DEVELOPMENT OF U-SHAPED TWO-PHASE HOLLOW FIBER-LIQUID-PHASE MICROEXTRACTION FOR FOOD MYCOTOXINS ANALYSIS BY USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Abstract

This study is focusing on the development and validation of an analytical method for the determination of Aflatoxins (AFB1, AFB2, AFG1, and AFG2) and Ochratoxin A (OTA) in food samples. The first part is the LC-ESI-QQQ-MS/MS optimization using response surface methodology combined to Quick, Easy, Cheap, Effective, Rugged, and Safe method (QuEChERS) for the determination of mycotoxins in the different food matrix. The mobile phase composition is optimized using univariate methodology. While the experimental methods were optimized using multivariate strategy, *i.e.* Plackett-Burman and Box Behnken designs (BBD). The optimized LCMS parameters were subjected to method validation, which revealed good repeatability (0.63-4.76%), IDL (1.41 -3.61 ng), and R^2 (0.9992-0.9999) in standard and spiked samples. The validated LC-MS/MS combined with QuEChERS method was successfully applied for the determination of Aflatoxins and Ochratoxins A in a variety of food matrices. The results demonstrated good sensitivity with LOD ranged from 0.05-0.10 µg/Kg, and LOQ ranged from 0.08-0.30 µg/Kg. The results have revealed a good intra-day and inter-day precision below the acceptance limit of the method (<20%) with recovery ranged between 81.94-101.67%.In the second part of the study, U-Shape hollow fiber liquid phase microextraction (U-Shape-HF-LPME) supported by extraction/dispersion solvent was developed for the analysis of Ochratoxin A (OTA) in a food sample using both univariate and multivariate optimization (central composite design (CCD)) strategy. In the last part of the study, the U-Shape hollow fiber liquid phase microextraction (U-Shape-HF-LPME) supported by extraction/dispersion solvent also was developed for the analysis of

Aflatoxins (AFB1, AFB2, AFG1, and AFG2) in a food sample using both univariate in fruit juice. Moreover, QuEChERS and UAE were optimized and combined with HF-LPME for nonliquid food samples. The developed methods were successfully applied for the analysis of OTA as well as Aflatoxins (AFB1, AFB2, AFG1, and AFG2) in liquid and non-liquid food samples, in which three food samples were shown to contained trace amount of OTA and five food samples also shown to contained trace amount of aflatoxins below the maximum level allowed in the European Legal Maximum Limit. The results revealed satisfactory performance in the term of linearity, LOD, LOQ, precision, and accuracy and fulfilled the method performance criteria of Commission Regulation (EC) No 401/2006 and Sanco, 2013. The correlation coefficient (R^2) is higher than 0.9906. The limit of detection was in the range of 0.04-0.09 μg/L, while the quantification limit ranged from 0.8- 0.2 μg/L. The method showed good precision (<14% RSD) with recovery from 75.38-107.39%.

Key word: Development, U-Shaped, hollow fiber liquid phase microextraction, mycotoxins analysis , liquid chromatography tandem mass spectrometry, experimental design.

PEMBANGUNAN EKSTRAKSI MIKRO FIBER BERONGGA DUA-FASA BENTUK-U UNTUK MAKANAN MIKOTOKSIN ANALISIS DENGAN MENGGUNAKAN KROMATOGRAFI CECAIR BERSAMA SPEKTROSCOPI JISIM.

Abstrak

Kajian ini memberi tumpuan kepada pembangunan dan pengesahan kaedah analisis untuk penentuan Aflatoksin (AFB1, AFB2, AFG1, dan AFG2) dan Ochratoxin A (OTA) dalam sampel makanan. Bahagian pertama adalah pengoptimuman LC-ESI-QQQ-MS/MS menggunakan metodologi permukaan tindak balas yang digabungkan dengan kaedah cepat, mudah, murah, tahan lasak dan selamat (QuECheRS) untuk penentuan mikotoksin dalam matrik makanan yang berlainan. Komposisi fasa mudah alih dioptimumkan menggunakan metodologi univariate, dan kaedah eksperimen dioptimumkan menggunakan strategi multivariate, iaitu reka-bentuk Plackett-Burman dan Box Behnken (BBD). Parameter LCMS yang dioptimumkan tertakluk kepada pengesahan kaedah yang menunjukkan keboleh-ulangan (0.63-4.76%), IDL (1.41 -3.61 ng), dan R^2 (0.9992-0.9999) dalam sampel standard dan sampel yang di "spiked". LC-MS/MS yang digabungkan dengan kaedah QuEChERS telah berjaya digunakan untuk menentukan kandungan Aflatoksin dan Okhratoksin A dalam pelbagai matrik makanan. Keputusan menunjukkan sensitiviti yang baik dengan LOD bernilai 0.05-0.10 µg/Kg, dan LOQ bernilai 0.08-0.30 µg/Kg. Keputusan yang diperolehi telah mendedahkan ketepatan perbandingan "dalam hari" dan "antara hari" di bawah batas penerimaan kaedah (<20%) dengan pemulihan berkisar dari 81.94-101.67%. Dalam bahagian kedua kajian ini, fasa mikro ektraksi serat cair berrongga berbentuk-U (HF-LPME berbentuk-U) yang disokong oleh pelarut ekstraksi/sebaran telah direka untuk analisis Okhratoksin A (OTA) dalam sampel makanan yang menggunakan kedua-dua strategi pengoptimuman univariat dan multivariat (reka bentuk komposit pusat (CCD). Di bahagian terakhir kajian ini,

kaedah yang dibangunkan telah digunakan untuk penentuan serentak Aflatoksin (AFB1, AFB2, AFG1, dan AFG2) dalam jus buah-buahan, buah-buahan kering, dan produk kacang dengan menggunakan kaedah univariat dan kaedah reka bentuk kerangka mudah (multivariat). Kaedah yang telah dibangunkan telah berjaya digunakan untuk analisis OTA serta analisis serentak bersama Aflatoksin (AFB1, AFB2, AFG1, dan AFG2) dalam sampel makanan cecair dan bukan cecair (pepejal), di mana tiga sampel makanan didapati mengandungi jumlah OTA yang boleh dikesan dan lima sampel makanan pula mengandungi jumlah aflatoksin di bawah tahap maksimum yang dibenarkan oleh had maksimum undang-undang Eropah. Hasil dapatan menunjukkan prestasi memuaskan dari segi lineariti, LOD, LOQ, ketepatan, dan memenuhi kriteria penilaian kaedah mengikut peraturan komision EC No. 401/2006 dan Sanco, 2013, dengan Koefisien korelasi (R^2) lebih tinggi dari 0.9906. Batasan pengesanan berada dalam lingkungan 0.04-0.09 µg/L, manakala had kuantifikasi adalah dari 0.8 - 0.2 µg/L. Kaedah ini menunjukkan ketepatan yang baik (<14% RSD) dengan kadar pemulihan dari 75.38-107.39%.

Kata kunci: Pembangunan, ekstraksi mikro fiber berongga , bentuk-U , mikotoksin analisis,kromatografi cecair bersama spektroscopi jisim, reka bentuk eksperimen.

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In addition, I would like to acknowledge the sacrifice made by my beloved wife, Maryam, for her support and words of encouragement. I also appreciate the love and support of my brothers and sisters, uncles, aunts, and other family members.

Finally, to my children, Fatima Alzahra, Esra, Mohamed, and Nurul Huda, I dedicate this work to them.

Dedication

First and foremost, I praise and thank Allah SWT for His greatness, blessing, kindness, inspiration and for giving me the strength and courage to complete this thesis. I dedicate this work to the spirit of my parents. Ask Allah for forgiveness and mercy. I also dedicate this work to my dear wife and children.

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LIST OF SYMBOLS AND ABBREVIATIONS

AFB1	:	Aflatoxin Blue 1
AFB2	:	Aflatoxin Blue 2
AFG1	:	Aflatoxin Green 1
AFG2	:	Aflatoxin Green 2
ANOVA	:	Analysis of variance
ASE	:	Accelerated Solvent Extraction
BBD	:	Box-Behnken Design
BTEX	:	Benzene, toluene, ethylbenzene, and xylene
CCD	:	Central Composite Design
CE	:	Capillary electrophoresis
CF	:	Continuous Flow
CPA	:	Chlorophenoxy acid
DAD	:	Diode array detector
DEHP	:	Di-(2-ethylhexyl) phosphate
DI	:	Direct immersion extraction
DON	:	Deoxynivalenol
ECD	:	Electron capture detector
EF	:	Enrichment Factor
EME	;	Electromembrane extraction
ESI	:	Electrospray ionization
ET-AAS	:	Electrothermal atomic absorption spectrometry
ETV-ICP	:	Electrothermal vaporization inductively coupled plasma
FAMEs	:	Fatty acid methyl esters
FAs	:	Fatty acids

FED	:	Fractional factorial design
FFD	:	Full factorial design
FFTSWV	:	Fast Fourier transform square wave voltammetry
FIA	:	Flow injection analysis
FID	:	Flame Ionization Detector
FLD	:	Fluorescence detector
FPD	:	Flame photometric detector
FTD	:	Flame thermionic detection
GC	:	Gas chromatography
HAAs	:	Haloacetic acids
HCAs	:	Heterocyclic amine
HF-DLLME	:	Hollow fiber dispersive liquid–liquid microextraction
HF-LPME	:	Hollow fiber based liquid-phase microextraction
HF-LLLME	:	Hollow fiber liquid-liquid-liquid phase microextraction
HPLC	:	High performance liquid chromatography
HRMS:	:	High resolution mass spectrometry
HS	:	Headspace extraction
IAC	:	Immunoaffinity Column
IARC	:	International Agency for Research Cancer
ICP	:	Inductively coupled plasma
IDL	:	Instrument detection limit
LC	:	Liquid Chromatography
ID	:	Internal diameter
LDS	:	Low-density solvent
LLE	:	Liquid-liquid extraction
LGLPME	:	Liquid–gas–liquid Phase microextraction

LOD	:	Limit of detection
LOQ	:	Limit of quantification
LPME	:	Liquid-phase microextraction
MAE	:	Microwave-assisted extraction
MALDI	:	Matrix-assisted laser desorption/ionization
ME	:	Matrix effect
MeCN	:	Acetonitrile
MEEKC	:	Microemulsion electrokinetic chromatography
MeOH	:	Methanol
MF	:	Molecular formula
MI	:	Molecular ion
MRT	:	Mean retention time
MS	:	Mass Spectrometry
MSTFA	:	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MW	:	Molecular weight
MWCNT	:	Multiwall carbon nanotube
NPD	:	Nitrogen-phosphorus detector
NPOE	:	2-Nitrophenyl octylether
NSAIDs	:	Nonsteroidal anti-inflammatory drugs
OCPs	:	Organochlorine pesticides
OFAT	:	One Factor at a Time
OPLC	:	Over Pressured-Layer Chromatography
OTA	:	Ochratoxin A
OTB	:	Ochratoxin B
OTC	:	Ochratoxin C
PAHs	:	Polycyclic aromatic hydrocarbons

PBDs : Plackett-Burman designs

PBDEs : Polybrominated diphenyl ethers

PCBs : Polychlorinated biphenyls

PDHID: : Gas chromatography pulsed discharge helium ionization detector

PI : Precursor ion

PSA : Primary secondary amine

QFED : Quarter factorial experimental design

QTOF : Quadrupole time of flight

QuEChERS : Quick Easy Cheap Effective Rugged and Safe

QQQ : Triple Quadrupole

RSD : Relative standard deviation

RSM : Response Surface Methodology

SBME : Solvent Bar Microextraction

SD : Standard deviation

SDME : Single drop microextraction

SDS : Sodium dodecyl sulfate

SD : Standard deviation

SFE : Supercritical Fluid Extraction

SLE : Solid Liquid Extraction

SLM : Supported Liquid Membrane

SPE : Solid-phase extraction

SPME : Solid-phase Microextraction

TCPA : Total chromatographic peak area

TDM : Therapeutic drug monitoring

TIC : Total ion chromatogram

TLC : Thin-layer Chromatography

TOAD	:	Taguchi orthogonal array design
TOPO	:	Trioctylphosphine oxide
UAE	:	Ultrasonic Assisted Extraction
ZEA	:	Zearalenone
VA	:	Vortex-assisted
VOCs	:	Volatile organic compounds

CHAPTER 1: INTRODUCTION

1.1 Mycotoxins

Mycotoxins are secondary metabolites produced by fungi and commonly found in food and feed (Bhat et al., 2010; Streit et al., 2013). Over 300 types of mycotoxins have been discovered with various chemical and physicochemical properties (Zain, 2011). Aflatoxins, ochratoxins, fumonisins, trichothecenes, zearalenone, patulin, and ergot are the most frequently occurring mycotoxins and have been noted to have adverse effects on humans and animals (Smith et al., 2016;Filazi et al., 2017). Mycotoxins have been shown to cause health problems such as nephrotoxic (Pfohl-Leszkowicz, 2009), teratogenic, genotoxic and immunotoxic (Bui-Klimke & Wu, 2015). The Agency for Research on Cancer (IARC) has classified Aflatoxin B1(AFB1) as a carcinogenic compound to humans (IARC, 2016;2018) and Ochratoxin A (OTA) in group 2B as a possibly carcinogenic compound (Bui-Klimke & Wu, 2015; IARC, 2011). In view of the health problems caused by the OTA to the consumers, the EU and other international organizations have passed some legislations to regulate the permitted maximum levels of OTA in food and feed (European Commission, 2006).

1.1.1 Aflatoxins

The aflatoxin type has about 20 chemically related toxic derivative compounds. Aflatoxins B1, B2, G1, and G2 are usually found in foods, mainly produced by foodborne moulds, such as *Aspergillus flavus* and *A. parasiticus*. Such aflatoxins contaminations in a variety of foods, such as nuts and maize are associated with health risks for consumers. AFB1 is known to be a particularly potent cancer-causing agent in both humans and animals (Afsah-Hejri et al., 2013).

In term of chemical properties, aflatoxins are crystalline, freely soluble substances in moderately polar solvents (e.g., methanol, chloroform, and dimethyl sulfoxide), and water soluble to the extent of 10-20 mg/L. Aflatoxins fluoresce under UV radiation. Light, especially ultraviolet radiation in the presence of moisture causes destruction of aflatoxins. Crystalline aflatoxins are not affected by temperatures exceeding 100 ºC in the absence of light, particularly UV radiation. They are also stable for years if the solution is prepared in chloroform or benzene and kept in a cold and dark place (Shvets & Baranovsky, 2015;MdQuadri et al., 2017).

1.1.2 Ochratoxins

OTA is a secondary metabolites of many filamentous species belonging to the genera *Aspergillus* and *Penicillium*. It is a pentaketide derived from the coupling of dihydrocoumarins to β-phenylalanine. Ochratoxin B (OTB) and ochratoxin C (OTC) are derivatives of OTA; Ochratoxin α (Otα) and its de-chloro analog, ochratoxin β (OTβ), have also been identified as metabolites related to OTA (Kőszegi & Poór, 2016;Gil-Serna et al., 2018).

OTA is a colorless crystal at room temperature under normal light but exhibits green and blue fluorescent shades under ultraviolet light. The free acid of OTA is moderately soluble in organic solvents, such as chloroform, ethanol, methanol, and xylene but insoluble in water. It is not degradable when stored in the dark in ethanol solutions, but it becomes unstable in light with high humid conditions. OTA is only partially degraded at normal cooking conditions (Gilbert & Şenyuva, 2009) and displayed fair stability toward heat (Kőszegi & Poór, 2016;Gil-Serna et al., 2018).The chemical structure of studied mycotoxins AFB1, AFB2, AFG1, AFG2, and OTA are shown in Figure 1.1.

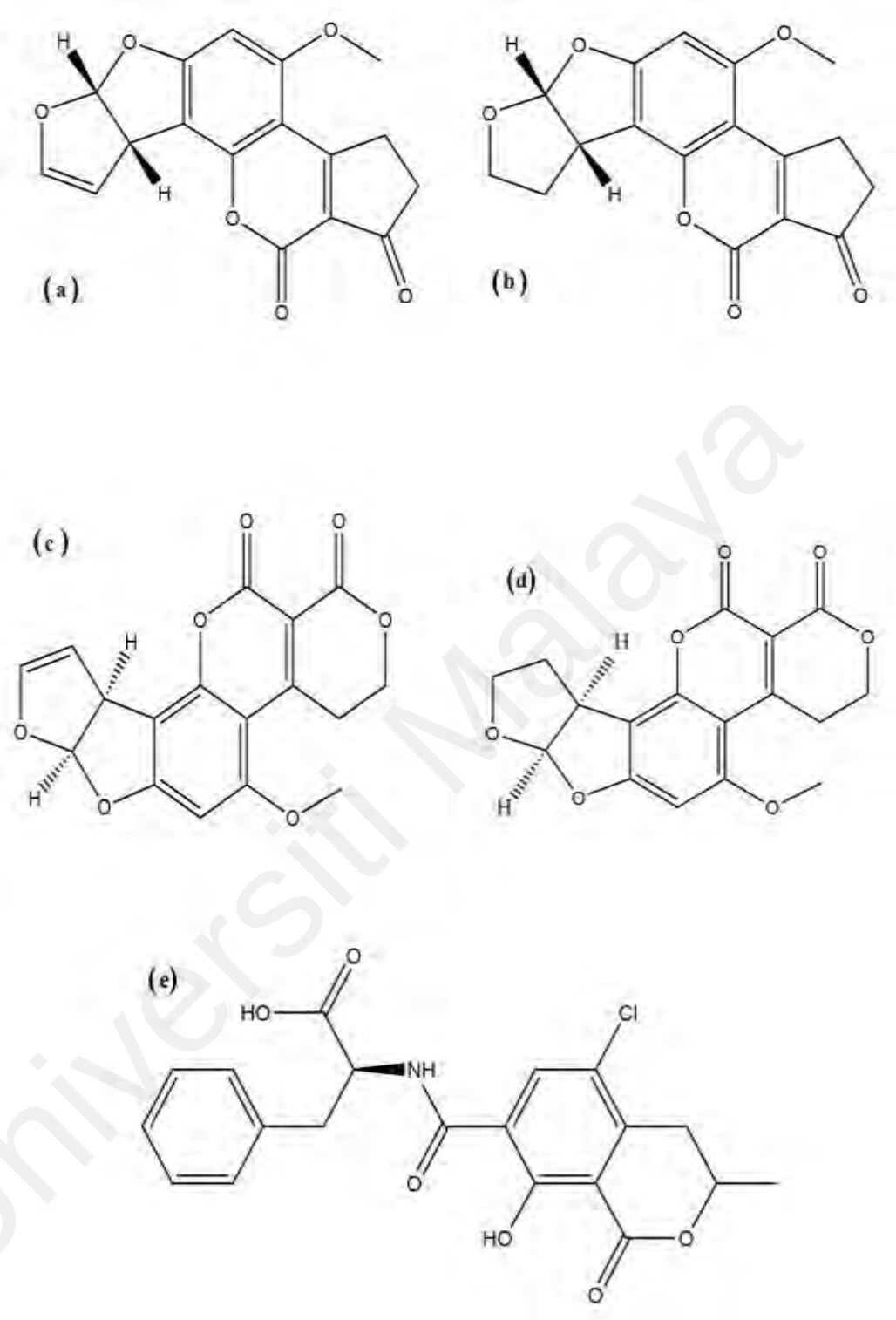

Figure 1.1: Chemical structure of studied mycotoxins; (a) AFB1, (b) AFB2, (c) AFG1, (d) AFG2, (e) OTA.

1.2 Analytical Methodology

1.2.1 Sampling

Sampling is a critical step in the mycotoxins analysis process. It is critical to obtain a representative sample due to the high heterogeneity of mycotoxins. Therefore, in any sampling program associated with mycotoxins, the main questions to be answered are "Why", "When", "Where", "What", and "How" to the sample and "Which" samples are to be collected ((Miraglia et al., 2005; Nollet & Toldrá, 2012). Previous studies on agricultural products, such as peanuts and shelled corn, showed that a minuscule part representing1% of the kernels in the lot was contaminated, and the concentration on a single kernel may be extremely high. Sampling protocols for mycotoxin detection specified how to take or select a representative sample of the total and the size of the sample. The sample selection procedures used to gather a suitable sample from a bulk lot is considered most important. Every individual item in the lot should have an equal chance of being chosen (known as random sampling) (Whitaker et al., 2010).

