QUANTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH4) IN ROASTED COCOA BEANS USING QUECHERS AND DISPERSIVE LIQUID-LIQUID MICROEXTRACTION COUPLED WITH HPLC-FLD

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By

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Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic contaminants found in cocoa beans and cocoa products during primary cocoa bean processing such as drying, storage, transportation and roasting. Currently, the determination of PAHs in cocoa product is conducted using the combination of QuEChERS (Quick Easy Cheap Effective Rugged Safe) method and dispersive liquid-liquid microextraction (DLLME) method. The combination of these two methods provides advantages such as simplicity of operation, lesser amounts of organic solvents used and high recovery for PAH quantification in cocoa products as compared to other methods such as Soxhlet extraction, accelerated solvent extraction (ASE), solid phase extraction (SPE), alkaline saponification and maceration extraction with mechanical agitation.

However, according to the preliminary study, PAH4 in cocoa beans was unable to be detected and quantified using QuEChERS and DLLME coupled with gas chromatography method. Therefore, the first objective of this study was to modify analytical methods using QuEChERS and DLLME coupled with high-performance liquid chromatography with fluorescence detector (HPLC-FLD) for PAH4 recovery in cocoa bean. The results showed that by using modified DLLME parameters; 8 mL acetonitrile (dispersive solvent), 2 mL chloroform (extractive solvent), 6 mL water, centrifuge at 6000 rpm for 10 min and finally quantification by HPLC-FLD provide better recovery (68.12 – 89.16%) of PAH4 in cocoa beans. The second objective of this research was to validate the modified method based on performance characteristics provided by Regulation (EU) No. 836/2011. The validation process has shown that the modified method had met the performance criteria required by Regulation (EU) No. 836/2011. Performance characteristics such as limit of detection (LOD, 0.04 - 0.14 ng/g), limit of quantification (LOQ, 0.13 - 0.48 ng/g), repeatability (HORRATr: 0.30 – 1.25), intermediate precision (HORRATR: 0.33 – 0.77), linearity (R^2 > 0.997) and recovery

(52.13 to 86.23%) were determined. The third objective was the application of the modified method on the quantification of PAH4 in cocoa bean roasted at different roasting conditions (whole cocoa bean and nib roasting, different temperatures varying from 110 to 190 °C and different duration ranging from 10 to 50 min). The roasted cocoa beans' analysis showed a significant ($p < 0.05$) increase in B[a]A (0.19 – 1.18 ng/g), Chrys (<0.48 – 2.60 ng/g) and B[a]P (<0.13 – 4.11 ng/g) contents with increasing temperatures (110 to 190 °C) and duration (10 to 50 min), whereas B[b]F was not detected until the temperature reached 190 °C. Total PAH4 (sum of total B[a]A, Chrys, B[b]F and B[a]P) showed a significant ($p < 0.05$) increase (0.19 – 7.73 ng/g) with increasing temperatures and duration. This study shows that whole cocoa bean or nib roasting methods are safe to be used since the maximum B[a]P (4.11 ng/g) and PAH4 (7.73 ng/g) presence are lower than maximum limit by Regulation EU No. 835/2011. In the present study, roasting conditions (whole cocoa beans or nibs roasting at different temperatures and duration) did significantly affect the PAH4 contents in the cocoa samples. The modified and validated method (QuEChERS and DLLME coupled with HPLC-FLD) was effective in detecting and quantifying the PAH4 in cocoa beans with high accuracy, sensitivity and precision.

