However, agricultural crops and commodities which are the main ingredients for the production of edible oils are especially prone to PAH contamination due to the multiple modes of pollution by air, water, and soil, where they are usually found as complex mixtures [40]. The study conducted by Ibanez et al. has shown that roughly 50% of PAH exposure through dietary intakes can be closely accountable for contaminated oils and fats [41].

PAHs are introduced into VOs via the incomplete combustion of organic matter (pyrolysis) such as wood, coal and oil and can reach the environment through fire, thermogenic events or volcanic eruptions, among other sources. These toxic gases frequently containing PAHs eventually condense and fall back to the earth, attaching themself to plant crops or getting absorbed into the soil and water that eventually makes its way into the roots and the whole plant. However, the contamination of PAHs is often an indirect result of human activities such as the use of fossil fuels, tobacco smoking and forest fires [7, 8]. Studies have frequently attributed anthropogenic

sources as the primary contamination route for PAHs [34, 42]. Moreover, the contamination of PAHs could also be attributed to food processing, preservation and packaging [9, 19]. The extent of PAHs contamination in VOs depends on the raw material, harvesting and mill procedures, as well as processes used to obtain the product [10]. An example to exemplify the contamination of agricultural commodities through anthropogenic sources would be the drying of seeds in the sun or open air which are already contaminated with PAHs, further spreading the contamination to uncontaminated seeds [43]. Furthermore, significant dissimilarities in the levels of PAHs are seen when seeds are directly exposed to exhaust gases during drying and/or during high-temperature oil extractions [21, 44].

The contamination of PAHs in VOs is not limited to processing and manufacturing practices. Astonishingly, PAHs are also known to be generated in situ during food preparation such as grilling, barbecuing, frying, grilling and smoking by means of the Maillard reaction [45, 46] and lipid oxidation [47]. As a result of the various modes of contamination either through anthropogenic factors or natural sources, PAHs are shown to be present in soybean oil [18-20], coconut oil [18, 21], sesame oil [20, 22, 23], sunflower oil [20, 24], olive oil [22, 23], perilla oil [22] and palm oil [24, 48].

2.1.2 Biological and toxicological effects of PAHs

PAHs are known causes of cell mutations (mutagenic), cancer (carcinogenic), birth defects (teratogenic), and immune problems (immunotoxic) in all forms of life, ranging from microorganisms to animals and humans. The severity of the toxic effects of PAHs is dependent on crucial factors such as the method, duration and dose of the

exposure [49]. A study by Zheng et al. found that the highest cancer risk from PAHs was through ingestion (98.1%-99.3%), while dermal contact and inhalation account for < 1.83% and < 0.04% respectively [50]. The severity of the biotoxic effects of PAHs on humans can be categorized into acute and chronic. Acute health consequences may include eye irritation, vomiting, diarrhoea, confusion, skin irritation, and inflammation while chronic effects often result in eye cataracts, kidney and liver damage, breathing difficulties, decreased immune system function, lung problems, and symptoms similar to asthma [51].

The phototoxicity of PAHs stems from their ability to absorb ultraviolet A (UVA) and visible light, producing excited PAHs species which form reactive intermediates with oxygen and other molecules in the cells. The aftermath of this reaction encompasses no less than cellular damage and the destruction of nucleic acid and proteins in the body. It has been shown that the toxicity of PAHs can be more than 100 times greater in the presence of light than in darkness [52]. In mammalian species, the liver is the primary site for detoxifying PAHs through the action of various oxidase enzymes, as well as cytochrome P450 which facilitates the formation of water-soluble epoxide glutathione conjugates. Be that as it may, certain PAH metabolites (e.g., quinones, diolepoxides, and hydroxyalkyls) are persistent and remains in the body [53]. These compounds often react with nucleic acids and lead to genotoxicity with the formation of tumours in the lung, skin, oesophagus, colon, pancreas, bladder and breast [51, 54].