1.2.2 Sample Preparation

The extraction of mycotoxins from a sample depends on a few factors, i.e. physicochemical properties of the sample matrix and the type and amount of mycotoxins. Generally, the samples are blended with a suitable extraction solvent in a high-speed blender or mechanical shaker. The slurry is then filtered and subjected to subsequent purification procedures. Diatomaceous earth is sometimes included in the solvent mixtures to enhance filtration efficiency (Fung & Matthews, 1991;De Vivo et al., 2008).

1.2.3 Extraction Methods

Extractions are usually accomplished by employing the liquid-liquid extraction (LLE) or solid phase extraction (SPE) approaches. In LLE, the extraction is performed by using two immiscible liquid phases, while SPE uses a solid and a liquid phase. In the extraction process, the target analyte and other compounds that share similar properties will migrate into the extraction solvent phase from the aqueous phase until equilibrium is achieved. The target analytes can be separated and concentrated in the solvent phase, and multiple extractions may be carried out to ensure exhaustive extraction of the target analytes. The semi-pure fractions are gathered for further treatment (clean-up) to remove the co-extractives. Ideally, the extraction solvent should separate the target analytes from the sample matrix. In this regard, the selection of the optimum solvents is a crucial step to separate as much target analytes as possible while the co-extractives are suppressed from migrating to the extraction solvent. Other desirable properties of the extraction solvent include nontoxic, easily recoverable, nonflammable, stable, transparent to UV light, volatile, and environmental-friendly (Zhang et al., 2012).

1.2.3.1 Liquid-liquid Extraction (LLE)

The principle of LLE lies on partitioning the compounds into two immiscible solvents until equilibrium is reached. Although LLE coupled with the chromatographic systems has been used in the separation of various organic compounds, it has many drawbacks, such as multiple handling steps (which complicates automation), time-consuming, and require large volumes of organic solvent (Rahmani et al., 2009; Reiter et al., 2009). This technique has been reported in the mycotoxins extraction from cereals, sweet pepper, maize, dried figs, rice, milk, and animal feed (Rahmani et al., 2009).

1.2.3.2 Solid-Phase Extraction (SPE)

The SPE has been routinely used for sample clean-up, sample concentration, and matrix removal. In the sample clean-up application, the solid phase retains the target analytes and allows impurities to pass through the column. In sample concentration applications, large sample volumes are passed through the column where the retained target analytes are concentrated, eluting them with a small volume of solvent. In the matrix removal application the interfering species are retinal but the target analytes are allowed to pass through the column (Betina, 1993). Basically, the technique is based on the partitioning of target analytes or interfering compounds between a mobile phase and a stationary phase. The stationary phase can be composed of a solid adsorbent or an immobilized (bonded) liquid phase. The common immobilized phases include octyl (C8), ethyl (C2), octadecyl (C18), cyclohexyl (CH) silane and ion-exchange phases (Betina, 1993). SPE is generally reproducible, more rapid, efficient, and a safer method when compared to LLE, apart from offering a broad range of selectivity (Ahadi et al., 2011).

1.2.3.3 Immunoaffinity Columns (IAC)

IAC is a highly specific technique used for sample purification and concentration. IAC can be applied to complex matrices and uses a smaller volume of organic solvent. The high specificity nature of IAC limits the application for simultaneous determination of different groups of analytes (Pascale & Visconti 2006). Depending on the type of antibody, cross-reactivity may potentially lead to overestimation. This phenomenon can be strategically exploited in the analysis of masked mycotoxins. In addition to the target compound, structurally related to the derivatives of the target compound can be bound. For instance, in cross-reactivity of Deoxynivalenol (DON) (dedicated as the DON prep columns or deoxynivalenol-3-glucoside), the major “masked” Fusarium toxin, can be isolated together with free DON (Kostelanska et al., 2011). The IACs are commercially

available for T-2, and HT-2 mycotoxins, fumonisins, zearalenone, aflatoxins, and OTA (Barug, 2006). Multi-mycotoxin IACs have been successfully used for the detection of AFB1, AFM1, and OTA in lyophilized 24-h diet samples (Bakker et al., 2009), aflatoxins and OTA in maize-based specimens (Chan et al., 2004), AFB1, AFB2, AFG1, AFG2, OTA and ZEA in rice, rye, maize, and wheat via liquid chromatography with fluorescence detection (LC/FD). New multi-analyte IAC (AOFZDT2 TM column) containing antibodies have been developed for the analysis of aflatoxins (AFB1, AFB2, AFG1, AFG2) and OTA, with other mycotoxins, such as fumonisins, DON, ZEA, HT-2, and T-2 (Zabe & Basker, 2007). Due to their high selectivity for target or groups of mycotoxins in different food matrices, the use of IACs as a clean-up method of food extracts has increased considerably during the last decade (Pascale & Visconti 2006). IACs can offer unsurpassed specificity but they are expensive, non-recyclable, have limited binding capacity (heavily contaminated samples have to be diluted and subjected to multi-cleanup process), and limited shelf life (Al-Hadithi et al., 2015).

1.2.3.4 Supercritical Fluid Extraction (SFE)

SFE separates the target analytes using supercritical fluids (CO_2) as the extracting solvent. Carbon dioxide (CO_2) can be used alone or with co-solvents, such as ethanol or methanol. The dual properties of supercritical CO_2 provide an ideal condition for extracting target analytes with a great recovery's degree within a short time. SFE has been used to determine trichothecene mycotoxins in wheat with good recoveries (De Saeger, 2011).

1.2.3.5 Accelerated Solvent Extraction (ASE)

The accelerated solvent extraction (ASE) is also known as pressurized liquid extraction (PLE) which uses elevated temperatures and pressures to decrease the

extraction time. In ASE, parameters such as temperature, pressure, static time, cell size, and solvent are most critical (Gentili et al., 2004). The solid sample is filled with extraction solvent in a sealed cell; the solubilizing capacity of the extraction solvents is increased with a higher temperature which leads to a rapid diffusion rate. In this context, elevated temperature modified the solvent viscosity to allow better penetration of the solvent into the pore of the sample. The use of high-pressure forces the solvent into the inner pore of the matrices and facilitate the extraction of analytes trapped in the matrix pores (Richter et al., 1996).The ASE technique has been used for the extraction of mycotoxins, such as aflatoxins (B1 and B2) in contaminated pistachio samples (Sheibani & Ghaziaskar, 2009), zearalenone in cereal flour sample (Pérez-Torrado et al., 2010) and OTA in bread samples (Osnaya et al., 2006).

1.2.3.6 Quick Easy Cheap Effective Rugged and Safe (QuEChERS)

In this method (QuEChERS), the samples are usually homogenized at a low temperature, followed by the addition of solvent and then later centrifuged (Anastassiades et al., 2002). To remove water and contaminant residuals, inorganic salt such as anhydrous magnesium sulfate (MgSO4) and PSA are added to the supernatant. The supernatant is then shaken and re-centrifuged before subjected to instrumental analysis. The choice of the extraction solvent is a crucial factor that decides QuEChERS performance (Sospedra et al., 2010), which commonly utilizes mixtures of several solvents of different ratios (Desmarchelier et al., 2010; Romero-González et al., 2011).

1.2.3.7 Ultrasonic Assisted Extraction (UAE)

UAE is an alternative for the other extraction technologies due to its advantages, e.g. moderate energy and solvent volume, safe, easy to use, economical and reproducible. The ultrasonic energy enhances the extraction efficiency by creating cavitation with high

temperatures and high pressures; furthermore, it minimizes the extraction time. This technique is especially beneficial in the solid sample preparation (Mandal et al., 2015;Gong et al., 2017; Medina-Torres et al., 2017).

1.2.3.8 Solid Phase Microextraction Methods (SPME)

The SPME was developed as a green sample preparation technique (solvent-free) (Spietelun et al., 2013). It does not need a solvent and combined sampling, isolation, enrichment, and concentration in one single step. These offer a shorter sample preparation step and solvent-free extraction, which requires a small sample volume and allows the concentration of analytes from solid, liquid or gaseous samples. SPME uses a chemically inert fused silica optical fiber where the extraction process is accomplished by exposing the fiber to the headspace (HS) above the sample solution or direct contact with the sample solution (direct immersion). The former model applies to volatile and semi-volatile species, while the latter can be used regardless of analyte volatility (Risticevic et al., 2010;Abdulra'uf et al., 2012).

1.2.3.9 Liquid Phase Microextraction (LPME)

LPME, which is also known as solvent microextraction (SME), uses a small quantity of the solvent during extraction (Theis et al., 2001). The LPME method is rapid and inexpensive and can be performed using a small volume of a water-immiscible solvent (acceptor phase) and the aqueous phase (donor phase), which contains the target analytes (Sarafraz-Yazdi & Amiri 2010). The acceptor phase can either be immersed directly in the sample matrix or suspended above the sample for extraction (Kataoka, 2010) and the volume of the acceptor phase is in microliter. LPME can be further classified into three categories: 1) single-drop microextraction (SDME); 2) hollow fiber liquid phase microextraction (HF-LPME); and 3) dispersive liquid-liquid microextraction (DLLME).

They are promising tools for the analysis of compounds in complex matrices due to their unique features of easy sample clean-up, low limits of detection, inexpensive, and environment-friendly, when compared to other methods.

(a) Single-Drop Microextraction (SDME)

SDME uses a micro-drop of immiscible organic solvent suspended in the form of a droplet. This method is first described by Liu and Dasgupta (Liu & Dasgupta 1996) and known as the drop-in-drop method. In the same year, Jeannot and Cantwell (Jeannot & Cantwell, 1996) developed a similar method, in which a micro-drop of organic solvent was hung at the end of a Teflon rod immersed into an aqueous sample solution and stirred by a magnetic stirrer. After the extraction, the droplet was retrieved by a micro-syringe, and a small part of it was injected into a GC for further analysis.

The static and dynamic SDME approaches were introduced by He and Lee. In the static mode, one μL of the solvent drop was suspended on the micro-syringe needle tip which was immersed into the aqueous sample solution. While, in the dynamic mode, a microsyringe was used as a micro-separator funnel. An aqueous sample was first withdrawn into the micro-syringe containing the extraction phase and subsequently pushed back into the bulk sample. This whole process was repeated formultiple cycles (usually 20 times) and the extract was injected directly into a GC. During extraction, the solvent was withdrawn into the micro-syringe and a thin film formed on the inner wall. Later, the analyte from the aqueous sample rapidly partitioned in the film. The analyte will then diffuse into the bulk organic solvent on the expulsion of the aqueous portion from the micro-syringe (He & Lee 1997). The static mode provides better reproducibility, but suffers from limited enrichment and demands longer extraction time. Meanwhile, the dynamic mode provides higher enrichment in a shorter period as compared to the static

mode, but its disadvantages include low reproducibility and repeatability due to manual operation (He & Lee 1997).

The direct immersion (DI) SDME is carried out in two modes (static mode and dynamic mode). The principle of DI-SDME is based on the suspension a solvent drop from the tip of a micro-syringe needle which is immersed in an aqueous sample solution. The main drawback of this technique is that the drop at the needle or capillary tip limits the use of extended extraction times, high stirring rates and sample temperatures, and samples should be filtered before use to avoid drop instability by suspended particulate matter (Ahmadi et al., 2006; Kataoka, 2010; Sarafraz-Yazdi & Amiri 2010). To overcome the drop loss, the dynamic mode is preferred because the inconvenience of accidentally losing the drop is eliminated and also results in higher enrichment of analytes and much faster extractions are achieved(Jain & Verma, 2011).

In headspace (HS) SDME, only volatile and semi-volatile compounds are extracted. The analyte is diffused between three phases: aqueous phase, HS, and solvent drop. The aqueous phase mass transfer is the rate-determining step in this technique(Theis et al., 2001; Xu et al., 2007;Han & Row, 2010). Since the droplet is not in direct contact with the sample, an organic solvent can be used as an acceptor in HS-SDME and results in good clean-up procedures for complex samples (Ahmadi et al., 2006). The drawbacks of this method are the vapor pressure of organic solvent must be low enough to avoid evaporation during extraction and at the same time must be compatible with GC analysis. In addition, the water-miscible solvent may lead to the increment of droplet size consequently resulting in falling of the droplet from the needle (Nováková & Vlčková, 2009).

Continuous flow (CF) SDME relies on continuous refreshing of the immobilized organic droplet surface extracting solvent. The extraction solvent drop is injected into a

glass chamber by a conventional micro-syringe and held at the outlet tip of a Polytetrafluoroethylene (PTFE) connecting tube. The sample solution flows right through the tube and the extraction glass unit to waste, while the solvent drop interacts continuously with the sample solution and extraction proceeds simultaneously. This tube acts as both a fluid delivery system and solvent holder in which the HPLC injection valve is used to control the droplet size. CF-SDME method avoids the introduction of undesirable air bubbles and shows a higher enrichment factor. Its effectiveness attributed to both diffusion and molecular momentum due to the mechanical forces (Liu & Lee, 2000). CF-SDME–HPLC has been reported for the extraction and determination of commonly used pesticides (fensulfothion, simazine, etridiazole, bensulide, and fipronil). The method gave good linearity between 25-250 ng/mL (R^2 ranged from 0.9879 to 0.9999). The detection limit was 4 ng/mL for all analytes (He & Lee, 2006).

Directly suspended droplet (DSD) SDME is another similar technique. In this technique, the aqueous sample is filled in a vial containing a stir bar to give a gentle vortex. The solvent drop is added on the top surface of the aqueous sample, and the single droplet is vortexed at or near the center of rotation. The mass transfer is believed to increase as an effect of the rotation of the droplet on the surface of the aqueous phase. This method provides greater flexibility in solvent volume and stirring speed as compared to the other SDME techniques (Yangcheng et al., 2006).

(b) Hollow Fiber Liquid Phase Microextraction (HF-LPME)

Pedersen-Bjergaard and Rasmussen described a new LPME method by using a low-cost and disposable permeable hollow fiber (HF) made of polypropylene. The solvent is impregnated in the fiber pore by dipping the membrane in the solvent for several seconds to form the supported liquid membrane (SLM) and the excess organic solvent is removed by flushing with air, followed by the addition of an acceptor phase inside the lumen. The

HF was then immersed into the sample solution for extraction. The SLM is separated between two aqueous phases, the donor phase which contains the target analytes and the acceptor phase which receives the target analytes and named as the three-phase mode. The acceptor phase also plays a role in concentrating and transferring the analytes into the instrument. Another mode of hollow fiber liquid microextraction is called the two-phase mode. In this technique, same organic solvent is used to fill the hollow fiber pores and lumen (acceptor phase).This is more compatible with gas chromatography(Jönsson & Mathiasson, 2000). In HF-LPME, many samples can be prepared simultaneously, while eliminating potential cross-contamination and their effects. (Ghambarian et al., 2012).

(c) Dispersive Liquid-Liquid Microextraction (DLLME).

DLLME was originally developed for the analysis of water samples. This method has also been applied to soil and food samples in combination with other techniques. The principle of DLLME is based on the difference of analytes affinities toward aqueous and organic phases. DLLME is performed by adding a small volume of the organic solvent together with a dispersive solvent into the aqueous sample and the sediments are collected after centrifugation (Rezaee et al., 2006). The dispersive solvent must be miscible with both the extraction solvent and the donor phase so as to aid the extraction of the target analyte(Jahromi et al., 2007). The most commonly used dispersive solvents are ethanol methanol, acetone, and acetonitrile. The extraction solvent requires the following properties: the ability to form small droplets in the donor phase, low solubility in water, ability to collect analytes, compatibility with the desired analytical instrument, and higher density than that of the sample. Halogenated hydrocarbons solvents (chlorobenzene, carbon disulfide, carbon tetrachloride, and chloroform) are mostly used in this method. The simplicity of the operation, rapidity, low cost, good recovery, and high enrichment

factors prove advantageous to the extraction process (Rezaee et al., 2006 ;Jahromi et al., 2007).

1.2.4 Chromatographic Techniques

1.2.4.1 Thin-layer Chromatography (TLC)

TLC is used for separation in purity assessments and the identification of organic compounds. In the one-dimensional mode, the TLC plate is placed vertically in the solvent tank. The capillary action of the TLC sorbent moves-up the solvent together with the samples and results in separation. The separated compounds are spotted with UV, fluorescence, staining reagent or other detection methods (Rahmani et al., 2009). In the two-dimensional mode, the TLC plate is dried after the first development (similar process as the one-dimension TLC), before being rotated 90° and subjected to similar/another solvent system.

1.2.4.2 Over Pressured-Layer Chromatography (OPLC)

The over pressured-layer chromatography (OPLC) is a forced-flow technique which applies external pressure on the chromatography plate that seals on the edges; where a pump is used to flow the mobile phase into the stationary phase. The advantages of the OPLC includes a smaller volume of the mobile phase, an off-line method, and allows faster examination with the possibility of parallel analyses (Móricz et al., 2007).

1.2.4.3 Gas Chromatography

The GC is a technique used to partition the analytes between the liquid stationary and the gas mobile phases. Derivatization procedures can be carried out to increase the volatility of analytes and improve their GC responses (Turner et al., 2009; Köppen et al., 2010). Common drawbacks of GC include the requirement of derivatization of certain

analytes prior to the analysis and their incompatibility in the GC method with non-volatile and polar analytes.

1.2.4.4 Liquid Chromatography (LC)

The LC technique separates a mixture of compounds by relative affinity of the compounds between stationary (column) and mobile phases. Derivatization (pre-column or post-column derivatization) is sometimes needed to improve the detection sensitivity. For example, on-line electrochemical bromination using a "Kobra" cell is a robust procedure to enhance the fluorescence of analytes before passing through the detector in the analysis of aflatoxins (Kok et al., 1984; Stroka et al., 2000).

1.3 Scope and Objectives of Study

Mycotoxins are secondary metabolites produced by fungi and commonly found in food and feed. They are classified as harmful compounds and cause health problems when ingested. Sample extraction, clean-up, and instrumental optimization are critical steps in the determination of mycotoxins. The mass spectrometer, when used as a detector of the chromatographic method, provides high sensitivity, versatility and selectivity compared to the other classic detectors, which give it an advantage for trace analysis of compound in samples. The development of mass spectrometric technologies for single or multi-analyte detections has been frequently attempted; the details are discussed in the following chapters. However, there are still challenges as it is difficult to carry out simultaneous purification for multi-analyte (mycotoxins) determination in which the target analytes and samples have very different properties. Failure to minimize the matrix effects of food samples during the preparation steps have resulted in low peak intensity (ion enhancement or ion suppression) and poor precision, which affect the accuracy in mycotoxin determination. Thus, it is necessary to develop a selective method by making

use of the advantage of mass spectrometry (especially for identification of isomers). Moreover, a more efficient sample preparation which is capable of handling food matrices with good recovery of diverse mycotoxins is needed.

This study aims to develop and validate an effective and efficient two phase-U-shaped HF-LPME method which is supported by extraction/dispersion solvent prior to liquid chromatography electrospray ionization triple quadrupole tandem mass spectrometry (LC-ESI-QQQ-MS/MS) for qualitative and quantitative analyses of aflatoxins and ochratoxin A in various food matrices. The optimization strategy was performed using the combined strategy of univariate and multivariate approaches. The objectives are illustrated below:

1) To develop and optimize the analytical performance of LC-ESI-QQQ-MS/MS method for the determination of multi-mycotoxins (AFB1, B2, G1, G2, and OTA).
2) To develop, optimize, and validate a U-Shaped HF-LPME method for the preconcentration of liquid and solid food samples in the determination of mycotoxins by LC-ESI-QQQ-MS/MS

CHAPTER 2: LITERATURE REVIEW

2.1 Efficiency of Hollow Fiber Liquid-Phase Microextraction Coupled to Chromatographic Instrument in Analysis.

Liquid-liquid extraction (LLE) is a popular technique for the separation of different analytes from various sample matrices. It works on the principle of partitioning between two immiscible solvents, namely the sample solution containing the analytes and the organic extraction solvent. The analytes will migrate from the sample solution into the extractive solvent (which has different properties such as polarity, and density) until equilibrium is achieved. This step is performed several times with fresh solvent to ensure exhaustive extraction of analytes. The drawbacks of LLE coupled with chromatography includes multiple-steps in handling, time-consuming, and requires large volumes of organic solvent (Kenkel, 2010). Although full automation of LLE is still a challenge, the automation of most steps is possible (Li et al., 2013). The presence of co-extractives such as macromolecules (protein, particles) in the extracts may have adverse effects on the chromatographic systems, for instance, the column lifetime, causing system blockage and damage to the hardware such as the flow lines, rotary injection valves, and inlet fits. In addition to the physical damages, the sample matrix can significantly affect the quantitative results in trace analysis. The use of mass spectrometry and chromatography can reduce of the matrix effect from the co-extractives as the mass detector is toward the target analytes from complex matrices, hence increasing the accuracy in identification and quantification performance (Majors, 2013).