Keywords: Cocoa bean process, DLLME, QuEChERS, HPLC, FLD, Cocoa roasting

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGKUANTITIAN POLISIKLIK AROMATIK HIDROKARBON (PAH4) DI DALAM BIJI KOKO PANGGANG MENGGUNAKAN QUECHERS DAN PENGEKSTRAKAN MIKRO CECAIR-CECAIR PENYEBAR BERSERTA HPLC-FLD

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Polisiklik aromatik hidrokarbons (PAHs) adalah bahan pencemar karsinogenik yang ditemui dalam biji koko dan produk koko semasa peringkat awal pemprosesan koko seperti pengeringan, penyimpanan, pengangkutan dan pemanggangan. Pada masa sekarang, pengenalpastian PAHs dalam produk koko dijalankan dengan menggunakan kaedah QuEChERS (*Safe Easy Efficient Safe Rugged Safe*) dan kaedah pengekstrakan mikro cecair-cecair penyebar (DLLME). Gabungan kedua-dua kaedah tersebut memberi kelebihan seperti kesederhanaan operasi, jumlah pelarut organik yang lebih rendah dan perolehan kuantiti PAHs yang tinggi dalam produk koko berbanding kaedah lain seperti pengekstrakan Soxhlet, pengekstrakan pelarut yang dipercepatkan (ASE), pengekstrakan fasa pepejal (SPE), saponifikasi alkali dan pengekstrakan secara rendaman dengan pengadukan mekanikal.

Walau bagaimanapun, menurut kajian awal, PAH4 dalam biji koko tidak dapat dikesan dan dikira menggunakan kaedah QuEChERS dan DLLME berserta kromatografi gas. Oleh itu objektif pertama kajian ini adalah untuk mengubah kaedah analisis menggunakan QuEChERS dan DLLME berserta kromatografi cecair berprestasi tinggi dengan pengesan pendarfluor (HPLC-FLD) untuk perolehan PAH4 dalam biji koko. Keputusan menunjukkan bahawa dengan menggunakan parameter DLLME yang telah diubahsuai; 8 mL asetonitril (pelarut penyebar), 2 mL kloroform (pelarut ekstraktif), 6 mL air, mengempar pada 6000 rpm selama 10 minit dan akhirnya pengkuantitian menggunakan HPLC-FLD memberikan perolehan PAH4 dalam biji koko yang lebih baik (68.12 - 89.16%). Objektif kedua penyelidikan ini adalah untuk mengesahkan kaedah yang diubah suai berdasarkan ciri-ciri prestasi yang disediakan oleh Peraturan (EU) No. 836/2011. Proses pengesahan telah menunjukkan bahawa kaedah yang diubah suai memenuhi kriteria prestasi yang diperlukan oleh Peraturan (EU) No. 836/2011. Ciri-ciri prestasi seperti had pengesanan (LOD, 0.04 - 0.14 ng / g), had pengkuantitian (LOQ,

0.13-0.48 ng / g), keterulangan (HORRATr: 0.30 - 1.25), kepersisan perantaraan (HORRATR: 0.33 – 0.77), kelinearan ($R^2 > 0.997$) dan perolehan (52.13 hingga 86.23%) telah dikenalpasti. Objektif ketiga ialah penggunaan kaedah yang diubahsuai untuk pengkuantitian PAH4 dalam biji koko yang dipanggang pada keadaan pemanggangan yang berlainan (penggunanaan keseluruhan biji koko dan nib panggang, suhu berbeza dari 110 hingga 190 °C dan tempoh yang berbeza antara 10 hingga 50 min). Analisa biji koko panggang memperlihatkan peningkatan B[a]A (0.19 - 1.18 ng/g), Chrys (<0.48 - 2.60 ng/g) dan B[a]P (<0.13 - 4.11 ng/g) yang ketara ($p < 0.05$) dengan peningkatan suhu (110 hingga 190 ° C) dan jangka masa (10 hingga 50 min), manakala B[b]F tidak dikesan sehingga suhu mencapai 190 °C. Jumlah PAH4 (jumlah B[a]A, Chrys, B[b]F dan B[a]P) juga menunjukkan peningkatan ($p < 0.05$) yang ketara dengan peningkatan suhu dan masa. Kajian ini menunjukkan kaedah pemanggangan keseluruhan biji koko atau nib adalah selamat untuk digunakan kerana kehadiran maksimum B[a]P (4.11 ng/g) dan PAH4 (7.73 ng/g) adalah lebih rendah daripada had maksimum oleh Peraturan (EU) No. 835/2011. Dalam kajian ini, keadaan pemanggangan (pemanggangan keseluruhan biji koko atau nib koko dengan suhu dan jangka masa yang berlainan) menunjukkan kesan yang ketara terhadap kandungan PAH4 dalam sampel koko. Kaedah yang diubah suai dan disahkan (QuEChERS dan DLLME berserta HPLC-FLD) adalah berkesan untuk mengesan dan menentukan jumlah PAH4 di dalam biji koko dengan ketepatan, kepekaan dan kepersisan yang tinggi.