The teratogenicity of PAHs is well documented. Reports have shown that PAHs are capable of acting as antiestrogen and/or anti-androgens by binding with their respective receptors. Moreover, exposure to PAHs can result in a variety of non-cancerous reproductive health problems for both males and females, including

alterations in sperm quality, testicular function, and egg viability, as well as damage to oocytes' DNA, harm to the ovaries, and other reproductive ailments [55]. Additionally, critical hormonal regulators of reproduction, including follicle-stimulating hormone, luteinizing hormone, gonadotrophin-releasing hormone, and the aromatase enzyme are also affected by the presence of PAHs in the human body [55]. Last but not least, PAHs possess immunotoxicity effects on humans, causing immune-system adverse effects. Some reported causes of immunotoxicity are the apoptosis of lymphoid tissues, disruption of myelopoiesis, hypersensitivity and autoimmunity [37, 51, 56]. PAH exposure can lead to significant health outcomes as it alters the structure and functioning of bone marrow, which is the central organ of haematopoiesis and a critical site for the generation of cells that make up the immune system [57].

2.1.3 Legislation concerning PAHs in food

The detrimental effects of PAHs have garnered the attention of various international bodies such as the International Program on Chemical Safety (IPCS), the Scientific Community on Food (SCF) and the coalition between the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) into the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The United States Environmental Protection Agency (US EPA) has previously designated 16 PAHs as high-priority pollutants and among them: BAA, BAP, BBF, CHR, DBahA, benzo(k)fluoranthene (BkF), and indeno(1,2,3-c,d)pyrene (IP) are listed as probable carcinogens, similar to the delegation by the International Agency for Research on Cancer (IARC) with the exception of CHR [58]. The IARC has further classified BAP as Group 1 carcinogens (compounds with proven

carcinogenic effect on humans), DBahA as Group 2A (compounds probably carcinogenic to humans) while BkF, IP, BAA, BBF and CHR are classified as Group 2B (compounds possibly carcinogenic to humans) [6]. Studies have shown that excessive exposure to PAHs can increase the tendency of developing cancer (predominantly lung cancer) compared to other known carcinogens [33].

With due consideration to the existence of various pernicious PAHs in a wide assortment of edible oils, the EU issued strict regulations, with the latest update in 2011 for the maximum level of PAH4 allowed in oils and fats to be 10 µg/kg for the sum of BAP, BAA, BBF and CHR. Furthermore, the concentration of BAP must be less than 2 µg/kg [5]. There is however an exception to that ordinance to coconut oil which the EU has allowed increased levels of PAH4 in coconut oil to 20 µg/kg [59]. Based on the currently available data relating to occurrence and toxicity, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) concluded that PAH4 and PAH8 (BAP, BAA, BBF, CHR, BkF, DBahA, BghiP and IP) are the most suitable indicators of PAHs in all food matrices, with PAH8 not providing much added value compared to PAH4 [60]. Table 2.1 summarizes the PAHs prioritized by the EU and US EPA in addition to PAH4 and PAH8.

Table 2.1**:** IARC classification of EPA and EU PAHs, PAH4 and PAH8.

PAHs (Abbreviation.)	**No. of rings**	**IARC**	**EPA**	**EU**	**PAH4**	**PAH8**
Naphthalene (Na)	2	-	✓			
Acenaphthene (Ace)	3	3	✓			
Acenaphthylene (Acy)	3	-	✓			
Fluorene (Flu)	3	3	✓			
Phenanthrene (Phe)	3	3	✓			
Anthracene (Ant)	3	3	✓			
Fluoranthene (Fla)	4	3	✓			
Pyrene (Pyr)	4	3	✓			
Benzo(a)anthracene (BAA)	4	2B	✓	✓	✓	✓
Chrysene (CHR)	4	2B	✓	✓	✓	✓
Benzo(b)fluoranthene (BBF)	5	2B	✓	✓	✓	✓
Benzo(a)pyrene (BAP)	5	1	✓	✓	✓	✓
Benzo(k)fluoranthene (BkF)	5	2B	✓	✓		✓
Dibenz(a,h)anthracene (DBahA)	5	2A	✓	✓		✓
Benzo(g,h,i)perylene (BghiP)	6	3	✓	✓		✓
Indeno(1,2,3-cd)pyrene (IP)	6	2B	✓	✓		✓
5-methylchrysene (5MCh)	4	2B		✓		
Cyclopenta(cd)pyrene (CPP)	5	2A		✓		
Benzo(c)fluorene (BcF)	5	3		✓		
Benzo(j)fluoranthene (BjF)	5	2B		✓		
Dibenzo(a,l)pyrene (DBalP)	6	2A		✓		
Dibenzo(a,e)pyrene (DBaeP)	6	3		✓		
Dibenzo(a,i)pyrene (DBaiP)	6	2B		✓		
Dibenzo(a,h)pyrene (DBahP)	6	2B		✓		