2.1 Type of Hollow Fiber Liquid-Phase Microextraction (HF-LPME)

LPME techniques such as HF-LPME that miniaturize the LLE scale by reducing the extraction solvent volume in relation to the sample volume, have eliminated some of the drawbacks associated with the conventional LLE method (Tan & Abdulra'uf, 2012). The HF-LPME method was introduced by Pedersen-Bjergaard and Rasmussen based on the

use of a cost-effective and disposable hollow fiber material to overcome the drawbacks associated with SDME (Pedersen-Bjergaard & Rasmussen, 1999). Many studies have been carried out to study the effect of membrane-module type used in the HF-LPME approach such as flat or hollow fibers (Jönsson & Mathiasson, 1999) .The cone-shaped modules (Sanagi et al., 2007) showed that one of their main drawbacks is that they consumed a large volume of organic solvent in the acceptor phase . Despite its advantages, HF-LPME is still facing limited availability in the commercial system and the difficulty of full automation for a high-throughput configuration. There are many review articles on HF-LPME or the modified LPME method available, which describe the theory, classification, advantages, disadvantages, and their applications for extraction and enrichment (Lee et al., 2008;Abulhassani et al., 2010; Pena-Pereira et al., 2010; Mahugo-Santana et al., 2011;Bello-López et al., 2012; Ghambarian et al., 2012; Zhang et al., 2012; Zuloaga et al., 2012;Cabaleiro et al., 2013; He, 2014; Yan et al., 2014; Carasek & Merib, 2015).

The HF-LPME is used to display higher extraction efficiency when compared to other LPME techniques, which can be attributed to the enhancement of the mass transfer process under vigorous stirring during extraction. The technique can be categorized into two modes: a two-phase mode and a three-phase mode (Pedersen-Bjergaard & Rasmussen, 1999). In the two-phase HF-LPME (HF(2)LPME), the target analytes were isolated from the aqueous solution (donor phase) and dispersed into the organic solvent impregnated in the pore wall (supported liquid membrane (SLM)) of the hollow fiber. The analytes are subsequently transferred into the organic solvent (acceptor phase) inside the hollow fiber lumen. In this particular approach, the key parameter is the partition coefficient between both phases. It is important to adjust the donor phase pH to maintain the target analytes of their insoluble state (Ho et al., 2003). The extraction efficiency in the static mode is controlled by sample agitation through minimizing the diffusion

distance between the analytes and acceptor phase. Extraction in the dynamic mode is executed by using a micro-syringe to drown out the acceptor phase from the hollow fiber lumen. The later mode has been shown to decrease the extraction time, and improve reproducibility and efficiency when compared to the former mode (Ho et al., 2003). In the analysis of polycyclic aromatic hydrocarbons (PAH) by GC-MS, the dynamic mode offered double enrichment with better reproducibility (~75-fold in 10 min and even better reproducibility ~3%) while the static mode showed ~35-fold enrichment in 10 min and reproducibility of ~4% (Zhao & Lee, 2002).

In direct-immersion HF(2) LPME, the target analytes in the aqueous sample are extracted into an organic solvent which is immiscible in an aqueous sample. The analyte is then restrained in the hollow fiber pores and subsequently moved into the lumen of the hollow fiber. The hollow fiber is submerged in a vial containing the stir bar and aqueous sample. Most two-phase applications are carried out by this technique (Pedersen-Bjergaard & Rasmussen, 1999). The headspace technique was developed by Jiang *et al.* (Jiang et al., 2005) who used the HF-HS-LPME-GC-MS method for the determination of PAHs in soil samples. Such technique is efficient for volatile compounds. The hollow fiber membrane is placed vertically in the headspace area of the specimen solution and a micro-syringe needle is then used to draw the extraction solvent from the hollow fiber. This reduced the extraction time when compared to other HF-LPME approaches (Jiang et al., 2005).

In the three-phase HF-LPME (HF(3)LPME), the analytes are extracted from the aqueous solution (1st phase) into a thin organic film (2nd phase) and restrained in the hollow fiber pores in their deionized forms. The analytes are subsequently converted to their ionized forms and moved to the organic solvent (3rd phase; a different organic solvent from the 2nd phase) residing in the hollow fiber lumen. Single-cycle extraction

gives good reproducibility where the minute pore size of the hollow fiber membrane restricts the uptake of undesired components (large particles and molecules. In addition, the immobilized solvent in the hollow fiber pores (2nd phase) also served as a barrier between the two phases (donor and acceptor phases) (Ho et al., 2002).

HF(3)LPME can be further broken down into two categories based on the properties of the acceptor phase. The first category uses an aqueous solution as the acceptor phase, i.e. 1st phase aqueous solution (donor phase); 2nd phase organic solvent (SLM); 3rd phase aqueous solution (acceptor phase). In this technique, the pH of all phases plays important roles in the extraction, especially for the extraction of acidic or alkaline analytes which possess ionizable functionality. The pH of the acceptor and donor phases are adjusted accordingly to the acidic or basic nature of the analytes (Pedersen-Bjergaard & Rasmussen, 1999). This technique is compatible with most liquid chromatographic instruments (Ghambarian et al., 2012).

The second category uses an organic solvent as the acceptor phase instead of an aqueous solution in the hollow fiber lumen, i.e. 1st phase aqueous solution (donor phase); 2nd phase organic solvent (SLM); 3rd phase organic solvent (acceptor phase). Based on the study by Ghambarian *et al.* , the target analyte was separated from the aqueous solution (donor phase) into the first organic solvent (SLM) and then moved into the acceptor phase (second organic solvent; acceptor phase) which is confined in the lumen of the hollow fiber. Methanol or acetonitrile is utilized as the acceptor phase, and n-dodecane is restrained in the hollow fiber pores as the SLM. The movement of analytes from the SLM to the acceptor phase is dependent on the concentration gradient between the two immiscible organic solvents. In this method, the hydrophilic and ionizable analytes can be extracted based on the active transport and pH gradient (Ghambarian et

al., 2010) . The extraction technique can be coupled with various chromatographic systems (Ghambarian et al., 2012).

Hollow fiber liquid-gas-liquid microextraction (HF-LGLME) is an organic solvent-free microextraction approach whereby only a few microliters (μL) of the aqueous solution is used to fill the polypropylene hollow fiber lumen. The aqueous acceptor phase in the channel of the HF was separated from the sample solution by the hydrophobic microporous HF wall with air inside its pores. Through gas diffusion, the analyte travels through the hollow fiber pores into the acceptor solution from the sample solution (donor phase) (Zhang et al., 2006).

2.1.1 The Principle of HF-LPME

In the HF(2)LPME technique, extraction of target analytes from the sample is determined by its concentration differences between the aqueous donor phase and the organic acceptor phase (inside the lumen of the HF membrane) as shown in Figure 2.1. When the target analyte is present at high concentration in the donor phase, it will not migrate to the acceptor phase until equilibrium is achieved. This process may be described by the following equation:

$$A\ (Donor\ phase) \leftrightarrow\ A\ (Acceptor\ phase) \quad \text{.....(2.1)}$$

where A is the target analyte. The distribution ratio of the target compound is defined by the following equation :

$$K\ o/s = Cs/\ Caq\ , s \text{.....(2.2)}$$

where Cs and Caq,s are the concentrations of the target analyte (A) at the equilibrium, in the donor phase and the acceptor phase. The initial amount of analyte (n_i) is equal to

the sum of the individual amount of target analyte present in the acceptor phase (n_o) and the donor phase (n_s) in the extraction process (Ouyang & Pawliszyn, 2006):

$$n_i = n_s + n_o \quad \text{.....(2.3)}$$

the extraction kinetic is obtained by the compound concentration variation in the organic acceptor phase to the extraction time $C_a(t)$ and can be expressed as:

$$C_a(t) = C_{eq,a}\,(1 - e^{-kt}) \quad \text{.....(2.4)}$$

where k is the rate constant and $C_{eq,a}$ is the target analyte concentration at equilibrium in the organic acceptor phase(Abdulra'uf 2013) . At equilibrium, Eq. (2.3) can be expressed as a function of the concentration of analyte and volume of the organic solvents acceptor phase and donor phase as in equation (2.5)

$$C_i V_s = C_{eq,s} V_s + C_{eq,o} V_o \quad \text{.....(2.5)}$$

C_i is the initial concentration of target analyte in the donor phase, while $C_{eq,s}$ and V_s are the concentration of target analyte in the donor phase in equilibrium and volume of the donor phase. $C_{eq,o}$ and V_o are the concentration of analyte in the organic solvent in equilibrium and the volume of organic solvent (in the pores of the porous wall and lumen of the HF).

The quantity of extracted target analyte can be expressed as (Pedersen-Bjergaard & Rasmussen, 2008):

$$n_{aq,o} = \frac{K_{o/s} V_O C_i V_s}{K_{o/s} V_o + V_s} \quad \text{.....(2.6)}$$

The recovery (RE) and enrichment factor (EF) for target analyte are expressed in equations (2.7) and (2.8) respectively (Pedersen-Bjergaard & Rasmussen, 2008; Gjelstad & Pedersen-Bjergaard, 2013):

$$RE = \frac{K_{o/s}V_O}{K_{o/s}V_o+V_s} \times 100\% \quad \text{..... (2.7)}$$

$$EF = \frac{C_o}{C_i} = \frac{V_s R}{100V_o} \text{.....(2.8)}$$

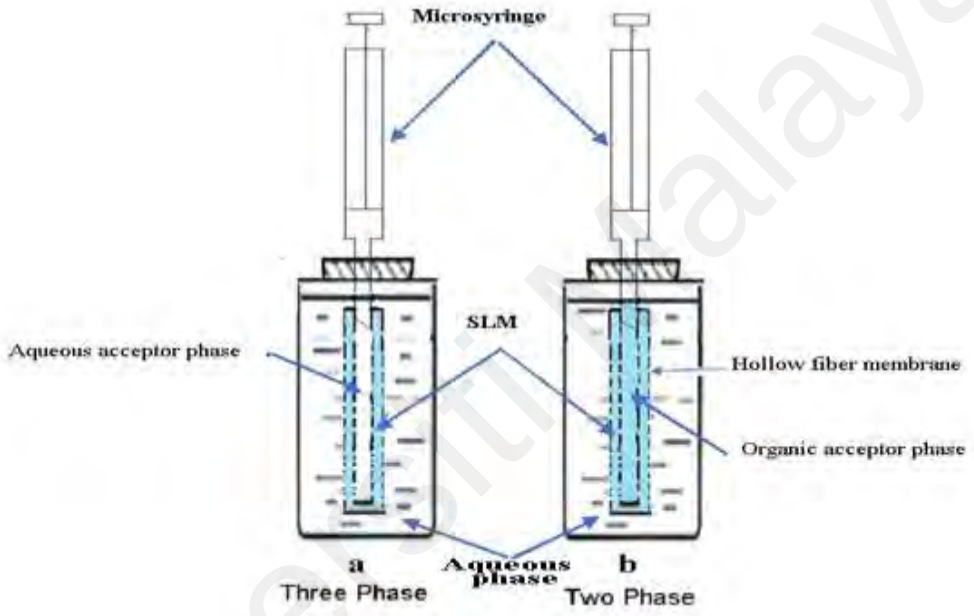

Figure 2.1:(a) Three Phase-Hollow Fiber-Liquid Phase Microextraction and (b) Two-Phase - Hollow Fiber-Liquid Phase Microextraction.

In HF(3)LPME, the target analyte moves from the donor phase to the acceptor phase through the SLM filed into the HF pores as shown in Figure 2.1. This process can be illustrated by equation (2.9) (Pedersen-Bjergaard & Rasmussen, 2008; Ghambarian et al., 2012).

$$A_{donor} \leftrightarrow A_{SLM} \leftrightarrow A_{acceptor} \text{.....(2.9)}$$

At equilibrium, the recovery is related to distribution ratios of the target analyte in SLM/the donor phase and acceptor phase/SLM (Pedersen-Bjergaard & Rasmussen, 2008; Ghambarian et al., 2012).

$$K_{o/s} = \frac{C_{eq,o}}{C_{eq,s}} \text{ .....(2.10)}$$

$$K_{a/o} = \frac{C_{eq,a}}{C_{eq,o}} \text{ .....(2.11)}$$

$K_{o/s}$ and $K_{a/o}$ are the distribution ratios of SLM (organic solvent)/donor phase (sample) and acceptor phase/SLM (organic phase), where $C_{eq,a}$, $C_{eq,o}$ and $C_{eq,a}$ are the concentrations of the target analyte in the acceptor phase, SLM, and donor phase, respectively. The partition constant between the acceptor phase and the donor phase can also be expressed as:

$$K_{a/s} = \frac{C_{aq,a}}{C_{aq,s}} = \frac{K_{o/s}}{K_{a/o}} \text{.....(2.12)}$$

The initial amount of analyte is equal to the sum of the amount of analyte found in all the three phases during the separation process (Ho et al., 2002) and expressed as

$$n_i = n_s + n_o + n_a \text{ .....(2.13)}$$

where n_o , n_s , and n_a are the amount of target analyte present at the initial donor phase, SLM and in the acceptor phase, respectively. At equilibrium equation can be expressed as below:

$$C_i V_s = C_{eq,s} V_s + C_{eq,o} V_o + C_{eq,a} V_a \text{.....(2.14)}$$

where $C_{eq,a}$ is the concentrations of the analyte in the acceptor phase and V_a is the acceptor phase volume at equilibrium. The quantity of target analyte extracted into the acceptor phase can then be expressed as (Ho et al., 2002):

$$n_{aq,a} = \frac{K_{a/s}V_O C_i V_S}{K_{a/s}V_a + K_{o/s}V_o + V_S} \quad \text{.....(2.15)}$$

The recovery (RE) and the enrichment factor (EF) of the analyte are, therefore:

$$RE = \frac{K_{a/s}V_a}{K_{a/s}V_a + K_{o/s}V_o + V_S} \times 100\% \quad \text{.....} \quad (2.16)$$

$$EF = \frac{C_a}{C_i} = \frac{V_S R}{100 V_a} \text{.....(2.17)}$$

Equation 2.17 has revealed that the analyte recovery by HF(3)LPME is dependent on both the distribution constants between SLM/donor phase and acceptor phase/SLM, and the volumes of the donor phase, SLM, and the acceptor phases (Pedersen-Bjergaard & Rasmussen, 2008).

2.1.2 Modified HF-LPME

Modified HF-LPME methods have been introduced to increase the efficiency, which aims at processing time reduction, increasing enrichment factor and recovery percentage, lowering limit of detection (LOD), and increasing extraction throughput (Gjelstad et al., 2012). Some of the modified HF-LPME approaches are discussed in brief as follows.

2.1.2.1 Ionic Liquid-Based Microextraction

Ionic liquid-based microextraction that uses an ionic liquid in place of the organic solvent provides many advantages, including low cost, simplicity of operation, precision and accuracy (Zhang et al., 2013). Because of the excellent properties of ILs, Peng and co-workers (Peng et al., 2007) used IL [C8MIM][PF6] as a membrane solvent for HF-LPME, which proved to be an effective method. Hollow fiber supported IL membrane microextraction (HF-ILMME) has since been applied in the fields of environmental analysis and drug/ pharmaceutical analysis, with applications such as lead and nickel (Abulhassani et al., 2010), sulphonamides (Tao et al., 2009), and BTEX components(Ma et al., 2011).

2.1.2.2 Hollow Fiber Solvent Bar Microextraction(SBME)

In the SBME approach, the extraction solvent (acceptor phase) fills the hollow fiber with both ends capped. This technique allows the hollow fiber solvent bar to move freely in the aqueous sample solution (donor phase) which increases the transfer of analytes to the extraction solvent. This method has been successfully applied in the analysis of pentachlorobenzene (PCB) and hexachlorobenzene (HCB) in a soil slurry sample (Jiang & Lee, 2004), non-steriodal anti-inflammatory drugs (NSAIDs) (Saleh et al., 2011), ephredrine in blood samples (Fotouhi et al., 2011), β-agonists and β-blockers in urine (Liu et al., 2009), and trihalomethanes in drinking water (Correa et al., 2015).

2.1.2.3 Electro Membrane Extraction (EME)

The electro membrane extraction (EME) method (based on electrokinetic migration) reduces the time required in the movement of analytes from the donor phase to the SLM, from the typical 20-60 min to approximately 5 min (Gjelstad et al., 2006). This approach can be used for the extraction of low-polar acidic or alkaline drugs. During extraction, the negative electrode is inserted into the acceptor solvent, while the positive conductor is inserted into the aqueous donor solution, and 300 V is applied across the SLM in the course of the extraction which produces higher analyte recapture (Gjelstad et al., 2006). Table 2.1 shows the different EME techniques that have been developed and coupled with various detectors such as CE, ESI-MS, GC, GC-MS, UPLC, and LC-MS.

2.1.2.4 Carrier-Mediated HF-LPME

The HF-LPME operates on the principle of passive diffusion to separate the target analytes from the donor phase to the acceptor phase through SLM. A carrier-mediated mode has been developed to enhance the diffusion process. The carrier can either be added to the donor phase (Kramer & Andrews, 2001) or combined with the solvent and impregnated in the pores of the hollow fiber (SLM) (Fontas et al., 2005). This mode uses

a hydrophobic ion-pair reagent which is water soluble. The ion-pairs form complexes with the target analytes in the donor phase, which are then extracted into the organic phase (SLM) and subsequently released into the acceptor solution. The excess counter-ions in the acceptor solution pair with the carriers and undergo back-extraction to be reused in the donor phase and the entire process is repeated. For basic drugs example, a high efficiency is achieved by adjusting pH into the alkaline region in the sample (typically pH 13 to deionize the analytes) and by acidifying the acceptor phase (typically pH 2 to ionize the analytes) (Rasmussen & Pedersen-Bjergaard, 2004; Pedersen-Bjergaard et al., 2005).

2.1.2.5 Graphene HF-LPME(G-HF-LPME)

The HF-LPME efficiency can also be improved by adding graphene. Graphene has good thermal, mechanical, and electronic properties besides being an excellent two-dimensional nanomaterial. The addition of graphene in the acceptor phase inside the hollow fiber lumen resulted in the increase of adsorption of analytes possessing benzene moiety, which can be attributed to the formation of a strong π-stacking interaction with the benzene ring of the analytes with the delocalized π-electron system of graphene (Wang et al., 2012). The graphene-reinforced HP-LPME (G-HF-LPME) has been used in the extraction of pollutants in food, such as carbamate pesticides in fruit samples (Ma et al., 2014a), phenylurea residues in milk samples (Sun et al., 2014), bisphenol A and 4-tert-butylphenol in bottled juice (Ma et al., 2014b), and chlorophenols in honey (Sun et al., 2014).

Table 2.1: The different techniques of EME.