Kata kunci: Proses biji koko, DLLME, QuEChERS, HPLC, FLD, Pemanggangan koko

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

PAH	Polycyclic aromatic hydrocarbon
B[a]P	Benzo[a]pyrene
B[a]A	Benz[a]anthracene
Chrys	Chrysene
B[b]F	Benzo[b]fluoranthene
PAH4	Sum of total B[a]A, Chrys, B[b]F and B[a]P
DLLME	Dispersive liquid-liquid microextraction
HPLC	High performance liquid chromatography
FLD	Fluorescence detector
LOD	Limit of detection
LOQ	Limit of quantification
QuEChERS	Quick Easy Cheap Effective Rugged Safe
ng/g	nano gram per gram
°C	Degree celcius
GC	Gas chromatography
MS	Mass spectrometry
µL	Micro litre
ng/mL	Nano gram per millimetre
PSA	Primary secondary amine
MgSO4	Magnesium sulphate
NaCl	Sodium chloride
CHCl3	Chloroform
ACN	Acetonitrile

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cocoa bean *(Theobroma cacao* L.*)* is a native species found in tropical humid forests in Andes, South America (Prabhakaran, 2010). At southwest of Mexico, cocoa is one of the main crops which has been cultivated since pre-Hispanic times (Rodriguez-Campos et al., 2012). The most important component of cocoa is the seed (cocoa bean) which is primarily used in confectionery industries. Cocoa is the fourth largest commodity crop in Malaysia after palm oil, rubber and timber (Ghazali, 2018). An Annual Report by Malaysian Cocoa Board (2017) showed that Malaysia is the largest cocoa grinder in the world after the Cote d'Ivoire, Netherlands, Indonesia, Germany, United States and Ghana. In 2018, the contribution of the cocoa industry to the country's revenue has increased by 9.4% to RM5.551 billion compared to RM5.029 billion in 2015. The major contributor to export earnings is cocoa butter with an export value of RM1.576 billion (Department of Statistics Malaysia, 2018).

Beckett et al. (2017) indicated that the steps involved in cocoa processing are cocoa bean cleaning, fermenting, drying, roasting, winnowing, grinding and producing cocoa butter and cocoa powder. Unfortunately, during the primary cocoa processing, several critical steps such as drying, storage, transportation and roasting might be susceptible to contamination with polycyclic aromatic hydrocarbons (PAHs). Previous studies reported that there were 0.07-7.86, 0.81-12.00 and 2.02-39.78 ng/g Benzo[a]pyrene (B[a]P) in cocoa butters from Indonesia, Germany and Ghana respectively which were formed during cocoa bean drying on asphalt, bitumen, under the sun or by direct firing (Lowor et al., 2012; Misnawi, 2012; Wandan, et al., 2011). Cocoa beans can also be contaminated with PAHs during storage and transport in jute or sisal bags that had been treated with batching oil (Grob et al., 1993). Moreover, studies on the effects of roasting conditions on PAHs content in cocoa beans sample showed an increase of total PAHs content with the increase of temperature from 130 to 150 °C (Zyzelewicz et al., 2016).

PAHs are a group of well-known chemical compounds distributed in the environment and formed by the incomplete combustion of organic compounds and geochemical processes (Barranco et al., 2003). PAHs have been the subject of much concern in recent years because of their toxic potentials and that they comprise of some well-known carcinogens. Benzo[a]pyrene (B[a]P) has been shown to be the most toxic and carcinogenic PAH and therefore is frequently used as a marker in PAHs contamination in food samples (EU, 2011a; SCF, 2002). In order to assure the consumers' safety of cocoa beans and cocoa products, the European Union has set a maximum limit of 5 ng/g for B[a]P and 30 ng/g for PAH4 (sum of four different PAHs: benz[a]anthracene (B[a]A), chrysene (Chrys), benzo[b]fluoranthene (B[b]F), and B[a]P in cocoa beans and derived products (EU, 2011a).