IARC classification is based on the IARC Monographs Programme (IARC, 2010) where class 1 compounds are determined to be carcinogenic to humans, class 2A compounds are probably carcinogenic to humans, class 2B are possibly carcinogenic to humans and class 3 are determined to be not classifiable as to their carcinogenicity to humans due to limited or inadequate data [6]. Figure 2.1 portrays the chemical structures of the 16 PAHs on the EPA priority list.

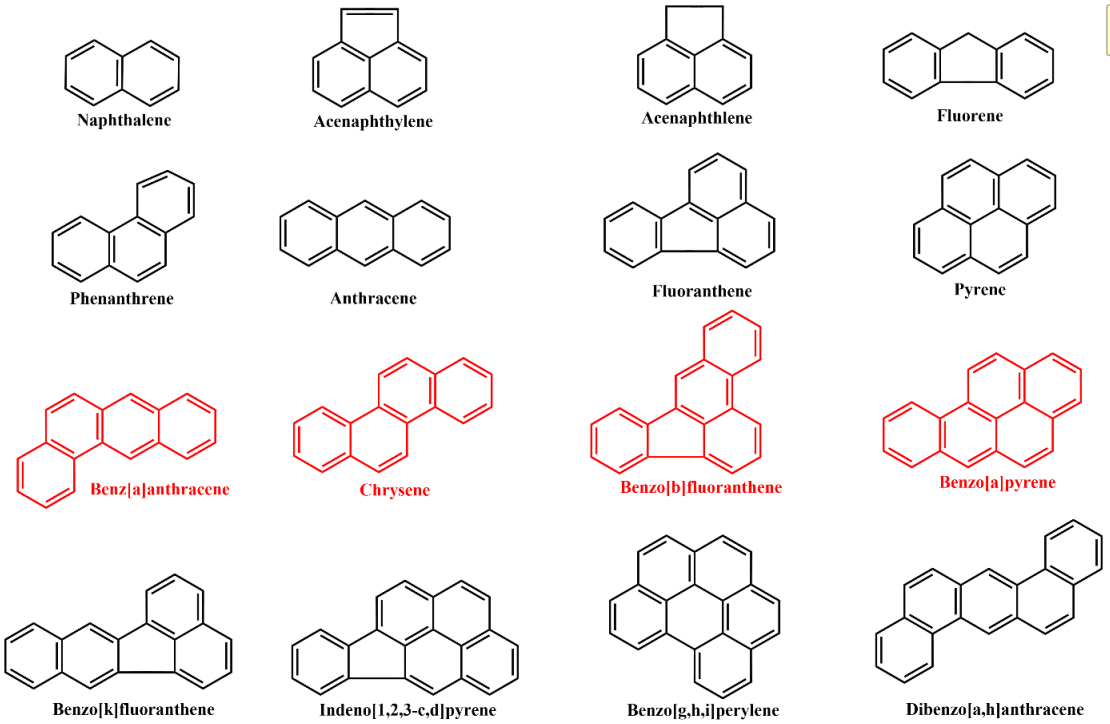


Figure 2.1**:** Chemical structures of 16 PAHs on the EPA priority list. PAHs highlighted in red correspond to PAH4 investigated in the current study.

2.1.4 Sample preparation procedures prior to chromatographic analysis.

Over the past 15 years, the combination of liquid-liquid extraction (LLE) and solid-phase extraction (SPE) has been used as the main sample preparation techniques for the determination of PAHs in vegetable oil samples. The sample preparation step is crucial to remove unwanted interfering compounds that might affect the chromatographic separation of PAHs. In particular, the high lipophilicity of PAHs,

exhibits a strong affinity towards oil matrices which further complicates their measurements [61]. Therefore prior to LLE, a saponification step is occasionally introduced to reduce the lipidic content of the sample. However, selected interfering molecules such as squalene are not removable and will remain in the unsaponifiable fraction, eventually making themself into the chromatographic separation system. Thus, additional preparation steps preceding saponification are quintessential [43].