Technique	Sample	Analyte	SLM	Voltage (V)	Time (min)	Detector	Refences
EME	water samples	Alkaline and acidic drugs	NPOE+DEHP in NPOE	300	5	CE	Gjelstad et al., 2006
low-voltage EME	human plasma, urine & breast milk	Alkaline drugs	1-isopropyl-4-nitrobenzene	9-10	5	CE	Kjelsen et al., 2008
Drop-to-drop EME	urine, plasma, & water	basic drugs	NPOE	15	5	CE	Petersen et al., 2009
On-chip EME	Human urine	Basic analyte	NPOE	15	10	ESI-MS	Petersen et al., 2010
EME-LDS-USAEME	water samples	Chlorophenols	1-octanol	50	10	GC-MS	Guo & Lee, 2012a
Single-well EME	human plasma	quetiapine, citalopram, amitriptyline, methadone and sertraline	NPOE	300	30	LC-MS	Huang et al., 2014
Pulsed EME	Human urine & plasma,	Nalt , Nalm	NPPE + 10% DEHP	100-350	2-20	HPLC	Rezazadeh et al., 2012.
Silver nanometallic-decorated hollow fibers-EME		(NSAIDs)	1-octanol	10	7-10	HPLC	Ramos-Payán et al., 2014
Parallel EME	water samples, human plasma,	Amitriptyline, fluoxetine, and haloperidol	NPOE	20	10	UPLC	Eibak et al., 2014
EME-probe	Drug metabolite	promethazine, amitriptyline&imipramine	NPOE	2.5	30	MS	Dugstad et al., 2014
Carbon-nanotube-assisted EME	plasma &urine samples	Tramadol and methadone	carbon nanotubes	50	20	GC	Hasheminasab et al., 2014
Carrier assisted electro membrane	urine samples	basic drugs	Nonionic surfactants	200	30	CZE-UV	Aladaghlo et al., 2017

Table 2.1, (cont'd)

Technique	Sample	Analyte	SLM	Voltage (V)	Time (min)	Detector	Refences
EME-SFFTCCV	whole blood	diclofenac	1-octanol	20 V	28	FFT	Mofidi et al., 2017
EME-FFTSWV	whole blood	Estradiol valerate	1-octanol	7 V	20	FFT	Mofidi et al., 2018
complexation-mediated EME	human urine sample	highly polar basic drugs	NPOE	50 V	15	(HPLC-UV	Fernández et al., 2017

CE: capillary electrophoresis, DEHP: Di-(2-ethylhexyl) phosphate, EME-FFTSWV: Electro membrane extraction combined with Fast Fourier transform square wave voltammetry, EME-SFFTCCV: Electro membrane extraction combined with FFT voltammetry technique, ESI-MS: electrospray ionization, GC: gas chromatography, GC-MS: gas chromatography mass spectrometry, HPLC: high performance liquid chromatography, LC-MS/MS: liquid chromatography tandem mass spectrometry, MS: mass spectrometry, NPOE: 2-Nitrophenyl octyl ether, NPPE: 2-Nitrophenyl pentyl ether, UPLC: ultra-performance liquid chromatography, NSAIDs: Non-steroidal anti-inflammatory drugs.

2.1.2.6 Ultrasound-Assisted Hollow Fiber Liquid Phase Microextraction (UA-HF-LPME),

In ultrasound-assisted hollow fiber liquid phase microextraction (UA-HF-LPME), combining HF-LPME with an ultrasound (US) source significantly shortens the extraction time and increases extraction performance with good repeatability (Szreniawa-Sztajnert et al., 2013). The UA-HF(2)LPME coupled to GC-Nitrogen-Phosphorous detector has been used in the extraction of metabolites and organophosphorus pesticides in wheat flour and baby food (González-Curbelo et al., 2013). An on-line UA-HF(3)LPME-HPLC was introduced by Chao *et al.* (2013) as a rapid method and has been successfully applied in the determination of chlorophenols in water samples and neutral analytes in plastic-bottled beverages. This method yielded good results with short extraction time, good accuracy and precision, and it is inexpensive. The method also showed a lower LOD due to the effective cleanup of the hollow fiber, efficient convection, and an increase of driving force for mass transfer of the target analytes (Chao et al., 2013).

2.1.2.7 Vortex-Assisted HF-LPME (VA-HF-LPME)

A vortex-assisted HF-LPME (VA-HF-LPME) was developed to boost the speed of extraction by enhances the rate of mass transfer to acceleration high recovery rate during the extraction processes (Wang et al., 2015). The VA-HF-LPME approach has been used in the determination of three synthetic endocrine-disrupting compounds in milk (Li et al., 2014) and estrogens in milk samples (Wang et al., 2015).

2.1.2.8 Surfactant-Enhanced HF-LPME

Yazdi *et al* (2014). developed a surfactant-enhanced HF-LPME coupled to HPLC for the determination of melamine in soil samples. The sodium dodecyl sulfate (SDS) was used to create an extractable ion-pair with the aqueous protonated melamine in acidic

solution. Melamine was first transformed into a protonated species and then paired with SDS to increase its efficiency (Yazdi et al., 2014).

2.1.3 HF-LPME Designs and Arrangements

There are four reported technical arrangements for the HF-LPME technique to date, i.e. rod-like arrangement, U-shape design, hollow fiber solvent bar, and knotted hollow fiber. The rod-like arrangement encompasses a direct fiber connection to a micro-syringe, which is the first introduced HF-LPME technical set-up. The syringe in the rod-like configuration function not only acts as a holder, but also to add and withdraw the acceptor phase, and directly injected it into the analytical system(Pereira, 2014). Figure 2.2 shows some HF-LPME technical arrangements

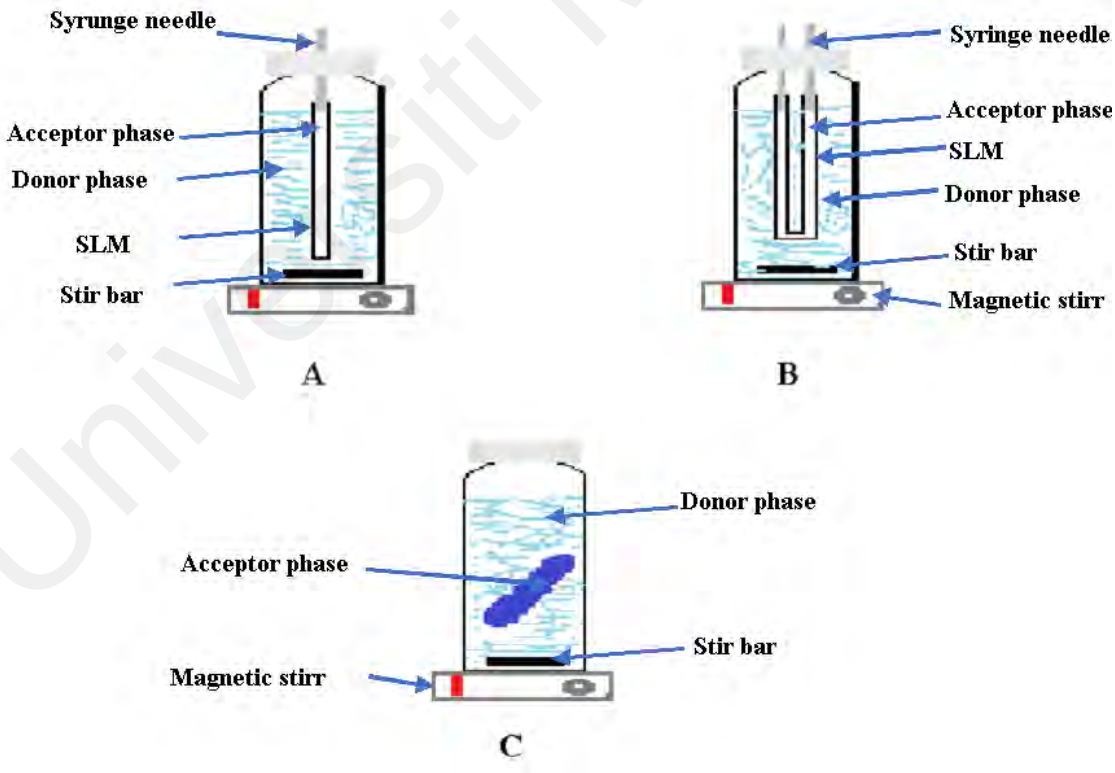

Figure 2.2: HF-LPME technical arrangements (A) Rod-Like configuration; (B) U-Shaped configuration;(C) Solvent bar configuration.

The U-shaped design has a large interaction area between the hollow fiber and the sample solution. The two ends of the U-shape are connected to the conserve needles. After extraction, one end of the U-shape is disconnected so that air is blown in to flow the extract into a vial (Pedersen-Bjergaard & Rasmussen, 1999). Modifications to the U-shaped device have been carried out to enhance extraction kinetics and reduce acceptor phase loss. For instance, a polypropylene hollow fiber piece was coiled around a paper clip to increase contact area and extraction kinetics (Pedersen-Bjergaard, et al., 2001), while Müller *et al*. developed an exit at the installation guide to reducing filling problems (Müller et al., 2003). Hollow fiber solvent bar microextraction is the third arrangement of HF-LPME (Jiang & Lee, 2004). The hollow fiber solvent bar microextraction technique prior to chromatographic analysis has been applied for the analysis of compounds in various samples as shown in Table 2.6.

The last arrangement is a knotted hollow fiber microextraction. Chen *et al.* introduced a headspace knotted hollow fiber microextraction prior to GC-MS to determine volatile organic compounds in water. The advantage of this method is good extraction efficiency due to larger extraction contact interface which resulted in an increase of the mass transfer of the target analytes (Chen et al., 2015).

2.1.4 The Attempts of HF-LPME Automations

HF-LPME is one of several microextraction methods having high extraction efficiency with low consumption of organic solvents (Zhao & Lee, 2002). However, inappropriate handling of the micro-syringe can lead to analyte losses which deny the advantages of the HF-LPME method. When used in a rod-like arrangement where one end of the hollow fiber was sealed, the air-gap within the lumen may cause difficulty in the process of filling and withdrawal of the acceptor phase. The small volume of the acceptor phase (maximum 3 μl) injected into the lumen can be easily lost through diffusion. In many occasions, the

sealed end of the hollow fiber may break and the acceptor phase could be completely lost (Yong et al., 2014). Despite attempts at the automation of HF-LPME, the development still has a long way to go. Although lab-built semi-automated HF-LMPE (non-commercial instruments) has been reported, a fully automated commercial system is still not available. Zhao and Lee (2002) reported the use of a syringe pump to repeatedly withdraw and discharge the acceptor phase from the hollow fiber. It can be regarded as a semi-automated HF-LPME. This semi-automated dynamic procedure was coupled to GC-MS for the analysis of PAHs compounds in an aqueous sample. (Zhao & Lee, 2002)

A CTC CombiPal autosampler has been employed to perform solvent filling, transfer and agitation of sample solution and solvent is included in the introduction of the extraction phase into the GC injector (Ouyang & Pawliszyn, 2006; Ouyang et al., 2007 ;Cui et al., 2009). Pezo *et al*. (2007) used a multichannel syringe pump which can process up to six samples simultaneously (Pezo et al., 2007). The dynamic experimental set-up for extraction with a high automatization degree to process up to six samples simultaneously by multiple channel syringe pump has played an important role in the achievement of better reliability, rapidness, and reproducibility compared to manual assays. The system has been applied for the first time for the determination of simulants of migrants in aqueous food from prototypes of active packaging to assess their safety before marketing (Pezo et al., 2007), and for specific migration analysis of new active food packaging material containing essential oils (migration of 43 compounds including terpenes, alkanes, plastic additives and degradation compounds) (Salafranca et al., 2009).

Esrafili et al., (2011 & 2012) reported on the use of TT-tube extractor for their semi-automated HF-LPME techniques for the quantitative determination of five chlorobenzenes in water samples (Esrafili et al., 2012), and chlorophenoxy acid herbicides in environmental samples (Esrafili et al., 2011). The device consists of a

polypropylene hollow fiber mounted inside a stainless steel tube. Toluene was the extraction solvent that filled the lumen and pores of the hydrophobic fiber when the flow was controlled by a programmable syringe pump. Esrafili group also developed an online HF-LPME when the system consists an electronic board with a microcontroller for storage of data and instrument programs, a sampling loop for online injection of the extract to the HPLC, a platform lift for moving the sample vial, and an automated syringe pump for loading the SLM and acceptor solvents. The system not only allowed an increase in the extraction efficiency but also decreased the amount of solvent used and the run time (Esrafili et al., 2012).

In 2015, Tajik *et al*. (2015) developed a fully automated HF-LPME-HPLC method (based on two immiscible organic solvents) which provides acceptable sensitivity and precision and low relative standard deviation values (RSD) in the determination two hormonal drugs in water and urine. The good pre-concentration factors obtained in this development demonstrated the capability of HF-LPME to serve as a clean-up procedure and low-cost sample pretreatment (Tajik et al., 2015).

2.1.5 Influencing Factors in HF-LPME

Many factors have to be considered in the optimization process in order to achieve efficient extraction. The selection of a suitable organic solvent is a critical step that depends on a number of factors, i.e. the affinity of the solvent toward the target analytes, miscibility (water miscible or immiscible), volatility (volatile, semi-volatile, or non-volatile), and stability during the extraction time, as well as compatibility with the chosen analytical instruments (detector) (Han & Row, 2012). In a similar contest, the volumes of the donor and acceptor phases affect the extraction efficiency. In general, the efficiency of the extraction and enrichment factor increases when the ratio is increased, but the enrichment factor deteriorates after achieving the maximum limit. Therefore, an optimum

volume should be maintained to obtain the required selectivity (Psillakis & Kalogerakis 2003 ; Han & Row, 2012).

LPME's efficiency(recovery) depends significantly on the extraction time in which the highest extraction efficiency is achieved at the equilibrium condition. In HF-LPME, the equilibrium time is usually 30–60 min without taking the organic solvent into account (Psillakis & Kalogerakis 2003). Although longer extraction times generally lead to higher recovery, the extension of extraction times is not always practical. In this regard, some approaches to accelerate the process via carrier-mediated extraction or use of electrokinetic migration (Han & Row, 2012).

pH influences the solubility, dissociation, and equilibrium of the basic or acidic analytes. In HF(2)LPME, high analyte response can be achieved by adjusting the pH of the donor solution. pH matching between the acceptor phase and donor phase in the HF(3)LMPE is a vital step that determines the recovery of target analytes, the enrichment factors, and distribution ratios. It is crucial to manipulate the pH of the donor solution. So as to adjust the solubility of target analytes towards desired transfer gradient. On the other hand, the pH of the acceptor solution needs to be controlled to retain the target analytes in these ionized form which avoids the trapping of these analytes inside the organic phase, and this facilitates the recoveries (Psillakis & Kalogerakis 2003).

Agitation can accelerate the extraction kinetics and increases extraction efficiency. Agitation causes continuous replacement of the exposed acceptor phase against the donor phase, therefore, the migration kinetics of the target analytes from the donor phase via the SLM to the acceptor phase is prorated. Agitation can be achieved by sample stirring, vibration, or sonication (Psillakis & Kalogerakis 2003).

The effect of salt addition on extraction efficiency may vary, depending on the properties of the target analytes. A specific concentration of salt can be added to facilitate the partition between the phases included in the HF-LPME. However, the high concentration of salt in the sample solution (donor phase) may alter the physical properties of the extraction film (SLM). This condition could reversed the diffusion of the analyte into the organic phase (Nováková & Vlčková, 2009).

Membrane type plays a significant role in the extraction process. The membrane should be compatible with the organic solvent (acceptor phase). Most commonly, extraction methods are carried out using polypropylene fiber membrane(Kokosa et al., 2009). Some researchers have also applied other membranes such as polyvinylidene difluoride which are not commonly used. According to the literature, polyvinylidene difluoride membranes have advantages such as faster extraction and operational accuracy (Wang et al., 2014;Huang et al., 2015).

In general, extraction by membrane-based techniques is performed at a temperature below 40 °C, where most reported cases were performed at room (ambient) temperature (Li et al., 2014;Akhavan et al., 2015). This is because the operation beyond this temperature may lead to undesirable consequences such as degradation of the SLM (Marothu et al., 2013). Previous reports have shown that no noticeable increase was recorded in extraction efficiency (recovery) by increasing the temperature range from 5 to 40 °C, even though the diffusion coefficient and flux were enhanced (Chimuka et al., 2009; Michel et al., 2009).It should be mentioned that elevated temperatures may have an adverse effect on the extraction efficiency (recovery) due to the organic solvent evaporation, increasing the solubility of organic solvent in the aqueous phase and formation of air bubbles adhering to the hollow fiber (Sharifi et al., 2016).

According to the study by Huang et al. (2015), extraction temperature above 40 ºC increased the diffusion coefficient of the analyte and improved the mass transfer rate. Nevertheless, too high a temperature led to raising the mass transfer parameters such as viscosity, diffusion coefficient and flux but decreased the extraction efficiency (recovery). The extraction efficiency increased sharply from 40 to 50 °C, then slightly decreased at higher temperatures (Huang et al., 2015). In another report by Chen et al., (2015), application of high temperature for the headspace method increased the extraction efficiency (recovery). Temperature ranging from 25 to 95 °C were tested for the determination of VOCs in water samples. It was found that all analytes have the highest extraction efficiency when the temperature was 95 °C. This was attributed to an increase in Henry's constant and the diffusion coefficients of the analytes in the headspace at a higher temperature (Chen et al., 2015).

2.1.6 HF-LPME and Derivatization

Some of the HF-LPME applications were prior to gas chromatography systems. Although the gas chromatography (GC) was used for the analysis, some organic compounds containing carboxyl, hydroxyl, and amino functional groups are difficult to detect due to their physicochemical properties, such as low volatility, high polarity, and high solubility in water. Furthermore, some compounds are volatile enough but tend to be adsorbed and decomposed on the columns, and readily give tailed peaks, ghosting phenomena, and low detector sensitivity. Furthermore, to obtain optimal analysis conditions, the derivatization procedures combined with HF-LPME is usually essential and the formation of derivatives which are easier to extract than the original analytes (Majors, 2013). Hence, HF-LPME techniques have attracted much attention as alternatives to classical extraction and sample-preparation procedures. (Farajzadeh et al., 2014). In this context, some efforts have been made to combine extraction procedures together and can synergistically derive benefits from individual microextractions (Sajid

& Płotka-Wasylka, 2018). This derivatization process can be accomplished in different ways:

(a) HF-LPME Coupled with Injection Port Derivatization

In this method Basheer & Lee (2004) connected the HF-LPME with injection port derivatization for the determination of endocrine disrupting alkylphenols. This method provided very high enrichment factors for the analysis of polar and semi-volatile analytes by using GC (Basheer & Lee, 2004).

(b) Pre-HF-LPME Derivatization

Derivatization can be performed before HF-LPME to enhance the analytes extractability. In this context, Ganjikhah et al, (2017) determined a trace amount of formaldehyde using HF-LPME based on derivatization by Hantzsch reaction. The derivatization reaction was supported by ultrasonic energy for 30 min at 70°C. This work revealed very good results (Ganjikhah et al., 2017). The same mode of derivatization was used to determine cocaine and its derivatives in hair samples (Pego et al., 2017)

(c) Pre-HF-LPME Derivatization

In this process, the target analytes were extracted by HF-LPME and followed by derivatization step before being injected in the instrument. It was applied in the detection of Phthalates and bisphenol A in urine samples. This method showed linearity, precision, limits of detection, and quantification suitable to analyze these compounds at low concentration levels in urine(Fernandez et al., 2017).

2.1.7 Application of HF-LPME

HF-LPME in its two- and three-phase modes (different designs and arrangements) have been utilized for the extraction of a variety of compounds (pharmaceuticals and organic compounds), and the following sections concentrate on the different applications

of HF-LPME techniques. However, their applications have not extended sufficiently in the determination of mycotoxins in food samples. Only four studies have been reported up to date; for the determination of ochratoxin A in wine (González-Peñas et al., 2004) and ochratoxin A and T-2 toxin in wine and beer (Romero-González et al., 2010), as well as for the detection of aflatoxin M1 in (Huang et al., 2015) and multiple aflatoxins in edible oil (Huang et al., 2017).The following sections will illustrate the appropriateness of the application of HF-LPME for the extraction of various analytes. Most of these studies demonstrated the efficiency of HF-LPME techniques in extracting various types of compounds from a variety of sample matrices (e.g. air, water, soil, food, and biological samples).