There has been an increase in studies and investigation related to the occurrence of PAHs contaminants in cocoa. There are many analytical methods developed for the determination of PAHs in cocoa. The method for PAH4 determination normally starts with sample extraction followed by clean-up and finally, PAH4 identification and quantification by analytical instruments (HPLC, GCMS and LCMS) (Sadowska-Rociek et al., 2015; Raters & Matissek, 2014; Kumari et al., 2012).

Several sample extraction and clean-up procedures have been proposed for PAHs determination in cocoa including Soxhlet extraction, accelerated solvent extraction (ASE), ultrasonic extraction, alkaline saponification, maceration extraction with mechanical agitation, liquid-liquid extraction (LLE) and solid phase extraction (SPE) (Belo et al., 2017; Raters & Matissek, 2014; Wandan et al., 2011; Ziegenhals et al., 2009; Zyzelewicz et al., 2016). However, these methods require a long preparation time, considerable investment for instrumentation and consume a large amount of solvents.

There are crucial aspects that need to be considered in choosing the right method to determine PAHs contaminants. The method must be able to thoroughly extract the PAHs from the complex cocoa matrices and ensure that there are no presence of co-extracted interfering compounds. As such, it is not a surprise to see more comprehensive procedures for PAHs determination in cocoa bean and its product have been developed. One of the most promising method to determine PAHs content is developed by Sadowska-Rociek et al. in 2015. This method is the combination of QuEChERS method and dispersive liquid-liquid microextraction (DLLME) and was applied on chocolate (white, milk and dark chocolate). This combination provided important advantages such as simplicity of operation, quickness, fewer amounts of organic solvents, high recovery and enrichment factor (Rai et al., 2016; Sadowska-Rociek et al., 2015; Melo et al., 2013).

1.2 Problem Statement

In the last few years, QuEChERS method has been widely used for analysis of contaminants in food products (vegetables, fruit juices, chocolate, canned seafood, canned vegetables and fruits). Using the combination of QuEChERS and DLLME method in contaminants extraction procedure yields clearer extracts with less or without interferent (such as micro components of cocoa beans such as fat, protein, cellulose and fiber) (Rai et al., 2016; Sadowska-Rociek et al., 2015; Cunha & Fernandes, 2013; Cunha et al., 2012). Sadowska-Rociek et al. (2015) have developed the combination of QuEChERS and DLLME for the determination of PAHs content in chocolate using GCMS. The method provides a lower quantification limit (0.42 - 0.50 ng/g) for PAHs quantification in chocolate.

However, there was limitation in applying the Sadowska-Rociek method using QuEChERS and DLLME for quantification of PAH4 in cocoa bean. Despite the advantages and its successful application in determining PAHs in chocolate, this method was unsuitable and unable to detect and quantify all of the PAH4 in cocoa bean matrix as determined in the preliminary study. On top of that, most of the previous studies on method determination of PAHs in cocoa also did not analyse the complete parameters to

evaluate the fitness of purpose (validation process) after modification of method has been made. According to EURACHEM (2014), ISO/IEC 17025 (2005) and IUPAC Technical Report (2002), the modified method must be validated in order to confirm that the method has consistent capabilities with the required application. Limit of detection (LOD), limit of quantification (LOQ), Horrat values and recovery rates are among performance characteristics usually referred to in the validation process (EU, 2011b).

In this research, a new modification of method to determine PAHs in cocoa bean was proposed using QuEChERS and DLLME coupled with HPLC-FLD at different parameters for PAH4 recovery in cocoa bean. This was followed by a validation step to ensure that this method conforms to the regulated standard. The method was then used to quantify the presence of PAH4 in cocoa bean roasted at different roasting conditions (whole cocoa bean and nib roasting at different temperatures and duration) to ensure that the method is feasible and suitable to be used for cocoa beans. The experimental flow of the present work is showed in Figure 1.1.