The goal of performing LLE is to gradually enrich the concentration of PAHs in a distinct fraction. Common solvent systems such as acetonitrile/acetone, water/dimethyl sulfoxide and acetonitrile are utilized for the LLE procedure. Ultrasound-assisted LLE made the scene in 2007 for the study of 15 EPA PAHs in assorted oil samples [62]. Since then, numerous other research teams have adopted ultrasound-assisted LLE for the extraction of PAHs in edible oil samples [18, 63-65]. On the other hand, Alarcón et al. presented a microwave-assisted LLE (MA-LLE) technique for the analysis of 7 EU PAHs in olive and sunflower oils [66]. Moreover, there were instances of incorporating a freezing step after the addition of organic solvent in the LLE procedure to precipitate the lipidic compounds. This innovation led by Payanan et al. was able to remove 94% of interfering lipids in the final extract while reducing the number of interfering peaks without significant loss of PAHs [24]. Other reported extraction methods include supercritical fluid extraction (SFE) [67, 68] which offers short extraction times and satisfactory recoveries (between 77-106%), and donor-acceptor complex chromatography, which is highly automated and reduces the need for human labour while also decreasing sources of error [69].

After the successful implementation of LLE, an additional purification step is sometimes necessitated. Gel permeation chromatography was proven to be highly effective in the removal of interfering components in oil matrices due to the notable

differences in molecular size between triacylglycerides (TAGs) and PAHs [70-72]. Although this technique necessitates the use of a large volume of solvents, its semi-automated nature and typically high recoveries are benefits that should be taken into account. Otherwise, SPE is most typically employed preceding LLE. The International Organization for Standardization (ISO 15753:2016) standard procedure recommends the application of a C18 SPE cartridge tandem to a Florisil cartridge preceding LLE for the determination of PAHs in various oil matrices [62, 63, 73, 74]. Yousefi et al. applied the ISO 15753 method to concentrate 13 PAHs in different types of edible oils (frying oil, sunflower oil, corn oil and canola oil). The study demonstrated outstanding results with recoveries ranging from 83.6% to 107.1% [63]. Innovations regarding the combination of various SPE phases have also been reported. Jiang et al. proposed a combination of Oasis HLB SPE cartridges which composes of a hydrophilic-lipophilic-balanced copolymer tandem to a typical Florisil cartridge, obtaining recoveries of 77.4%-127.2% for 15 EU PAHs [75]. Belo and colleagues used a new C18 SPE method in combination with silica-based SPE, demonstrating satisfactory recovery (54%-115%) for 8 PAHs, which proved sufficient with accordance to the European Union Commission Regulation No 835/2011 [5, 76].

For extractions using SPE without prior cleanup (LLE/saponification), samples are usually diluted with solvent such as hexane [77] or isooctane/cyclohexane (1:2 v/v) [78] prior to extraction and purification. There are distinct cases where dilution was not performed by directly channelling the sample into the cartridge [79, 80]. A study on the use of dual-layer SPE without prior sample preparation can be exemplified by the study performed by Bogusz et al. in 2004 [79]. Bogusz et al. suggested the use of a two-layer SPE cartridge, which has a lower layer of C18 and an upper layer of Florisil, to extract BAP directly without requiring any prior dilution or partitioning. The authors

conducted a comparison between the proposed dual-layer SPE cartridge method and the dispersive SPE (dSPE) method. They found that the dual-layer SPE method was more efficient, as it provided higher recoveries and was quicker, less complex, and more reproducible [79].

2.1.5 Liquid chromatographic techniques for the analysis of PAHs in oil samples.

LC has been a popular separation technique for PAHs since the early 2000s, due to the latent ability of PAHs to fluoresce enabling the pairing of LC with fluorescence detector (FLD) [68]. The first occasion however was in the early 80s, in which Ogan et al. reported the separation of 12 PAHs using HPLC-FLD [81]. Octyldecylsilane (C18) columns are by far the most commonly employed column to effect the separation of PAHs with a few instances of disparate dimensions. A solvent gradient of acetonitrile/water is the preferred choice of mobile phase that starts with 40%-50% acetonitrile (ACN) and is increased at a constant rate to 89%-100% in around 45 minutes as recommended by the International Standardization for Organization (ISO) 15753:2016 [74]. Nonetheless, there are also a number of researchers who reported an isocratic elution which often results in an increment of analysis times [67, 82, 83].