2.1.7.1 Applications of Two-Phase HF-LPME

Since the selected acceptor solvent is usually an organic solvent in two-phase HF-LPME, its applications have been extensively used on environmental applications compared to pharmaceutical and drug analyses(Ghambarian et al., 2012).The HF(2)LPME approach is used for the extraction of organochlorine, organophosphorus, organosulfate pesticides, polychlorinated biphenyls, triazine herbicides, aromatic amines and BTEX (benzene, toluene, ethylbenzene, and xylene(, as well as mycotoxins and many other contaminants (Table 2.2).Hence, HF-LPME techniques have attracted much attention as alternatives to classical extraction and sample-preparation procedures. In this context, some efforts have been made to combine extraction procedures together and can synergistically derive benefits from individual microextractions such as combined two LPME techniques (HF-LPME and DLLME) in the analysis of aflatoxins in soy juice which is called the HF-DLLME by (Simão et al., 2016).

The most common approach is the HF(2)LPME procedure applied with direct immersion. Headspace method has also been reported for extraction of volatile and semi-

volatile compounds, such as in the analysis of PAHs by the dynamic hollow fiber headspace prior to GC-MS for soil samples (Jiang et al., 2005), and in the analysis of organochlorine pesticides by GC-MS (Huang & Huang, 2007). Table 2.3, shows the applications of HF(2)LPME combined with LC–MS, GC–MS, HPLC and GC for drug and pharmaceutical extraction and analysis.

2.1.7.1 Applications of Three-Phase HF-LPME

Most studies have focused on the HF(3)LPME prior to LC-MS and HPLC with different detectors. However, with the use of two immiscible organic solvents, the HF(3)LPME approach is also compatible with GC or GC-MS as the detection method (Ghambarian et al., 2010). Based on the nature of the target analytes, i.e. polarity and ionizability, the mechanism of the HF(3)LPME could be performed by utilizing the pH gradient, carrier transport, and EME. Furthermore, some of the solvents used as SLMs in the HF(3)LPME method typically have higher polarity than those employed in the HF(2)LPME method. HF(3)LPME has been used to extract pesticide residues and organic pollutants from food, biological, and environmental samples. Table 2.4 summarizes some applications of the HF(3)LPME for pesticides and organic pollutants.

The HF(3)LPME has been extensively used for the extraction and determination of drugs from different samples as shown in Table 2.5, i.e. environmental (sewage, wastewater, and tap water) and biological (human urine, whole blood, plasma, breast milk, pharmaceuticals, and rat plasma). The SLM in most cases varied between dihexyl ether and octanol. However, studies have shown the use of other solvents as SLM with good stability for a prolonged period, e.g. silicone oil, plant oils, and dodecyl acetate, octanol.

Table 2.2: Applications of HF(2)LPME: Pesticides, mycotoxins, and other organic pollutants

Analyte	Sample	SLM	Detector	LOD	Reference
Acidic herbicide	Water	1-Octanol	GC-MS	0.5–14 ng/L	Wu & Lee, 2006a
Ochratoxin A	Wine	1-Octanol	HPLC-FLD	0.2 ng/mL	González-Peñaset al., 2004)
Aromatic amines	Water	1-Octanol	MEEKC	0.0021–0.0048 µg/L	Zhao et al., 2002
Ochratoxin A, T-2	Wine & Beer	1-Octanol	LC-MS/MS	as LOQ 0.1 g/L	Romero-González et al., 2010
Carbamates	Tap and drain water	1-Octanol	GC-MS	0.2–0.8 µg/L	Zhang & Lee, 2006
Chlorophenoxy acid	Water	1-Octanol	HPLC	0.2–0.5 µg/L	Esrafili et al., 2011
Chlorobenzenes	Water	1-Octanol	GC-ECD	10-100 ng/L	Esrafili et al., 2012
Fatty acids	Water	1-Octanol	GC-MS	0.0093–0.015 µg/L	Wu & Lee, 2006b
Fungicides	Farm water	Toluene	GC-ECD	0.004–0.025 µg/L	Pan & Ho, 2004
Organic pollutants	Marine sediments	Toluene	GC-MS	0.07–0.7 ng/L	Basheer et al., 2005
Aflatoxin M1	Milk	1-Octanol	LC-MS/MS	0.06 g/ kg	Huang et al., 2015
OCPs	Soil	Toluene	GC-MS	0.05–0.1 µg/L	Hou & Lee, 2004
OPPs	Lake water	Cyclohexane	GC-MS	0.006–0.2 µg/L	Chen & Huang, 2006
PAHs	Soil slurry	1-Octanol	GC-MS	0.0059–0.072 µg/L	Jiang et al., 2005
Phenols	Seawater	Toluene	GC-MS	0.005–0.015 µg/L	Zhang et al., 2006
Phthalate ester	Water	Toluene	GC-MS	0.005–0.015 µg/L	Psillakis & Kalogerakis, 2003
PBDEs	Tap and river water	n-Undecane	GC-MS	0.3–1.1 µg/L	Fontanals et al., 2006
PCBs	Plasma	Toluene	GC-MS	0.07–0.94 µg/L	Basheer et al., 2004
Triazine herbicides	Soil slurry	Toluene	GC-MS	0.007–0.063 µg/L	Shen & Lee, 2002
chlorophenols	Urine		GC-MS	0.1–0.2 ng/ ml	Ito et al., 2008
AFB1, B2, G1& G2*	Soya juice	1-octanol	HPLC-FLD	0.01 - 0.03 µg/ L	Simão et al., 2016
Benzimidazole	Soil	1-Octanol	HPLC-FLD	0.001–6.94 ng/g	Asensio-Ramos et al., 2012
OPPs	Fish tissue	o-xylene	GC-MS	2.1–4.5 ng/g	Sun et al., 2011
Carbamate	Fruit samples	1-octanol	HPLC-DAD	0.2 to 1.0 ng/ g	Ma et al., 2014a
Pyrethroid	Fruits and vegetable	1-octanol	LC-MS	0.02–0.07 ng/mL	Arvand et al., 2013
Pesticides	Cucumbers	n-octanol	LC-MS/MS	0.01- 0.31 µg /Kg	Wang et al., 2012

Table 2.2, (continued)

Analyte	Sample	SLM	Detector	LOD	Reference
Organochlorine pesticides	Ecological textiles	n-octanol	GC/MS	0.06–2.30 ng/g	Cai et al., 2016
Phthalic acid esters	Water	1-octanol	GC-MS/MS	-	González-Sálamo et al., 2018
Emerging contaminants	water	1-octanol	LC-MS/MS	1.09 to 98.15 ng/L	Salvatierra-stamp et al., 2018
Allergenic coumarin	Children's toys	n-octanol	UPLC-MS/MS	1-5 µg /Kg	Hu et al., 2017
Fatty acid methyl ester	Bio-liquid	n-octanol	GC/MS	-	Choi et al., 2017
Isophthalaldehyde	Food packaging materials	toluene	GC-MS	10 ng g	Osorio et al., 2018
AFB1, B2, G1& G2	Edible oil	pure water	LC-MS/MS	0.02-0.32 ng Kg	Huang et al., 2017

AFB1, B2, G1& G2: Aflatoxins B1, B2, G1& G2; BTEX:benzene, toluene, ethylbenzene, and xylenes, GC-ECD: gas chromatography electron captured detector, GC-MS: gas chromatography mass spectrometry, HF(2)LPME: two phase hollow fiber liquid-phase microextraction, HF-DLLME: hollow fiber dispersive liquid–liquid microextraction; HPLC-FLD: high performance liquid chromatography fluorescence detection; HPLC-DAD: high performance liquid chromatography diode array detection; LC-MS: Liquid chromatograph mass spectrometry; LC-MS/MS: liquid chromatography tandem mass spectrometry; LOD: limit of detection; MEEKC: microemulsion electrokinetic chromatography; OCPs: organochlorine pesticides; OPPs: organophosphorus pesticides; PAHs: polycyclic aromatic hydrocarbons; PBDEs: polybrominated diphenyl ethers; PCBs: polychlorinated biphenyl; SLM: supported liquid membrane.*HF-DLLME Technique,-:not reported.

Table 2.3: Applications of HF(2)LPME : Clinical and pharmaceutical compounds

Analyte	Sample	SLM	Detector	LOD	Reference
Cocaine +metabolites	Saliva	Chloroform	GC-PDHID	6–280 µg/L	de Jager & Andrews, 2002
Mitrazapine	Plasma	Toluene	HPLC-UV	3 µg/L	de Santana et al., 2005
Non-steroidal drugs	Water	1-Octanol	GC-FID	1–2 µg/L	Es'haghi, 2009
Different anabolic steroids	Urine	Dihexyl ether	GC-MS	2 µg/L	Leinonen et al., 2006
Benzophenone and derivatives	Urine	Toluene	GC-MS	5–10 ng/L	Kawaguchi et al., 2009
β-Agonists and β-blockers	Urine	Methylbenzol	GC-MS	0.08–0.1 µg/L	Liu et al., 2009
Narcotic drugs	Plasma, urine	Hexylacetate	GC-NPD	5–8 ng/L	Saraji & Boroujeni, 2011
Phenothiazines	Urine	Toluene	GC-FPD	1.4- 203.4µg/L	Xiao & Hu, 2010
Progesterone	Human serum	Toluene	GC-MS	0.5 µg/L	Kawaguchi & Takatsu, 2009
Emodin & its metabolites	Plasma,urine	n-octanol	HPLC-UV	0.1-3.0 µg/L	Tian et al., 2012
Nonsteroidal drugs	Wastewater	1-Octanol	GC-MS	1.6–5.6 ng/L	Manso et al., 2014
Acidic Nonsteroidal drugs	Water, juice	1-Octanol	LC-MS/MS	0.5-1.25 mg/Kg	Zhang et al., 2013
Echinacoside	Rat plasma	n-Octanol	HPLC-UV	2.0 ng/mL	Zhou et al., 2013
Antidiabetic drugs	Plasma , urine	Dihexyl ether	HPLC-UV	0.12, 1.0 ng/ mL	Ben-Hander et al., 2015
Oestrogens+metabolite	Dairy	1-Octanol	HPLC-DAD/FLD	0.23-72.9 µg /Kg	Socas-Rodríguez et al., 2014
Echinacoside, Tubuloside B	Rat plasma	n-octanol	HPLC-UV	8–15 pg/ mL	Zhou et al., 2014
benzodiazepine	Urine	n-dodecane	HPLC–MS	0.3-0.7g/L	Nazaripour et al., 2016

GC-FID: Gas Chromatography – Flame Ionization Detector; GC-FPD: Gas Chromatography – Flame Photometric Detector; GC-MS: gas chromatography mass spectrometry; GC-NPD: Gas Chromatography – Nitrogen Phosphorous Detector; GC-PDHID: gas chromatography pulsed discharge helium ionization detector; HPLC- DAD/FLD: high performance liquid chromatography diode array-fluorescence detection; HPLC-UV: high performance liquid chromatography ultraviolet detector; HF(2)LPME: hollow fiber two-phase liquid-phase microextraction; LC-MS/MS: liquid chromatography tandem mass spectrometry; LOD: limit of detection: SLM: supported liquid membrane

Table 2.4: Applications of HF(3)LPME for detection the pesticides and organic pollutants

Analyte	Samples	SLM	Detector	LOD	Reference
Anilines	Water sample	Toluene	HPLC-UV	0.01–0.1 mg/L	Sarafraz-Yazdi et al., 2009
Aromatic amines	Water	Dihexyl ether	HPLC-UV	0.05–0.10 mg/L	Zhao et al., 2002
Dinitrophenols	Plasma	Dihexyl ether	HPLC/MS	0.05–0.1 mg/mL	Hansson et al., 2010
HCAs	Urine	Octanol	LC-MS/MS	2–50 pg/g	Busquets et al., 2009
Chlorophenols	Water	Dodecane	GC-ECD	0.006–0.2 ng/mL	Ghambarian et al., 2010
HCAs	Human plasma	1-octanol	LC-MS/MS	2–5 pg/mL	Cooper et al., 2014

GC-ECD: gas chromatography electron captured detector; HPLC-UV: high performance liquid chromatography ultraviolet detector; LC-MS/MS: liquid chromatography tandem mass spectrometry; HCAs: heterocyclic amine; HF(3)LPME: hollow fiber three-phase liquid-phase microextraction; LOD: limit of detection; SLM: supported liquid membrane.

Table 2.5: Drugs, Clinical and pharmaceutical compounds extractions by HF(3)LPME

Samples	Analyte	Solvent (SLM)	Detector	LOD	Reference
Echinophora platyloba DC.,Mentha piperita	flavonoids	1-Octanol	HPLC-UV	0.5-7.0 ng /mL	Hadjmohammadi et al., 2013
Polygonum hydropiper L	flavonoids	ethyl acetate	LC-MS	< 0.170 µg/L	Yang et al., 2011
Raceanisodamine , belladonna	four hyoscyamines	Dimethylbenzene /n-Heptanol	HPLC-UV	0.01-0.03 mg/L	Xi et al., 2010
traditional Chinese medicines	Phenylpropionic acids	n-enathol	HPLC-UV	,0.004-0.250 g/L	WANG et al., 2009
Valeriana officinalis	valerenic acid	dihexyl ether	HPLC-UV	2.5 µg/ L	Mirzaei & Dinpanah, 2011
Human plasma	Atropine,scopoamine	n-heptanol +dimethyl benzene	HPLC-UV	-	XI et al., 2011
wastewater	ibuprofen	1-octanol	HPLC-UV	≤100 ng/L	Wen et al., 2004
Whole blood and urine	Amphetamines	Dihexyl ether	FIA-MS-MS	0.4–100 ng/ mL	Pedersen-Bjergaard, et al., 2001
Sophora flavescens Ait.	Oxymatrine and Matrine	ispropyl alcohol	HPLC	1.0 mg/L	Xiao-Hong et al., 2008
seawater and sewage	serotonin reuptake inhibitors+ metabolites	Dihexyl ether	LC-MS	17 pg/L ,618 ng/L	Vasskog et al., 2008
human urine	ibuprofen, diclofenac and salicylic acid	Dihexyl ether	HPLC-DAD/FLD	2 -53 ng /mL	Payán et al., 2009
urine using	aristolochic acid	1-Octanol	HPLC-PAD	0.01 µg/L	Yang et al., 2010

Table 2.5, (continued)

Samples	Analyte	Solvent (SLM)	Detector	LOD	Reference
Tap water , plasma, urine	pioglitazone	di hexyl ether	HPLC-UV	1.0 µg/ L	Tahmasebi et al., 2009
plasma and urine	Cabergoline	n-octanol	HPLC-UV	0.01 µg /L	Piroozi et al., 2014
human plasma	naloxone, buprenorphine and norbuprenorphine	1-octanol/chloroform /toluene(2/4/4)	LC-MS/MS	0.025-0.05 ng/mL	Sun et al., 2014
Pollen	Caffeic, Ferulic, Cinnamic acid	n-Octanol	HPLC	0.015 µg/L	Chen et al., 2012
medicinal materials	hypoxanthine , xanthine, and adenine	1-octanol	HPLC-UV	97- 173 ng/mL	Liu et al., 2014
biological fluids	Vitamin D3	n-dodecane	HPLC-UV	0.9 ng /mL	Saber-Tehrani et al., 2014
Urine	anticancer	n-Dodecane	HPLC- UV–Vis	0.3, -0.6 µg/L	Nazaripour et al., 2018
Benzodiazepine	urine sample	n-dodecane	HPLC–MS	0.2-0.4 g/ L	Nazaripour et al., 2016
Antihypertensive peptide	human plasma		HPLC-DAD	68.5 ng /mL	Wang et al., 2018

FIA-MS-MS: flow injection analysis tandem mass spectrometry; HF(3)LPME: hollow fiber three-phase liquid-phase microextraction; HPLC: high performance liquid chromatography; HPLC-UV: high performance liquid chromatography ultra violet detector; LC-MS: liquid chromatography mass spectrometry; LC-MS/MS: liquid chromatography tandem mass spectrometry: LOD: limit of detection; SLM: supported liquid membrane. -:not reported;Applications of HF-SBME

As illustrated in Table 2.6, hollow fiber solvent bar microextraction (HF-SBME) mode which has been successfully used to separate from complex sample matrices such as aliphatic amines, ionizable organic complexes, plasma proteins, clenbuterol and pesticides.

2.1.7.2 Applications of Carrier-Mediated HF-LPME

Carrier-mediated HF-LPME has been used in the extraction of some pharmaceuticals compounds and pesticides from biological fluids as well as from environmental water samples as shown in Table 2.7. Two different techniques of carrier-mediated HF-LPME are used in both the two- and three-phase modes: the first is by adding into the donor phase (e.g. octonal),and the second is by dissolving into an organic solvent, e.g. dihexyl ether which is loaded into the pores of the hollow fiber membrane. In both cases, good performance was achieved in terms of sensitivity, preconcentration factor, precision and accuracy. In some cases, the recovery can be affected by factors such as drug-protein interactions in plasma containing samples. However, the lowering of the plasma concentration and its alkalinity may eliminate such drawbacks(Pedersen-Bjergaard et al., 2005). In addition, the studied matrix effect by adding humic acid to the sample did not affect the extraction efficiency (Tao et al., 2009).

Carrier-mediated hollow fiber coupled to LC-MS/MS can be applied to determine multiple classes of drugs (antibiotics) in water samples. Acceptable results in terms of linearity, enrichment factor (156 times), and relative recovery between 79-118% were recorded (Yudthavorasit et al., 2011). A comparison of the carrier-mediated technique with other methods (i.e. HF-LPME, SPE, and other LLE techniques) demonstrated high sensitivity, large preconcentration factors and precision when in the determination of highly hydrophilic tetracycline in different sample matrices (bovine milk, human plasma and water samples) (Shariati et al., 2009).

The most common sample pretreatment methods are removing the particulates from the sample by the filtration, centrifugation, and sedimentation. However, the disposable nature of the hollow fiber eliminates the possibility of sample carryover and ensures high reproducibility. Moreover, the wall pores of hollow fiber provide good selectivity by preventing the extraction of macromolecules (e.g. protein, particles) from the sample matrix. Therefore, HF-LPME is no longer just a preconcentration technique, it can provide sample cleanup and thus can be used for complex sample matrices

2.1.8 Analytical Performance of HF-LPME Methods

The comparison of the HF-LPME performance with other sample extraction methods has been reported by Sarafraz-Yazdi and Haghi (2006) . Based on this study, HF-LPME technique showed higher RSD and lower recovery when compared with the SDME technique which was attributed to the memory effect (residual target analyte remains in the porous hollow fiber). Despite this, HF-LPME yielded better LOD, high enrichment factors, and had a longer lifetime in terms of fiber usage. On the other hand, the SDME technique is simple, low-cost, and permits rapid extraction. However, it requires careful and complex manual operations and has poor sensitivity and precision. When SDME is used in complex extraction processes, an extra filtration step is necessary (Sarafraz-Yazdi & Es' Haghi, 2006).

Xiong and Hu (2008) showed that dispersive liquid-liquid microextraction (DLLME) had a shorter extraction time, higher extraction capacity, and is suitable for simultaneous batch sample pretreatment compared to HF-LPME (Xiong & Hu, 2008). However, in the analysis of a complex sample, the HF-LPME was found to be more robust, sensitive, and repeatable than DLLME. HF-LPME procedure is generally less of a hassle when compared to that of DLLME which usually carried out centrifugation/sedimentation of the post-extraction solvent.