1.3 Research Aims and Objectives

Therefore, the objectives of the present study were:

1. To modify analytical methods using QuEChERS and DLLME coupled with HPLC-FLD at different parameters for PAH4 recovery in cocoa bean.

2. To validate the modified method based on Regulation (EU) No. 836/2011 performance characteristics.

3. To quantify the presence of PAH4 in cocoa bean roasted at different roasting conditions (whole cocoa bean and nib roasting at different temperatures and duration) using the modified method.

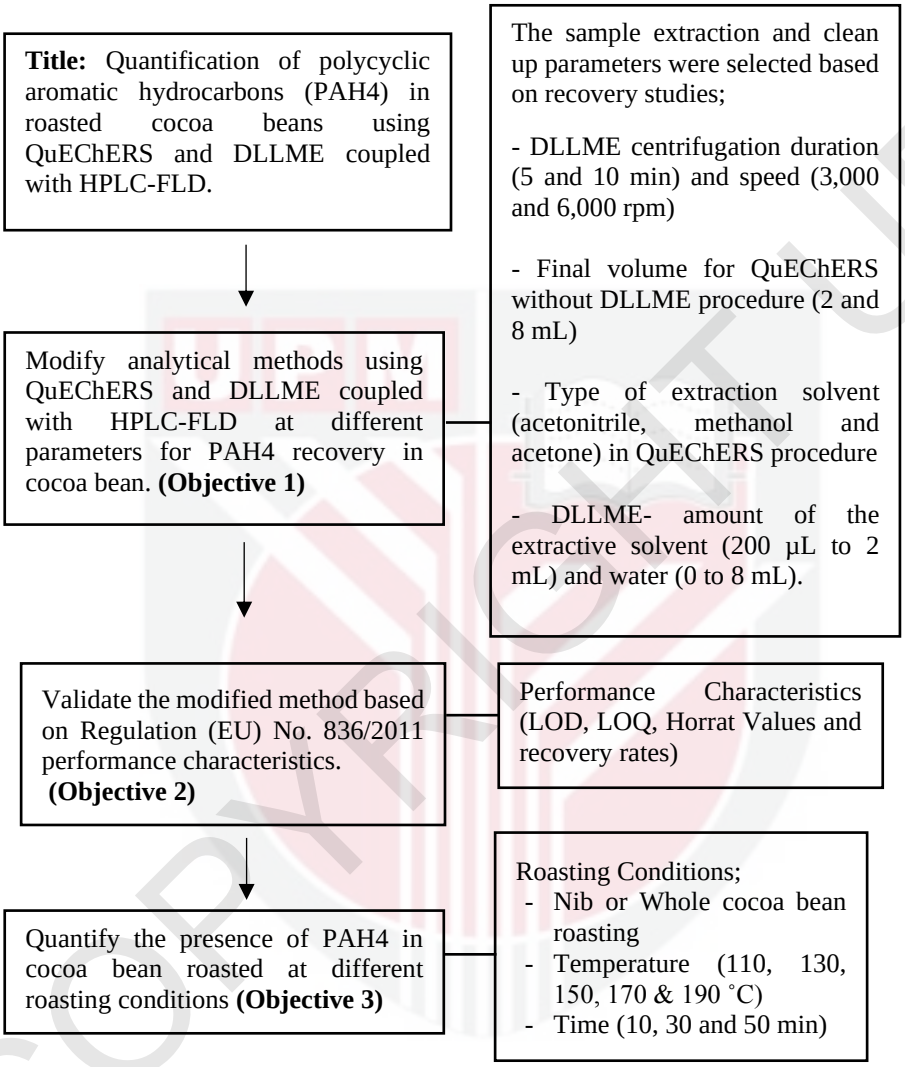

Figure 1.1: Experimental Flow of The Present Work

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