Detection of PAHs using FLD detectors is popular among researchers due to the innate ability of PAHs to fluoresce, producing characteristic excitation and emission wavelengths, making detection ideal with respect to sensitivity [43]. However, in terms of selectivity, the overlapping of fluorescent bands poses a great hurdle for the resolution of targeted PAHs in a complex matrix. For instance, the Acy molecule does not conjure a good signal in FLD detection, especially in cases where

researchers intend to analyze all 16 of the EPA PAHs. This issue however can be resolved by applying a diode-array detector (DAD) in tandem with the FLD [71, 84]. MS detectors are less frequently seen in tandem with LC separation due to the low ionization efficiency of the non-polar PAHs particularly with prevalent ion sources namely the electrospray ionization (EI) and atmospheric pressure chemical ionization (APCI). Hollosi et al. conducted an extensive study on the various ionization methods and discover that the atmospheric pressure photoionization on the MS produced the best signal intensities when operating in the positive mode [69]. At the same time, the authors studied the effects of various dopants in increasing the ionization efficiency of the PAHs. Dopants are ionizable species that transfer energy from the photons to the sample molecules, minimizing the loss of energy in the process. The study concluded that anisole was the best dopant due to high proton affinities, increasing the lifetime in the ionization source and thus expediting the ionization of the targeted PAH molecules. The mobile phase however must be compensated with methanol instead of the standard ACN solutions, due to ion suppression that generates low signals [69]. The use of toluene as a dopant was also found to be favourable to achieving low detection limits (0.006 – 0.156 µg/kg) [85]. Figure 2.2 presents a representative chromatogram for the chromatographic separation of 15 PAHs in oil samples using HPLC-FLD [86].

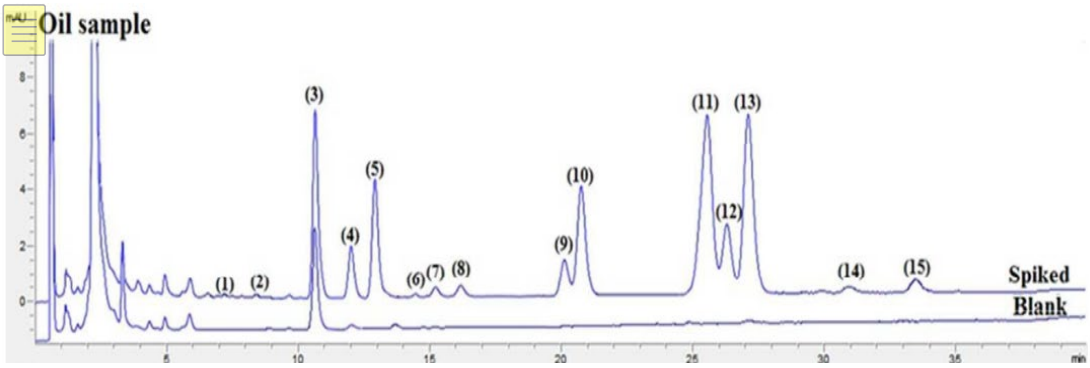


Figure 2.2: Representative chromatogram of PAHs in edible oil sample analyzed using HPLC-FLD. (1) Na, (2) Ace, (3) Flu, (4) Phe, (5) Ant, (6) Fla, (7) Pyr, (8) Pery, (9) BaA, (10) CHR, (11) BbF, (12) BkF, (13) BaP, (14) DBahA and (15) BghiP. Retrieved from Shi et al. [85].