Table 2.6: Applications of SBME for extraction of different analytes

Analyte	sample	SLM	Detector	LOD	Reference
Aliphatic amines	Waste water	n-nonanol	GC-FID	0.01 - 0.06 µg /L	Kamarei et al., 2010
tramadol	Plasma and urine	n-nonanol	GC-MS	0.02 µg /L	Ghasemi, 2012
anabolic steroids	Urine and hair	toluene	GC-MS	lower than 0.10 ng/mL	Liu et al., 2012
Phthalate esters	Mineral water, ice red tea, red wine, and human urine	xylene	GC-MS	5–100 ng /L	Huang et al., 2012
Methadone	Human urine & plasma	1-Undecanol	GC-FID	2.7-7 µg /L	Ebrahimzadeh et al., 2014
Organophosphorus pesticides	Fruit juice	1-octanol	GC-MS	0.018–0.096 µg /L	Wu et al., 2015
Chlorophenols	Water	Ethyl acetate :toluene	GC-MS	14.8 ng/L- 22.9 ng/L	Alcudia-León et al., 2011
Naproxen, ibuprofen, ketoprofen, propranolol, diclofenac, and alprenolol	Drain water	1-octanol	GC-MS	0.006 - 0.022 µg /L	Guo & Lee, 2012b
Chlorobenzenes	Soil samples	1-octanol	GC-MS	0.7–27.3 ng/ g	Wang et al., 2012
Tanshinones &Salvianolic Acids	Radix Salvia miltiorrhiza	octanol	HPLC-UV	lower than 1.11 ng/ mL	Ma et al., 2014
Herbicides	Pear	chloroform	HPLC-UV	7.15–8.26 ng/g	Shi et al., 2014
Oleanolic & Ursolic Acid	Chinese medicinal herbs	Decanol	HPLC-UV	e 1.3 ng/ mL and 1.5 ng/ mL	Hao et al., 2014
Organochloride and Triazine Pesticides	Seawater, freshwater	toluene	GC-ECD	0.005- 0.086 µg/ L	Vergel et al., 2014
phthalate esters	Environmental and drinking water	1-octanol	HPLC–UV	0.012- 0.03 ng/ mL	Bandforuzi & Hadjmohammadi, 2018
Sarcosine	Human urine	toluene	HPLC–UV	0.02 µm/L	Huang et al., 2018

Table 2.6, (continued)

Analyte	sample	SLM	Detector	LOD	Reference
Warfarin	Human plasma	1-octanol	HPLC–UV	0.3 ng/ mL	Hadjmohammadi, 2017
Triazine herbicides	Water samples	[C4MIM][PF6]	HPLC–UV	0.14–0.48 µg/ L	Wang et al., 2017
Vincristine	Plasma and urine	1-octanol	HPLC-UV	0.015 mg /L	Kiani et al., 2018

GC-ECD: gas chromatography electron captured detector; GC-FID: gas chromatography flame ionization detector; GC-MS: gas chromatography mass spectrometry; HPLC-UV: high performance liquid chromatography ultraviolet detector; LC-MS/MS: liquid chromatography tandem mass spectrometry; LOD: limit of detection: SBME: solvent bar microextraction; SLM: supported liquid membrane.

Table 2.7: Applications of carrier-mediated HF-LPME for extraction different analytes

Analyte	Sample	SLM	Meditation	Detector	LOD	Reference
Macrolide, sulfonamide, tetracycline, and quinolone	Water	Dihexyl ether	Dissolved SLM	LC-MS/MS	10–250 ng/L	Yudthavorasit et al., 2011
Sulfonamides	Differed water sample	[C8MIM][PF6]	Dissolved SLM	HPLC-UV	0.1–0.4 µg/L	Tao et al., 2009
Polar drug	Human plasma	Octanol	Donor phase	LC-MS	-	Pedersen-Bjergaard et al., 2005
Estrogenic hormones	Seawater, tap &sewage water	Dihexyl ether	Dissolved SLM	GC-MS	1.6–10 ng/L	Zorita et al., 2008
Dicocodimethylammonium chloride	Tap and process water	Octanol	Donor phase	LC-MS	0.9 µg/L	Hultgren et al., 2009
Phenoxy acid herbicides and phenol	Differed water sample	Dihexyl ether	Dissolved SLM	HPLC-UV	0.4–1.2µg/L	Hu et al., 2010
Aromatic amines	Differed water sample	Dihexyl ether	Dissolved SLM	HPLC-UV	0.5–1.5 µg/L	Tao et al., 2009
11-nor-D9 -tetrahydro cannabinol-9-carboxylic acid	Human urine	*N*,*O*-bis (trimethylsilyl) Trifluoroacetamide/octane	Donor phase	GC-PDHID	1 ng/mL	Kramer & Andrews, 2001

Table 2.7, (continued)

Analyte	Sample	SLM	Meditation	Detector	LOD	Reference
Tetracyclines	Bovine milk, human plasma	Octanol	Dissolved SLM	HPLC-UV	0.5–1.0 µg/L	Shariati et al., 2009
Pesticides	Natural and tap water	Dihexyl ether	Dissolved SLM	LC-MS/MS	0.026–0.081 µg/mL	Trtić-Petrović et al., 2010
Aminophosphonic pesticide	Ground water	Dihexyl ether	Dissolved SLM	HPLC-FLD	0.22–3.40 µg/L	Piriyapittaya et al., 2008
Nicotinic acid, amoxicillin, hippuric acid and salicylic acid.	Human urine	1-octanol	Dissolved SLM	HPLC-DAD	112.0-234.5 µg/L	Román-Hidalgo et al., 2017
Antibacterial residues	Lamb and chicken (muscles and the liver), fish, honey, and milk	1-octanol	Dissolved SLM	HPLC-DAD	0.5 – 20 ng /g	Tajabadi et al., 2016
Methyl hippuric acids	Aqueous samples	1-octanol	Dissolved SLM	HPLC-UV	2-3 µg/ L	Ghamari et al., 2017
Hippuric Acid and Mandelic Acid	Urine samples	1-octanol	Dissolved SLM	HPLC-UV	0.007–0.009 mg/L	Bahrami et al., 2017

GC-FID: gas chromatography flame ionization detector; GC-MS: gas chromatography mass spectrometry; GC-PDHID: gas chromatography pulsed discharge helium ionization detector; HF-LPME: hollow fiber liquid-phase microextraction; HPLC-FLD: high performance liquid chromatography fluorescence detector; HPLC-UV: high performance liquid chromatography ultra violet detector; LC-MS: liquid chromatography mass spectrometry; LC-MS/MS: liquid chromatography tandem mass spectrometry; SLM: supported liquid membrane.

Thus, HF-LPME used to have a higher recovery rate than DLLME and readily semi-automated. Although DLLME shows lower LOD (due to better analyte enrichment), HF-LPME has better inter- and intra-day RSD due to its simplicity (Xiong & Hu, 2008).

In the determination for drugs of abuse, Meng et al. found that both HF-LPME and ultrasound-assisted low-density solvent DLLME (UA-LDS-DLLME) were rapid and convenient approaches which consume very little solvent. The UA-LDS-DLLME was slightly more sensitive (enabled higher enrichment of analytes) than HF-LPME. However, the RSD of HF-LPME was better than that of UA-LDS-DLLME. Nevertheless, good linearity was obtained for both extraction techniques. When extraction time is one of the comparative factors, the extraction time for UA-LDS-DLLME is 3 min compared to 15 min for HF-LPME, and thus UA-LDS-DLLME has higher extraction efficiency than HF-LPME. Also, HF-LPME extraction has fewer impurity peaks than UA-LDS-DLLME, and thus HF-LPME has an excellent sample purification effect and is a robust and suitable technique for various sample matrices(Meng et al., 2015).

The HF-LPME methods are shown to be more sensitive for drug analysis than SPE and SPME. In comparing HF-LPME with the LLE and SPE methods in the determination of pyrethroid metabolites in urine samples, Lin et al. found that HF-LPME had a lower LOD than LLE. Also, the HF-LPME method consumed less extraction solvent and was less time-consuming (15 min) compared to the LLE and SPE methods which ranged from 2 to 4 hours (Lin et al., 2011). Frenich et al,. (2011), compared the performance of SPME with HF-LPME for the determination of pesticides in drinking water using GC–MS/MS. It was found that SPME was less complicated (easy to use), amenable to automation and had a lower LOD compared to HF-LPME. SPME also showed better sensitivity where a total of 77 pesticide compounds were successfully recovered, but HF-LPME only managed to detect 56 of the

pesticides. This can be explained by taking the injection volume into account, where the SPME fiber was exposed directly into the GC, while only 10 µL (a 1/25 fraction of the extract) of HF-LPME was introduced. Lastly, HF-LPME showed higher intra-day and inter-day precision compared to SPME; however, the feasibility of SPME to be automated makes it the preferred method in this case (Frenich et al., 2011).

These comparative studies showed that HF-LPME has advantages in the extraction of certain analytes from different types of sample matrices. However, as reported in many review articles, the automated HF-LPME is still not being commercially available in a high-throughput configuration due to the drawbacks i.e long extraction time and low efficiency for highly polar compounds. However, it is possible to overcome some of these drawbacks and improve HF-LPME performance through innovation and optimization. Its efficiency can be enhanced by employing modern developments such as parallel artificial liquid membrane extraction (PALME) (Gjelstad et al., 2013).

HF-LPME has been a successful technique used for the clean-up of different analytes such as organic pollutants, pesticide residues, as well as pharmaceutical compounds. The determination of various analytes at different concentrations and matrices can be successfully achieved by utilizing a number of HF-LPME techniques. However, it is necessary to optimize simultaneously the influencing factors to achieve desirable results. The HF-LPME techniques are compatible with many chromatographic systems and have been extensively utilized in combination with HPLC, LC–MS, LC–MS/MS, GC, GC–MS, and GC–MS/MS. However, the application of HF-LPME in solid and semi-solid samples such as food, soils, and sediments matrices is not as successful as in liquid samples, and requires more extensive development. Future development of the HF-LPME methods will most likely be focused on

developing a commercial system with good efficiency, and ones that are compatible with various samples and chromatography systems.

2.2 Optimization Methodology

The classical or univariate optimization approach (also known as one-factor-at-a-time (OFAT) or one-variable-at-a-time (OVAT)) involves monitoring the effect of one variable at a time while maintaining the other variables constant. The major disadvantage of this method is disregarding the interactive effects between variables. Moreover, the approach requires numerous experiments which leads to an increase in time, expenses, and consumption of reagents and materials (Czitrom, 1999; Liyana-Pathirana & Shahidi, 2005; Varmuza & Filzmoser, 2016). Despite the above disadvantages, many researchers are still relying on OFAT especially non-experts, and in cases where the data is cheap and plentiful (Czitrom, 1999). In cases where data is valuable and must be analyzed carefully, it is always better to study several factors simultaneously and systematically using multivariate experimental designs. In this case, statistical–mathematical methods (which combines experimental design, regression modelling techniques, and optimization tool) are used to achieve the objectives that may not be attained by the conventional approach (OFAT). The process of experimental design involves the selection of critical parameters for further study by removing those with little or no effect altogether and predicting the optimum condition to give a targeted response. Collecting meaningful data for analysis estimate the method performance within the constraints such as overall analysis time, chemicals, and equipment (Figard & Abbott Park, 2009; Nor et al., 2017).

2.2.1 Problem Formulation

The first step in an experimental design is understanding the process requirements to determine the inputs, outputs and the process objectives. The criteria will select the other process requirements such as factors and models to support the objectives, collect the data from the design, perform the analysis, and verify the model (Figard & Abbott Park, 2009). There are two kinds of chemical problems that need an experimental design for their solution. The first is to investigate which factors have a significant effect on the response, and the second is to find adequate factor values that optimize the response. One of the DOE objectives is to perform a minimum number of experiments on the maximum number of factors, where the minimum number of levels is two. An optimum analytical response depends on the wider problem with considerations such as time, cost, and required measurement uncertainty that is used to which the analytical information is to be placed. An advantage of DoE is that multiple responses can be measured, for example, resolution, time, peak area, and peak height. The models developed arrive at the desired overall optimum without extra experiments. As an example, for chromatographic separations, it is important to have an acceptable response that meets minimum criteria and so the aim is often to locate that region rather than find the absolute optimum. This makes DoE very powerful when the polynomial function does not fit the data perfectly but does describe the response sufficiently to locate an acceptable region. This means, within the span of the values of factors in an experiment, there will be better response values and also not so good ones. Optimization is the process of discovering where the best values lie. There is not always ideal single maximum of a function that can be discovered. Often the response plateaus and there is an area of response surface with approximately the same value. Sometimes , the function shows a saddle with maximum values at the edges. In the optimization process, the method, sample

preparation or instruments, successive steps are taken to reach the desired goal (Hibbert, 2012).

2.2.2 Screening Design

The available information and details about a study are often limited in the preliminary stage. Hence, a researcher may have to consider many potential factors, even though some of the factors could be insignificant in the process. Therefore, the first step is to screen for critical factors with a small experimental run. A two-level experimental design is most suitable at this stage. There are many different types of two-level experimental designs, but the factorial and Plackett-Burman designs (PBDs) are the most common. When considering a large number of factors, the number of experiments required by the full factorial design and high order models are not practical.One of the alternatives in this situation is the Plackett-Burman design (Ebrahimi-Najafabadi et al., 2014a).

2.2.2.1 Two-Level Factorial Design

The two-level full factorial design is commonly employed for screening of the key factors. The number of experiments run is expressed as $2^{\mathbf{k}}$, where k is the number of factors studied. This technique reveals the main effect of each factor and interactions among the factors. However, this design becomes untenable when the number of factors becomes too large. Hence, fractional factorial design can be carried out to minimize use of fewer experiment runs, to support lower order models (Ferreira et al., 2017).

2.2.2.2 Plackett-Burman Design (PBD)

The Plackett-Burman design (PBD) is carried out in either 12-, 20-, 24-, or 28-run (and higher) designs and it is a reasonable tool for preliminary evaluation of main effect (Ebrahimi-Najafabadi et al., 2014a)). As the number of runs increases, the maximum number

of factors can be accordingly increased. For example, in a 12-runs experiment, it is possible to screen up to 11 factors, a 20-runs experiment can screen to 19 factors, etc. (Andrade-Garda, 2013). Plackett-Burman designs are basically a saturated main effect design with resolution III designs because all degrees of freedom are utilized to estimate the main effects. Thus, the statistics do not indicate if a factor has a significant effect or not, as the data collected may not provide enough information.

2.2.3 Response Surface Methodology (RSM)

2.2.3.1 Central Composite Designs (CCD)

The central composite designs are widely used for process optimization possessing two or more factors.CCD consists of a two-level full or fractional factorial design, a star design, and a central point (Sudha et al., 2017). One characteristic of CCD is that the full two-level factorial design can be performed in preliminary evaluations and the result feed in the RSM (Ferreira et al., 2018).

2.2.3.2 Box-Behnken Designs (BBD)

Box-Behnken designs are an important RSM design for experiments involving three factors and more. They are an incomplete three-level full factorial and rotatable or nearly rotatable second order designs with central points and middle points at the edges. BBDs are an alternative to CCD and are more efficient compared to other three-level factorial designs (Ferreira et al., 2007).

2.2.3.3 Doehlert Design (DD)

The Doehlert designs are suitable for experiments with a small number of factors. For example, in two factor experiments, three to five levels designs are used, while three factors experiments are accommodated with three, five and seven-levels designs. Such non-rotatable

designs can clearly identify the main effects, quadratic effects, and first-order interactions (Roussel et al., 2014; Sudha et al., 2017).

2.2.3.4 Three-Level Full Factorial Designs (3FFD)

Three-level full factorial designs (3FFD) has low applicability. They require a large number of runs and consider all factors at both positive and negative level with center points, which increase the processing time and cost. However, they provide information on the interactions (Ebrahimi-Najafabadi et al., 2014a).

2.2.3.5 D-Optimal Design

The D-optimal designs is a popular method and are used mainly when the factor space cannot be accessed uniformly as a result of the solute concentration, and solvent composition is not possible to combine. Another benefit is that the number of the experiments is specified. This minimize the necessity to the coefficient detection of the model effect. The D-optimal design was generated for multi-factor experiments as quantitative and qualitative factors. The factors can have a mixed number of levels. For example, this procedure can be used to establish an experiment with quantitative and qualitative multi-levels factor. This design (D-optimal designs) is found to reduce the generalized variance of the analyzed regression coefficients by maximizing the determinant of X'X. (X the matrix of design points). The X matrix is used to represent the data matrix of independent variables. The design is also used in case there is not enough budget, and cannot be run for everything replicated in a factorial design (Hoskins et al., 2004).

2.2.4 Mixture Designs

Mixed designs have been used for formulation optimization, e.g. the mobile phase of liquid chromatography composition or extraction solvents for sample preparation methods.

A mixed design allows determination of the optimum composition of each mixture component, with the purpose of achieving a response with the best features. It has been utilized in analytical chemistry for the preparation of chemical mixture solutions where the components can be measured in volumes or weight. The component proportions are established by linear, quadratic or cubic models. There are many variations in mixed designs:- the most common are simplex-centroid and simplex-lattice designs. Simplex-centroid design is not compatible with the full cubic model, but able to estimate a special cubic model, while simplex-lattice has enough points to estimate the full cubic model (Ferreira et al., 2007; Şahin et al., 2016; Sudha et al., 2017).

2.2.5 Model Adequacy

The model adequacy can be examined by applying different statistical tools. ANOVA checks the adequacy of the regression model using the lack-of-fit test and compares variation due to random errors as a result of the measurements of the response, and evaluates regression significance. The comparison is expressed as F-value and in proportion to the model's mean-square and residual error. If the F-value is higher than the critical value, it indicates a good fit between the model and experimental results (Stalikas et al., 2009). A model with good adequacy will have non-significant lack-of-fit, significant regression model, correlation coefficient and adjusted correlation coefficient value of close to 1.0, and low residual analysis. Student's t-test is applied to check the significance of the regression coefficients. If the regression coefficient of factors/interactions differs significantly from 0, its effect is significant based on the estimated P- value. Generally, when the t-value is high, the P-value will be small (Stalikas et al., 2009; Sudha et al., 2017).

2.3 Experimental Design for HF-LPME & LC-MS Optimization

Experimental design is widely applied in the field of analytical chemistry, where many review articles have been published in the last two decades focusing on sample preparation and instrument optimization (Bezerra et al., 2008; Stalikas et al., 2009;Horstkotte et al., 2010;Araujo & Janagap, 2012;Callao, 2014; Candioti et al., 2014; Ebrahimi-Najafabadi et al., 2014a; 2014b; Kumar et al., 2014; Reinholds et al., 2015;Ferreira et al., 2017;Sudha et al., 2017;Ferreira et al., 2018). Response surface methodology (RSM) is widely used in the optimization of microextraction due to its advantage's comparison to the classical univariate optimization approach. Table 2.8 summarizes the types of RSM methods used extensively in the optimization of HF-LPME for the detection of various compounds by chromatographic technologies. Table 2.9 summarizes the experiment design for LC-MS optimization.