2.1.6 Gas chromatographic techniques for the analysis of PAHs in oil samples.

On the other hand, GC strategies are immensely popular among researchers in more recent times [65, 87, 88], possessing high resolving powers and frequent accompaniment of MS detectors which works in tandem impeccably with GC systems, that allow the separation of overlapping compounds with characteristic ion masses. Low polarity stationary phase (5%-phenyl)-methylpolysiloxane with typical dimensions of 30 m × 0.25 mm I.D. and 0.25 μm film thickness, is customarily adopted for the chromatographic separation of non-polar PAHs. However, there is a discrepancy in using low polarity stationary phases for the separation of PAHs of similar properties i.e., 15+1 EU PAHs. The PAH molecules being examined by the EU are larger and more alike in structure than the EPA PAHs. Consequently, distinguishing between some of the compounds may be difficult as they have the potential to co-elute during chromatographic separation. This is especially significant for PAH isomers that cannot be differentiated by extracting their respective *m/z* traces or shared fragments. The most critical groups are CPP– BAA–CHR, BBF–BjF–BkF, and DBahA–IP. Gómez et al. evaluated the effect of stationary phase polarity on the separation of 15+1 EU PAHs. Results show that using a longer column (60 m) with a mid-polar (50%-phenyl)-methylpolysiloxane phase was ideal for the separation of the PAH isomers [89].

With regards to the detection of PAHs, using MS detectors provides a key advantage to analysts, that is the capability of utilizing isotope dilution using deuterated or isotope-labeled standards, providing accurate quantification and identification [90, 91]. Quadrupole mass spectrometer operating in the selected ion monitoring (SIM) mode with electron impact ionization mode is the preferred detection method for PAHs analysis using GC [92, 93]. It is also worth noting that

sophisticated higher orders mass analyzers are also utilized for the detection of PAHs such as the triple quadrupole mass spectrometer (MS/MS) operating in multiple reaction monitoring (MRM) modes [88]. A combination of detection modes is reported by Arrebola et al., utilising both SIM and MS/MS modes, providing decent sensitivity when monitoring a specific ion mass while the complimentary MS/MS acquires the product ions [94]. Ion trap MS has also been reported albeit obscure among the studies of PAHs in edible oils [18, 92]. Figure 2.3 presents a representative chromatogram for the determination of PAH4 in an olive oil sample using GC-MS [65].

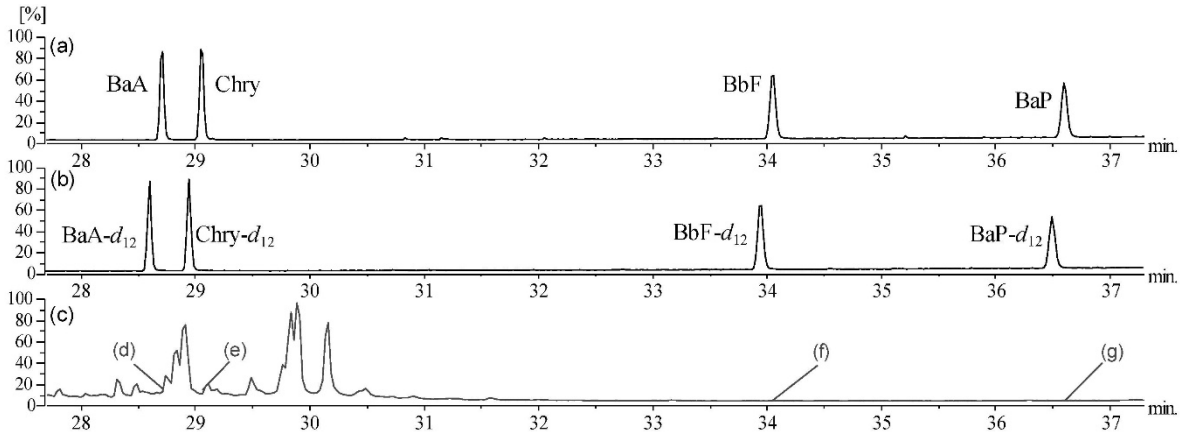


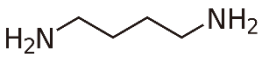
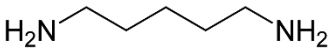
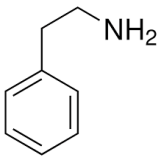
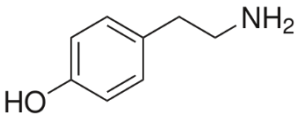
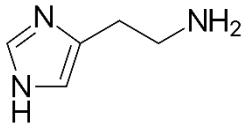
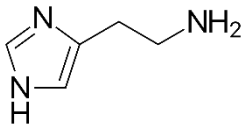
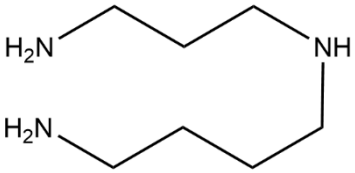
Figure 2.3: Total ion chromatogram (TIC) of PAH4 in (a) native standard, (b) deuterated standard, (c) olive oil sample, (d) BAA, (e) CHR, (f) BBF, and (g) BAP. Retrieved from Ju et al. [65].

2.2 Biogenic amines

BAs are basic organic compounds that have a low molecular weight and contains the amino (-NH_2) group, which may be aliphatic, aromatic, or heterocyclic. The term "biogenic" is commonly used to refer to a class of substances produced through metabolic or chemical processes from flora or fauna [95]. Chemically, BAs are mainly formed by the decarboxylation of their precursor amino acids, catalyzed by substrate-specific microbial decarboxylases (carboxylase enzyme commission number: 4.1.1.1), resulting in the corresponding amines [96, 97]. However, BAs can also accumulate from the amination and transamination of aldehydes and ketones [98]. BAs

are introduced into the human body primarily through the consumption of protein-rich plant and animal products that are formed during processing, preparation, and storage. The formation of BAs during food processing is influenced by various factors, including the availability of amino acids, decarboxylating microorganisms, enzymatic activity, and favourable bacterial growth conditions [99]. Figure 2.4 presents the chemical structures of the BAs investigated in this study.

Table 2.2: Abbreviation and chemical structures of BAs investigated in the current study.

Name	Abbreviation	Structure
Putrescine	PUT	
Cadaverine	CAD	
Phenylethylamine	PEA	
Tyramine	TYR	
Tryptamine	TRP	
Histamine	HIS	
Spermidine	SPD	

2.2.1 Occurrence of BAs in soy sauce

Soy sauce is known to produce from soybean and wheat via specific fermentation processes. Although the fermentation methods used may vary among countries, it is accepted that majority of the method employs a two-step fermentation process known as the koji (solid-state fermentation) and moromi (brine fermentation). The koji process uses mold spores such as *Aspergillus oryzae* or *Aspergillus sojae* to biologically "transform" soybean and wheat into simpler molecules and nutrients that can be readily utilized by the following fermentation processes. The process starts with cooking soybean flakes and grit at a high temperature, then adding roasted and cracked wheat that varied according to the types of soy sauce being made. Mold spores are then inoculated into the mixture, and after three days of fermentation, the substrate becomes a green-yellow mush. This 3-day fermentation period is where BAs can be formed as a result of the breakdown of amino acids and peptides from protease excretions by the mold [100]. These processes of breaking down amino acids become the precursor to the formation of BAs.

The subsequent process involves the addition of salt water to the koji, leading to the formation of the soy sauce mash (moromi) and the start of brine fermentation. The lactic acid bacteria (LAB) takes over the fermentation process from the koji mold during the early stages, leading to the production of lactic and acetic acid, which decreases the pH of the mash to pH 4.0-5.0 and terminates prokaryotic metabolism [101]. Moromi yeasts such as *Zygosaccharomyces rouxii*, *Candida versatilis*, and *Candida etchellsii* will increase in population and are responsible for the secondary fermentation process that leads to the production of distinctive volatile compounds. These brewing processes indirectly resulted in the production of BAs, noting that the variation of the ingredients also influenced the formation of BAs. For instance,

Japanese soy sauces have been reported to contain higher amounts of TYR and HIS, while Thai soy sauces contain higher levels of PUT and CAD [16, 17]