Table 2.8: Experiment design used in the HF-LPME optimization

Analyte	Technique	Experimental design	Detector	Reference
Acidic and basic drugs	EME	CCD	HPLC-UV	Seidi et al., 2012
Tramadol	Three phase HF-LPME	BBD	GC-MS	Ghambarian et al., 2011
Pyrethroid pesticides	Two-phase HF-LPME	CCD	GC-MS	Arvand et al., 2013
Chloropheniramine maleate & dextromethorphan ydrobromide	Three phase HF-LPME	CCD	HPLC-UV	Ebrahimzadeh et al., 2012
Volatile organic compounds of selenium	HS-HF-LPME	PBD&BBD	GC–MS	Ghasemi et al., 2011
Warfarin	SBME	CCD	HPLC-UV	Hadjmohammadi, 2017
Morphine, xymorphone, and methylmorphine	EME	CCD	HPLC-UV-Vis	Yamini et al., 2014
PAH	MWCNT-HF-LPME	QFED and CCD	HPLC–UV	Hamedi & Hadjmohammadi, 2017
Vincristine	SBME	BBD	HPLC–UV	Kiani et al., 2018
Dexamethasone sodium phosphate	SBME	CCD	HPLC–UV	Ara et al., 2013
Anilines	Two-phase HF-LPME	BBD	HPLC–UV	Moradi et al., 2012
Emerging contaminants	Two-phase HF-LPME	FED	LC-MS/MS	Salvatierra-stamp et al., 2018
Phthalate esters	Two-phase HF-LPME	BBD	HPLC–UV	Yamini et al., 2016
Muscimol	Two-phase HF-LPME	CCD	HPLC -UV	Ncube et al., 2016
Aflatoxins	HF-DLLME	CCD	HPLC-FD	Simão et al., 2016

BBD: Box Behnken design;CCD : Central composite design ;FD: Fluorescence Detector; EME: Electro membrane extraction ; HF-DLLME: Hollow fiber dispersive liquid-liquid microextraction; HS: Headspace; MWCNT: Multiwall carbon nanotube; PAH: Polycyclic aromatic hydrocarbon; QFED: Quarter factorial experimental design ; SBME: Solvent bar microextraction; UV: Ultraviolet

Table 2.9: Experimental design applications for LC-MS optimization

Analyte	Sample	Experimental Design	Detector	Reference
Pesticides	Drinking Water	CCD	LC-ESI-QQQ-MS/MS	Müller et al., 2007
Zwitterionic antiepilepticdrugs	plasma samples	BBD	LC- ESI-QQQ-MS/MS	Kostić et al., 2013
Irgarol, Diuron, & its degradation products	Environmental Samples	FED&CCD	LC–ESI–MS/MS , LC–APCI– MS/MS	Maragou et al., 2011
Hydroxamate siderophores	Pure standard	CCD	LC/ESI-ITMS	Moberg et al., 2006
Estriol,ibuprofen ,and morphine	urine	CCD	LC-ESI-MS	Moberg et al., 2000
Acidic compounds	Pure standard	MFED	LC-ESI-QQQ-MS/MS	Seto et al., 2002
Genotoxic 4-dimethylaminopyridine	glucocorticoid drug	CCD&FED	LC-ESI-QQQ-MS/MS	Székely et al., 2012
Metabolite	urine sample and Xylem tissue	CCD	LC-ESI-QTOF-MS/MS	Eliasson et al., 2012
Contaminants	water sample	CCD	LC-HRMS	Meng Hu et al., 2016
Metabolites	urine sample	PBD&CCD	LC-ESI-QTOF-MS/M	Zheng et al., 2013
Estrogenic compounds	beer	FFD	LC-ESI-QQQ-MS/MS	Maragou et al., 2008
GXG tripeptides	Pure standard	FFD	LC- ESI-MS	Raji & Schug, 2009
Pharmaceutical	Pure standard	FED&CCD	LC-MS	de Sousa et al., 2008
Alkaloids	Meconopsi species	BBD	LC-ESI-QTOF-MS/MS	Zhou et al., 2009
Pesticides	Fruit and Vegetables	PBD&BBD	LC-ESI-QQQ-MS/MS	Lawal et al., 2018
NSAIDs and analgesic drugs	Pure standard	PBD&CCD	LC-ESI-QQQ-MS/MS	Paíga et al., 2016
Fluoxetine	Human Plasma	BBD	LC-ESI-QQQ-MS/MS	Hasnain et al., 2016
human urinary amino acids	urine sample	BBD	LC-ESI-QQQ-MS/MS	Peris-Díaz et al., 2018
Guanidinoacetate and creatine	human urine and plasma.	BBD	LC-ESI-QQQ-MS/MS	Ovanov et al., 2018
Genotoxic impurities	meropenem	BBD	LC-ESI-QQQ-MS/MS	Grigori et al., 2017
Enzalutamide	plasma samples	TOAD&CCD	LC-ESI-QQQ-MS/MS	Sankar et al., 2017
Sildenafil	human plasma	BBD	LC-ESI-QQQ-MS/MS	Hasnain et al., 2017
Dexmedetomidine	Human blood	CCD& PBD	LC-ESI-QQQ-MS/MS	Szerkus et al., 2017
Levofloxacin and ciprofloxacin	Prostate samples	PBD	LC-ESI-QQQ-MS/MS	Szerkus et al., 2017

BBD: Box Behnken design;CCD : Central composite design; ESI: Electrospray ionization source; FED: Fractional factorial design;FFD: full factorial design;HRMS: high resolution mass spectrometry; LC: Liquid chromatography; MFFD: modified fractional factorial design; MS/MS: Tandem mass spectrometer; NSAID: Nonsteroidal anti-inflammatory drugs ; PBD:Plackett–Burman designs ; QTOF: quadrupole time of flight;QQQ: Triple quadrupole ;TOAD: Taguchi orthogonal array design.

CHAPTER 3: EXPERIMENTAL

3.1 Develop and optimize the analytical performance of LC-ESI-QQQ-MS/MS method for the determination of multi-mycotoxins (AFB1, B2, G1, G2, and OTA).

3.1.1 General Reagents and Materials

Aflatoxins with standard mixture of AFB1 (1 mg/L), AFG1 (1 mg/L), AFB2 (0.3 mg/L), and AFG2 (0.3 mg/L) in MeOH, and Ochratoxin (OTA) standard (50 mg/L), glacial acetic acid, and formic acid were obtained from Sigma-Aldrich (Darmstadt, Germany). Ammonium formate and ammonium acetate were obtained from Agilent Technologies (Santa Clara, CA, USA). Sodium chloride and anhydrous magnesium sulfate, primary secondary amine (PSA), C18 sorbent, and LCMS-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultra-pure water (ELGA) was used throughout this study.

3.1.2 Food Sample

3.1.2.1 Liquid Food Sample

Fruit juices (apple, grape, orange, and pomegranate) were purchased and used in this study, i.e., Sunkist brands from Malaysia, Judi brand from Libya, Cesar and Original brands from Saudi Arabia, and Pfanner brand from Austria. All packages were kept in at 4 ^{o}C until analysis.

3.1.2.2 Solid Food Sample

Solid food samples, i.e. high sugar and low water content (raisin & fig), high oil content and low water content (peanut and pistachio) were purchased from Kuala Lumpur market. Dried fruits and nuts samples were pulverized to obtain fine homogeneous granules using an electric spice and nut grinder to reduce the particle size. The homogenized samples were then kept in a tightly closed vial and stored at 4 ^{o}C until

analysis. Then, a subsample is removed for mycotoxin extraction. It is assumed that the mycotoxin distribution among contaminated particles in the sample has closer distribution among contaminated kernels found in the lot (Whitaker, 2006). Three replicates of each subsample were analyzed for mycotoxins content.

3.1.3 Sample Preparation

3.1.3.1 Non- and Low-Fat Content Samples Using QuEChERS Method for LC-MS Study

The extraction of mycotoxins from various food samples (fruit juices, dried figs, dried raisins, wheat flour, barley flour, chili and spices) was performed according to the QuEChERS method (Vaclavik et al., 2010). Briefly, 2.0 g of homogenized solid food (or 2 mL liquid sample) was weighed and transferred into a 50 mL polypropylene tube. 10 mL of acetonitrile (acidified with 1% acetic acid) and 7.5 mL of cold water added to the tube, shaken for 1 min and vortexed for 4 min. 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride were then added to the mixture and shaken for 3 minutes followed by centrifugation for 6 min at 7500 rpm. 4 mL from the upper organic phase was pipetted into another 15 mL centrifuge tube that contained 0.2 g PSA and 0.6 g of fine powder anhydrous magnesium sulfate. The extract was further shaken for 2 min and centrifuged at 4000 rpm for 5 min. 2.5 mL of the supernatant was evaporated to dryness by a rotary evaporator and reconstituted with 1 mL of methanol , and filtered through a 0.22 μm nylon syringe filter prior to the LCMS analysis.

3.1.3.2 High-Fat Content Sample Using QuEChERS Method for LCMS Study

The extraction of mycotoxins from nut samples (non-roasted peanut and roasted pistachio) was performed according to the QuEChERS method described by (Liu et al., 2014). 2.5 g of the homogeneous nuts sample was weighed and transferred into a 50 mL polypropylene tube. 20 mL of acetonitrile aqueous solution (80:20, v/v) containing 0.1%

formic acid was added to the mixture and shaken for 30 min at 300 rpm. The mixture was then centrifuged for 5 min at 8000 rpm and the supernatant was transferred into a clean vial. The residue was extracted again with 5 mL acetonitrile aqueous solution (20:80, v/v) containing 0.1% formic acid for 5 min followed by centrifuging at 8,000 rpm for another 5 min. The supernatant was pipetted out and combined with the earlier supernatant. 4 g of magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate and 0.5 g of sodium hydrogen citrate sesquihydrate were added to the combined supernatant and shaken for 1 min.

The fat content was removed by treating the extracts with 20 mL of hexane (2 times), vortexing for 1 min and standing for 5 min to separate the hexane layer from the extract. For dSPE clean-up, the bottom layer was transferred into a clean tube containing 150 mg of C18 and 900 mg of magnesium sulfate to ensure the removal of any fat remains. The cloudy solution was shaken for 1 min and centrifuged at 8,000 rpm for 5 min. The supernatant was transferred to a clean tube and washed twice with 5 mL of acetonitrile. The mixture was evaporated to dryness by a rotary evaporator and reconstituted with 1 mL of methanol and filtered through a 0.22 μm nylon syringe filter prior to LCMS analysis.

3.1.4 LC-MS

Liquid chromatography was performed on a 1290 series (Agilent Technologies, Waldbronn, Germany). Separation of mycotoxins was achieved on a reversed-phase C18 column (ODS) (15 x 2.1 mm; 5μm), held at 30 °C with an injection volume of 4 μl. The mobile phase consisted of (A) 5 Mm of ammonium formate in water and adjusting the pH by adding the formic acid and (B) MeOH. LC Gradient program: 0 min, (A) 40% and (B) 60% with flow rate of 0.195 mL/min; 2 min, (A) 75% and (B) 25% at flow rate 0.195 mL/min; 4-6 min, (A) 0% and (B)100% with flow rate 0.250 mL/min; 6-8 min, (A) 10%

and (B) 90% at flow rate 0.200 mL/min. The system was re-equilibrated with the initial composition for 5 min, prior to the next injection. Mass spectrometry was performed on a 6490 QqQ system (Agilent Technologies-Singapore) equipped with an Agilent Jet-Stream ESI interface. Data acquisition, processing, and instrument control were performed through the Mass Hunter Workstation B.06.01 software. The mass spectra were acquired over the mass range of 100 to 1000 m/z. The ionization source was operated in positive mode at the following conditions: gas flow = 13.65 L/min and temperature = 170.20 °C, nebulizer gas pressure = 20 psi, sheath gas flow = 11 L/min and temperature = 250 °C. The ESI setting was optimized using a mycotoxins standard solution containing 100 µg/L AFB1, AFG1, and OTA, and 30 µg/L of AFB2 and AFG2.

3.1.5 Strategy of LC-MS/MS Optimization

The development of an LC-MS/MS method for the determination of different compounds can be a tedious task because numerous parameters are involved, affecting the response in various ways. Moreover, different manufacturers of instruments have special settings as a result of specific electronics, geometrical designs and demand. Numerous LC-MS/MS conditions have been reported for analysis of mycotoxins. There do not appear to be universal conditions for all mycotoxins and the selection is dependent on the target mycotoxin. According to the Analytical Methods Committee in the optimization of LC-MS/MS (Sargent, 2013) and the factors and their respective upper and lower levels within the instrument operation parameters as recommended by the manufacturer (Agilent, 2014), the parameters that improve the separation and the ionization process have to be investigated. Moreover, some reports on LC-MS/MS optimization have mentioned the most significant parameter (Moberg et al., 2006;Diana Di Mavungu et al., 2009; Rubert et al., 2012). With reference to this, the following parameters were selected for this study:

(a) HPLC parameters: - Mobile phase (Additives, pH, organic solvent %, flow Rate), column temperature, injection volume.

(b) Mass Detector parameters: -Sheath gas temperature and flow, drying gas flow and temperature, nebulizer pressure, and collision energy.

3.1.5.2 Univariate Optimization of LC-MS/MS

The univariate method was used to optimize instrument parameters mentioned in the Section (3.1.5) and also considered as a preliminary approach prior to multivariate approach (Chen et al., 2015). In this method, one factor value was changed, and others kept constant. All univariate experiments were performed using 100 µg/L of aflatoxin B1, G1, and Ochratoxin A, and 30 µg/L of aflatoxins B2 and G2. In addition to helping in the selection of factors and their respective upper and lower levels, the univariate method was used to compare the results with multivariate study and their effects on the parameters of LC-MS/MS. The univariate optimization of LC-MS/MS parameters and their levels are summarized in Table 3.1

Table 3.1: LC-MS/MS parameters ranges for univariate method.

Factor	Levels	
	Minimum	**Maximum**
Mobile phase additives	Acetic acid &formic acid and ammonium formate & acetate	
pH	3	6
Organic Solvent (OS)%	30	60
Flow Rate (FR) (mL/min)	0.100	0.200
Column Temperature (CT) (°C)	25	35
Injection Volume (IV) (µL)	2	5
Sheath Gas Temperature (SGT) (°C)	150	250
Sheath Gas Flow (SGF) (L/min)	9	12
Gas Flow (GF) (L/min)	12	18
Gas Temperature (G T) (°C)	150	250
Nebulizer pressure (NP (psi)	20	35
Collision Energy (CE) (eV)	25	40

3.1.5.3 Multivariate Optimization for LC-MS/MS

(a) Plackett-Burman Design (PBD)

Experimental plan and data interpretation were performed using MINITAB® Release 17 Statistical Software (State College, PA, USA). Plackett-Burman Design provides information on each factor with a minimum number of the experiment (Khodadoust & Hadjmohammadi, 2011). In this study, PBD optimization was carried out to evaluate 11 factors and determined which factors have a significant effect on the total chromatographic peak area (TCPA) and mean retention time (MRT) (Stalikas et al., 2009) on the mycotoxins (AFB1, AFB2, AFG1, AFG2, and OTA). All experiments were carried out using mycotoxins mixture containing 100 ppb of aflatoxin B1, G1 and Ochratoxin A; and 30 ppb of aflatoxins B2 and G2 in 24 experiments

Table 3.2: Factors and levels of variables for PBD

Factors	Levels	
	Low(-1)	High(+1)
pH	3	6
Organic Solvent (OS)%	30	60
Flow Rate (FR) (mL/min)	0.100	0.200
Column Temperature (CT) (ºC)	25	35
Injection Volume (IV) (µL)	2	5
Sheath Gas Temperature (SGT) (ºC)	150	250
Sheath Gas Flow (SGF) (L/min)	9	12
Gas Flow (GF) (L/min)	12	18
Gas Temperature (G T) (ºC)	150	250
Nebulizer pressure (NP (psi)	20	35
Collision Energy (CE) (eV)	25	40

The 24 experimental runs format was selected over other format (i.e., 12, 16 or 20 experimental runs) because it can accommodate more parameters for evaluation and provide better results, especially in evaluating interactions between two factors , offer more precise estimates of the factor effects (Schoen et al., 2017) , and lead to a resolution design in which the main effects are no longer confounded with two-factor interactions (Bell et al., 2009). It was observed in the OFAT study that the peaks area and the retention time of all studied mycotoxins were changed in parallel with any change in the LC-MS/MS parameters. Moreover, the TCPA was reported as a response in many published articles, for example, Lukman and Tan (Abdulra'uf & Tan, 2015) or by using mean recovery obtained (%) for the analytes as a response(Manav et al., 2019). And lastly, to decrease the data yielded from using the experimental design for all five mycotoxins. Therefore, the total chromatographic peak area (TCPA) and the mean retention time (MRT) corresponding to the five mycotoxins (AFB1, AFB2, AFG1, AFG2, and OTA) responses were evaluated. The retention time was used as a second response (Schwartz, 2014). The factors and their respective upper and lower levels have been pre-selected using a univariate method which is within the instrument operation parameters as recommended by the manufacturer. Tables 3.2 shows PBD factors and levels. Minitab 17 software was used to create a design matrix (Table 3.3). The data model adequacy was detected by variance analysis (ANOVA), and the results are illustrated in Table 4.5. ANOVA analysis was used to evaluate the model adequacy and identify significant variables using F-test (Bordagaray et al., 2011). The fit quality of the polynomial model was expressed by the coefficient of determination (Paíga et al., 2016) with the lowest value for R^2 >0.8 (Saguy & Graf, 1990).

Table 3.3: Plackett-Burman design matrix

Run	pH	OS	FR	CT	IV	SGT	SGF	GF	GT	NP	CE
1	+1	-1	+1	-1	-1	+1	+1	-1	-1	+1	+1
2	-1	+1	-1	+1	-1	-1	+1	+1	-1	-1	+1
3	-1	-1	-1	+1	-1	+1	-1	-1	+1	+1	-1
4	-1	+1	+1	+1	+1	+1	-1	-1	-1	-1	+1
5	-1	+1	-1	+1	+1	+1	+1	+1	-1	-1	-1
6	+1	-1	+1	+1	+1	+1	+1	-1	-1	-1	-1
7	+1	+1	+1	+1	+1	-1	-1	-1	-1	+1	-1
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
9	+1	-1	-1	-1	-1	+1	-1	+1	-1	-1	+1
10	+1	+1	+1	+1	-1	-1	-1	-1	+1	-1	+1
11	-1	+1	+1	-1	+1	-1	+1	+1	+1	+1	+1
12	+1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1
13	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1	-1
14	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1	+1
15	+1	+1	-1	-1	+1	+1	-1	+1	-1	+1	+1
16	+1	-1	-1	+1	+1	-1	-1	+1	+1	-1	+1
17	+1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1
18	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	-1
19	-1	-1	-1	-1	+1	-1	+1	-1	-1	+1	+1
20	-1	-1	+1	+1	-1	-1	+1	+1	-1	+1	-1
21	-1	+1	+1	-1	-1	+1	+1	-1	+1	-1	+1
22	+1	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1
23	-1	+1	-1	-1	+1	+1	-1	-1	+1	+1	-1
24	-1	-1	+1	-1	+1	-1	-1	+1	+1	-1	-1

CE: collision energy, CT: column temperature, FR: flow rate, GF: gas flow, GT: gas temperature, IV: injection volume, NP: nebulizer pressure, OS: organic solvent percentage, SGF: sheath gas flow, SGT: sheath gas temperature.

.

(b) Behnken Design (BBD)

LC-MS/MS parameters were optimized using the Box-Behnken Design (BBD) which was carried out in 46 runs under 2 block factors using same MINITAB® Release 17 Statistical Software (State College, PA, USA). All BBD experiments were carried out using samples containing mixtures with same amount of mycotoxins (100 µg/L concentration for each of the aflatoxin B1, G1, and Ochratoxin A, and 30 µg/L concentration for each of aflatoxins B2, and G2). Moreover, the same responses (TCPA & MRT) corresponding to the five mycotoxins (AFB1, AFB2, AFG1, AFG2, and OTA) were used for contour and desirability surface plot (Abdulra'uf & Tan, 2015). The variables used in the generation of BBD experimental levels and design matrix are summarized in Tables 3.4 and 3.5 respectively. The main effect was estimated for each factor applying least square regression which indicates the significance of the response.

Table 3.4: BBD factors level.

Level	OS%	FR (mL/min)	IV(µL)	GT (°C)	GF (L/min)
-1	40	0.100	2	150	12
0	50	0.150	3	200	14
+1	60	0.200	4	250	16

FR: Flow rate, GF: gas flow, GT: gas temperature, IV: injection volume, OS%: organic solvent per cent.