2.2.2 Biological and toxicological effects of BAs

Of the many BAs present in fermented food matrices such as soy sauce, TYR and HIS are the most extensively studied due to their potential danger to human health [102]. Histaminosis is a condition caused by the ingestion of histamine-rich food that releases HIS or blocks the diamine oxidase (DAO) responsible for regulating HIS in the human body. Symptoms of Histaminosis resemble an allergic reaction, accompanied by hypotension, flushing and headache. In addition to its other effects, HIS can cause the muscles in the gastrointestinal tract to contract, resulting in symptoms such as abdominal cramps, diarrhea, nausea, and vomiting [103]. TYR, PEA, and TRP are associated with neuromodulatory functions and may be involved in various human disorders such as schizophrenia, depression, attention deficit disorder, and Parkinson's disease. TYR is also linked to the “cheese reaction”, a pathological condition that is commonly associated with excess tyramine in the blood. Symptoms include hypertensive crises, caused by the release of excess noradrenaline and an increase in blood pressure [3]. Excessive consumption of TYR and HIS can lead to various illnesses such as hypotension, allergic reactions, hypertension, tachycardia, hypercapnia, hyperglycemia, and chronic migraines [12]. However, the health risk of consuming BAs is not due to a sole contributor, but rather a combined synergistic effect of various types of BAs. There have been reports suggesting that certain amines such as CAD and PUT can increase the toxicity of HIS in the human body [103, 104]. Therefore, less commonly known and detectable BAs should also be taken seriously

and require close monitoring due to their potential health risks. Additionally, the presence of PUT and CAD increases the risk of toxicity as they have the potential to react with nitrites to form carcinogenic nitrosamines [13]. SPD on the other hand have been linked to food allergies while TRP is commonly associated to hypertension [105, 106].

Normally, the human body has the ability to oxidize BAs in the intestinal tract through various enzyme oxidases, such as the monoamine oxidase (MAO; CE 1.4.3.4), diamine oxidase (DAO; CE 1.4.3.6), and polyamine oxidase (PAO; 1.5.3.11) [102, 107]. However, this detoxification process can be obstructed by various factors, including the consumption of drugs that inhibit amine oxidase (mono/diamine oxidase inhibitor (MAOI/DAOI)), intake of alcohol beverages, immune deficiency, gastrointestinal disorders, and excessive consumption of BAs from fermented or spoiled foods [99, 108, 109].

2.2.3 Legislation concerning BAs in food and soy sauce

Determining the toxic limit of BAs is a challenging task since their toxicity can vary significantly depending on an individual's tolerance and response [104]. In 2011, the European Food Safety Authority (EFSA) expressed concern over the health risks associated with BAs found in fermented foods, including soy sauce. Furthermore, the report by the EFSA in 2011 states that the currently available information is inadequate to provide a quantitative risk assessment [102]. Although toxic limits for BAs in food have not been established, some researchers have proposed maximum allowed limits, including 1000 mg/kg for total BAs content, 30 mg/kg for PEA, 100-800 mg/kg for TYR, and 100 mg/kg for HIS. These proposed limits suggested by Brink et al. are

frequently used to evaluate the risks of exposure to BAs as no specific guidelines have been reported so far other than the maximum limit of HIS in fish [110, 111].

2.2.4 Sample preparation procedures prior to chromatographic analysis

Due to the low concentration levels of BAs and associated complexity of soy sauce, appropriate sample preparation approaches need to be implemented prior to instrumental analysis. Chemically, soy sauce contains large amounts of lipophilic components that originate from the soy and wheat precursor materials as well as high levels of NaCl and melanoidins which are expected to interfere with the measurement of BAs. Other potential interfering compounds include phenols and esters [112]. Thus, the key roles of sample preparation are to eliminate such interferences, preconcentrate BAs where applicable, and convert (via derivatization) these nitrogenous compounds to appropriate forms for chromatographic separation and/or detection. Technically, two important preparation procedures are generally applied for the quantitation of BAs in soy sauce, namely extraction and derivatization. Nevertheless, the high polarity of BAs renders them soluble in aqueous solutions, making extraction an arduous task to perform. Therefore, there is a need to administer appropriate extraction strategies to discern the nitrogenous compounds (BAs) from complex food matrices such as soy sauce.

2.2.4(a) Liquid phase microextraction techniques

Microextraction techniques are acknowledged by their low solvent, sample and reagent consumption, which generates significantly less waste. Several advantages of microextraction approaches in comparison with LLE include a high enrichment factor,