Table 3.5: BBD matrix

RunOrder	Blocks	O S	F R	I V	G T	G F
1	1	0	0	0	0	0
2	1	-1	-1	0	0	0
3	1	0	0	0	+1	-1
4	1	+1	0	-1	0	0
5	1	+1	-1	0	0	0
6	1	0	-1	0	0	+1
7	1	-1	0	-1	0	0
8	1	-1	+1	0	0	0
9	1	0	0	0	-1	+1
10	1	0	0	0	0	0
11	1	0	0	0	-1	-1
12	1	0	+1	0	0	+1
13	1	0	+1	0	0	-1
14	1	-1	0	+1	0	0
15	1	0	0	0	0	0
16	1	0	-1	0	0	-1
17	1	0	0	+1	+1	0
18	1	+1	0	+1	0	0
19	1	0	0	0	+1	+1
20	1	0	0	-1	-1	0
21	1	+1	+1	0	0	0
22	1	0	0	-1	+1	0
23	1	0	0	+1	-1	0
24	2	+1	0	0	+1	0
25	2	+1	0	0	-1	0
26	2	0	0	0	0	0
27	2	0	+1	-1	0	0
28	2	0	-1	0	+1	0
29	2	0	-1	0	-1	0
30	2	0	-1	+1	0	0
31	2	0	0	-1	0	+1
32	2	0	+1	0	0	0
33	2	0	-1	-1	0	0
34	2	0	0	0	0	0
35	2	0	0	+1	0	+1
36	2	0	+1	0	-1	0
37	2	+1	0	0	0	-1
38	2	0	0	-1	0	-1
39	2	-1	0	0	0	-1
40	2	-1	0	0	-1	0
41	2	-1	0	0	+1	0
42	2	0	0	+1	0	-1
43	2	0	+1	0	+1	0
44	2	0	0	0	0	0
45	2	-1	0	0	0	+1
46	2	+1	0	0	0	+1

FR: Flow rate, GF: gas flow, GT: gas temperature, IV: injection volume, OS: organic solvent

3.2 Develop and optimize a U-Shaped HF-LPME Method for the Preconcentration of Liquid and Solid Food Samples in the Determination of Mycotoxins by LC-ESI-QQQ-MS/MS

3.2.1 General Reagents, Chemicals, Apparatus and Equipment

3.2.1.1 General Reagents and Chemicals.

Aflatoxins were mixed in methanol at 1 mg/L for aflatoxins B1 and G1 and 0.3 mg/L for aflatoxins B2 and G2 and ochratoxin A at 10 mg/L in acetonitrile from Sigma-Aldrich (Darmstadt, Germany). HPLC grade formic acid and ammonium formate for mass spectrometry (_99.0%) were employed. The 1-octanol, 1-decanol, dihexyl ether and 2-hexyl-1-decanol were purchased from Sigma–Aldrich (Darmstadt, Germany). Acetonitrile and methanol (LC-MS grade) were used in liquid chromatography, and the dispersive solvent (HPLC grade chloroform) was purchased from Merck (Darmstadt, Germany). Ultra-pure water from a Milli-Q water purification system (MA, USA) was used throughout this study. The working standard solution was prepared by diluting the stock solution in methanol.

3.2.1.2 Apparatus and Equipment

Q3/2 Accurel PP hydrophobic polypropylene hollow fiber tubing (200 mm wall thickness,600 mm i.d., and 0.2 mm pore size) was obtained from Membrana GmbH (Wuppertal, Germany). A hot plate with a magnetic stirrer and a stir bar were obtained from Fisher Scientific. An ultrasonic bath (Branson 3200) was obtained from Branson

3.2.1.3 Food Samples for OTA Analysis

The validated HF-LPME method for monitoring of OTA content was applied to qualify and quantify of OTA in 60 types of liquid and solid food samples (five samples for each food type). All samples were purchased from Kuala Lumpur between April and May 2017. Each sample was prepared in triplicate according to the sample extraction and clean-up procedures under the optimized conditions. juice (apple, grape, orange, and

pomegranate), dried fruits (raisin and Fig), flour (wheat and barley), nuts (non-roasted peanut and roasted pistachio), and spice (chili and mixed spice).

3.2.1.4 Food Samples for Aflatoxins Analysis

The validated HF-LPME method for monitoring of aflatoxins (AFB1, AFB2, AFG1, and AFG2) content was applied to detect the target analytes in 60 types of liquid and solid food samples. 10 samples from each sample kind (apple juice, grape juice, raisin, dried fig, non-roasted peanut, and roasted pistachio) were purchased from Kuala Lumpur between April and May 2017.

3.2.2 U-Shape HF-LPME

3.2.2.1 Ochratoxin Analysis

The two-phase U-shaped HF-LPME polypropylene hollow fibers membrane were cut into 6 cm length pieces. To avoid contamination, each hollow fiber was sonicated in acetone (10 min) and dried by air flow. Each piece was used once only. The hollow fiber was first soaked in octanol (supported liquid membrane solvent) for 10 s to fill the pores and then washed with ultra-pure water (10 s) to remove the extra octanol. Both ends of the hollow fiber (6 cm) were connected to the micro-syringe needle and filled with approximately 16 µL of octanol (extraction/acceptor phase solvent). The extraction phase was then injected to fill the channel and pores of the hollow fiber, followed by introducing the aqueous sample (12 mL) to the U-shaped configuration in a closed vial. This aqueous sample consisted of 10 µg/L of OTA at pH of 2.66 and contained 7% NaCl. Prior to introducing the hollow fiber membrane to the donor phase, 200 µL of the extraction/dispersion solvent was added and mixed quickly with the donor phase. OTA was extracted for 70 min at a stirring speed of 700 rpm. At the end of the extraction period, the hollow fiber membrane was sonicated in 4 mL of acetonitrile to desorb the analyte from membrane and acceptor phase. After that, the extraction phase was evaporated under

vacuum by rotary evaporator to dryness and reconstituted in 1 mL of the mobile phase for LC-MS/MS analysis.

3.2.2.2 Aflatoxins Analysis

The method is similar to the one in section 3.2.3.1 except for some changes in the following parameters a: The donor phase consists of pH 8 with 2% of NaCl. Prior to introducing the hollow fiber membrane to the donor phase, 150 µL of the extraction/dispersion solvent was added and mixed quickly with the donor phase. Aflatoxins were extracted for 50 min at a stirring speed of 700 rpm.

3.2.3 Solid Food Sample Using QuEChERS Method for HF-LPME Study

Food samples were extracted similarly to the published method by Sirhan et al (Sirhan et al., 2012). 0.5 g of homogenized food sample was weighed into a 50 mL polypropylene tube. 5 mL of mixture 10:70:20 (MeOH: ACN:H_2O) was added to the tube and shaken for 1 min. A mixture of 1.32 g of anhydrous $MgSO_4$ and 0.25 g of NaCl was added into the sample tube and shaken again for 1 min. This was to ensure a complete partition of the target analyte into the organic layer and followed by 5 min of centrifugation at 4000 rpm. To remove the fat in high-fat content samples, 2 mL of the upper organic layer was mixed with 2 mL of hexane and vortexed for 1 min, followed by standing for 5 min. 1 mL of the lower layer was pipetted out for subsequent clean-up procedure using a two-phase U-Shaped HF-LPME prior to LC-ESI-QQQ-MS for OTA analysis.

3.2.4 Solid Food Sample Using Ultrasound Assisted Solid-Liquid Extraction (UASLE) Method for HF-LPME Study

Food samples were extracted according to the method published by Li et al. (Li et al., 2016). 1.0 g of grounded and homogenized sample (dried fruit and nut) were weighed into a 50 mL polypropylene tube. 8 mL of 10% methanol, 63.33 % acetonitrile and 26.67% of water containing 1% acetic acid were added and vortexed for 2 min. The

mixture was exposed for 15 min in an ultrasonic bath and centrifuged at 4500 rpm for 5 min. The supernatant was mixed with 15 mL hexane and vortexed for 1 min to remove the fats from the sample. After standing for 5 min, the supernatant was immediately transferred out for a subsequent clean-up process using a U-Shaped hollow fiber liquid-liquid microextraction prior to LC-ESI-QQQ-MS for OTA analysis.

3.2.5 Optimization Strategy

The use of multivariate approaches has not been widely applied by all scientists due to many technical reasons (Nunes et al., 2015). However, both univariate and multivariate used in this study provided good results for the optimization processes. The experimental designs have advantages of revealing interactions among variables, and the non-linear relationships with the responses. Based on these reasons, the univariate method and central composite design (CCD) approach were used for studying the optimal variables in this HF-LPME for OTA. In addition, another multivariate approach was used for the extraction of solvent components called the simple lattice design.

3.2.5.1 Univariate Optimization for HF-LPME Study

(a) Ochratoxin Analysis

The combined two LPME methods (Two phase-HF-LPME & DLLME) is able to reduce the drawbacks and improve the HF-LPME performance. According to published reports (Prosen, 2014; Sharifi et al., 2016), the are specific critical HF-LPME parameters which are SLM solvent, donor phase pH, ionic strength, extraction time and agitation speed. Four HF-LPME parameters were optimized using the central composite design, while the other parameters were studied using the univariate approach(one-factor at a time). The variables assessed for ochratoxin (OTA) analysis using univariate method are: the type of organic solvent as a supported liquid membrane (SLM), acceptor solvent, and extraction/dispersion (ratio and volume). The experiments were carried out using

samples containing 10 µg/L OTA in pure water. Excel 365 was used for statistical analysis.

(b) Aflatoxin Analysis

The variables assessed for aflatoxin analysis using the univariate method are: type of organic solvents (SLM), length of the membrane, pH of donor phase, the percentage of NaCl, extraction time, agitation speed,dispersion/extraction (ratio, volume), and desorption time. The experiments were carried out using samples containing 5 µg/L of AFB1 and AFG1, and 1.5 µg/L of AFG2 and AFB2 in pure water. Excel 365 was used for statistical analysis.

3.2.5.2 Multivariate Optimization for HF-LPME Study for OTA Analysis.

Second-order central composite design (CCD) with response surface methodology (RSM) were used to optimize four extraction variables, i.e., pH of donor phase, the percentage of NaCl, extraction time, and agitation speed. To minimize the effect of the uncontrolled variables, the CCD experiments were run in a random manner. The CCD design included 16 cube points, 4 center points in a cube, 8 axial points and 2 center point in axial with $\alpha = 2$ and a total of 30 randomized runs with two blocks. The points number in CCD is the factorial run of 2k, axial runs of 2k and center point runs. The selected variables involved in the generation of CCD and levels are shown in Table 3.6 Design generation and statistical analysis were performed using MINITAB® Release 17 Statistical Software (State College, PA, USA) (Table 3.7).

Table 3.6: Factors and levels in CCD design

Factors	Levels			Star points	
	Low (-1)	**Central (0)**	**High (+1)**	**-α**	**+α**
pH (A)	3.0	5.5	8.0	0.5	10.5
Time (min)(B)	40	70	100	10	130
Agitation speed (rpm)(C)	400	700	1000	100	1300
NaCl (%)(D)	4	6	8	2	10

The total experimental runs (N) of CCD is given by N = 2k + 2k + Co, where k and Co are the numbers of variables and center points, respectively (Stalikas et al., 2009). CCD allows regression variables to fit a second-degree polynomial model. As in equation 3.1, a polynomial determines the relationships among the quantified response y and some experimental variables x1…xk, where **k** is the factors number, **β** is the regressors and **Ɛ** is an error associated with the model

$$y = \beta_o + \beta_i x_i + \beta_i^2 x_i^2 + \beta_{ij} x_i x_j + \varepsilon \text{……..(3.1)}$$

The mean peak area of OTA was chosen as the experimental response. The fit quality of the polynomial model was expressed by the coefficient of determination(Paíga et al., 2016), and the lowest value acceptable is R^2>0.8 (Saguy & Graf, 1990). In the present study, the ANOVA results (Table 4.16) revealed R^2 = 0.93 with p<0.05 for linear and square terms, and no significant interacting effects among the investigated factors.

Table 3.7: Central composite design (CCD) matrix

RunOrder	Blocks	pH	NaCl %	EX.T(min)	St.S(rpm)
1	1	+1	-1	+1	+1
2	1	+1	-1	+1	-1
3	1	-1	-1	+1	+1
4	1	0	0	0	0
5	1	+1	-1	-1	+1
6	1	+1	-1	-1	-1
7	1	0	0	0	0
8	1	0	0	0	0
9	1	-1	-1	+1	-1
10	1	-1	+1	+1	+1
11	1	+1	+1	+1	-1
12	1	-1	-1	-1	+1
13	1	+1	+1	-1	-1
14	1	+1	+1	-1	+1
15	1	-1	+1	+1	-1
16	1	0	0	0	0
17	1	-1	+1	-1	+1
18	1	+1	+1	+1	+1
19	1	-1	-1	-1	-1
20	1	-1	+1	-1	-1
21	2	0	0	-α	0
22	2	0	0	0	0
23	2	0	0	0	0
24	2	+α	0	0	0
25	2	0	-α	0	0
26	2	0	0	0	-α
27	2	0	+α	0	0
28	2	0	0	+α	0
29	2	0	0	0	+α
30	2	-α	0	0	0

EX.T:Extraction time in minute; NaCl %:Percent of sodium chloride; St.S(rpm):Stirring speed

3.2.5.3 Simple Lattice Design for QuEChERS and Ultrasound Assisted Solid-Liquid Extraction (UASLE) Optimization

Extraction of mycotoxins from solid food samples into a liquid phase is the first step in sample preparation. For a solid sample, using pure organic solvent or pure water is not recommended for the extraction of mycotoxins due to low recovery (Berthiller et al., 2013). Most mycotoxins are highly soluble in organic solvents (methanol, acetonitrile, acetone), but hardly soluble in water. A mixture of organic solvents with water or acidic buffer is used to extract mycotoxins. The water enhance the penetration of the organic

solvents in the food matrix, while adding the acid to the extraction mixture breakup the strong bond between the analyte with other components, and leading to increase extraction efficiency (Alshannaq & Yu, 2017). Solvents assessed in the study are MeOH, ACN, and H_2O. Optimization of the extraction solvent composition was investigated by applying the simple lattice design. The simple lattice design is an alternative to simplex centroid designs when reducing the number of experiments is one of the objectives. It is represented as a triangle in which each corner represents one of the main component and the center of each side be regarded as a mixture of the ingredients at the corners connected by the side (Brereton, 2007).

The simple lattice design matrix was constructed using MINITAB® Release 17 Statistical Software (State College, PA, USA). The bonds of mixture components were adjusted to accommodate specified constraints by the Minitab software. Therefore the default total was not equal to 1.0 (Smith 2005) as illustrated in the Table 3.8 and Figure 3.1 for simple design plot of the solvent mixture. Moreover, inverse component terms included in the analysis allows extreme changes in the response as the proportion of one or more components nears its lower boundary (Minitab 2017). The mixture's contour and surface plots, and optimized response plot were constructed to indicate recovery efficiency for each solvent. In this study, the peanut was used as a representative sample for QuEChERS and UASLE solvents optimization. The design matrix is illustrated in Table 3.9. In high lipid content sample, co-extracts such as weak polar lipids could interfere with mycotoxins detection and which resulted in lower extraction yield and cause matrix effects in detectors (Zhou et al., 2017;Zhang et al., 2018). Non-contaminated peanut was selected as a representative for solid food. The peanut was grounded to powdery fine granules and spiked with 1.5 µg/Kg of aflatoxin G2 and 10 µg/Kg of OTA.

Table 3.8: The bonds of mixture components

Compound	Amount		Proportion		Pseudocomponent	
	Lower	Upper	Lower	Upper	Lower	Upper
MeOH	0.1	0.7	0.1	0.7	0.0	1.0
MeCN	0.1	0.7	0.1	0.7	0.0	1.0
H_2O	0.2	0.8	0.2	0.8	0.0	1.0

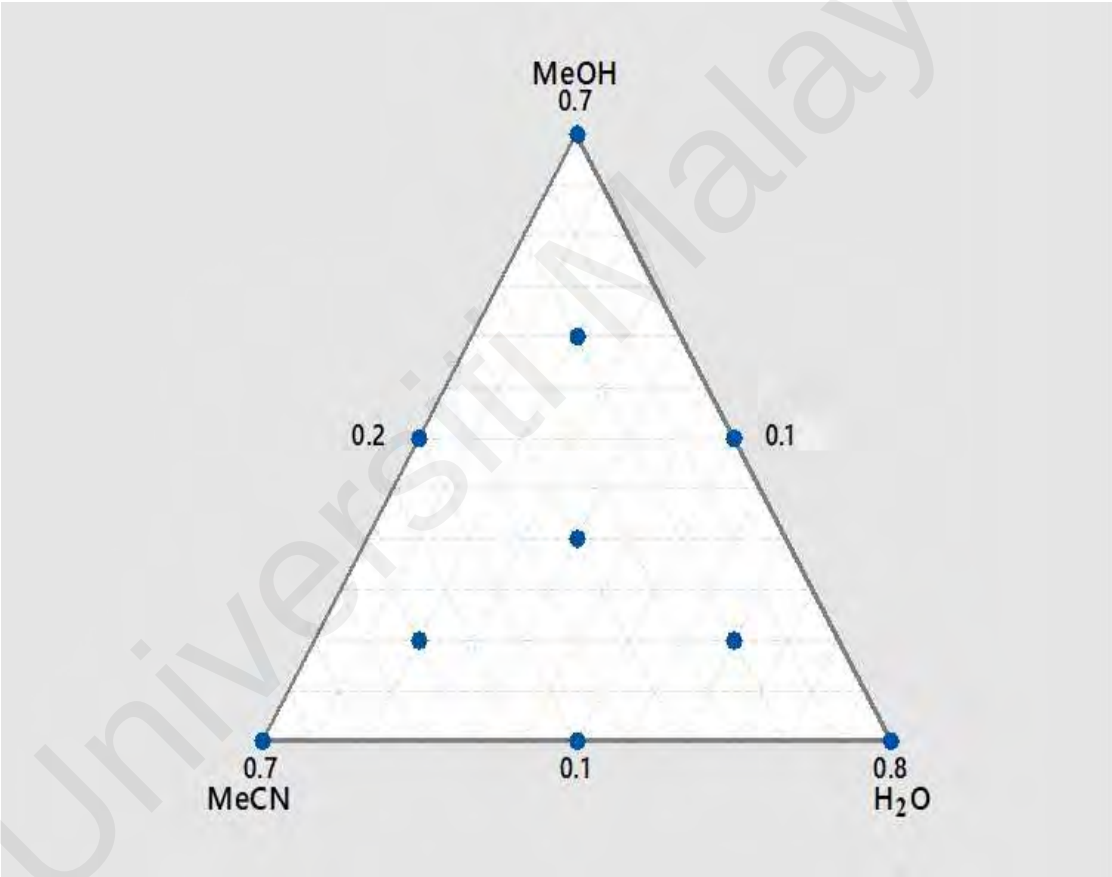

Figure 3.1: Simple design plot for solvent mixture.

Table 3.9: Simple lattice design matrix

RunOrder	Blocks	MeOH	MeCN	H_2O	X1
1	1	0.5	0.2	0.3	-1
2	1	0.3	0.3	0.4	1
3	1	0.2	0.5	0.3	-1
4	1	0.1	0.7	0.2	1
5	1	0.1	0.1	0.8	-1
6	1	0.4	0.4	0.2	-1
7	1	0.3	0.3	0.4	-1
8	1	0.1	0.7	0.2	-1
9	1	0.4	0.1	0.5	-1
10	1	0.5	0.2	0.3	1
11	1	0.1	0.1	0.8	1
12	1	0.7	0.1	0.2	1
13	1	0.1	0.4	0.5	-1
14	1	0.2	0.2	0.6	1
15	1	0.2	0.5	0.3	1
16	1	0.2	0.2	0.6	-1
17	1	0.1	0.4	0.5	1
18	1	0.4	0.1	0.5	1
19	1	0.4	0.4	0.2	1
20	1	0.7	0.1	0.2	-1

3.3 Method Performance

3.3.1 Instrument Validation

The optimized LC-MS/MS method was validated according to the International Conference on Harmonization (ICH) guidelines by assessing several methods of parameters performance such as range, linearity, instrument detection limit (IDL), and intra-day and inter-day precision used to assess the method performance since there are

no official guidelines for LC-MS/MS procedure validation as reported by Kruve et al. (Kruve et al., 2015).

3.3.1.1 Linearity

To study the linearity of the calibration curves, standard solutions were prepared at various concentrations levels in the mobile phase and in blank food matrix extracts and were injected into the LC-MS/MS system. Linearity is determined by a series injection of five or more standards. The response (peak area) should be directly proportional to the amount of analytes (Huber, 2007).

3.3.1.2 Instrument Detection Limit (IDL)

IDL is the minimum amount of analyte required for producing a signal which can be statistically distinguished from the background noise level within a specified confidence level. The IDL is the confidence factor (t) multiply with the standard deviation (SD) of the response of the replicates in term of analyte concentration as illustrated in the following equation (3.2):

$$IDL = t \times SD \quad (3.2)$$

Determination of the confidence factor (t) is achieved by applying Student t-distribution with a 99 % confidence level with n–1 (n = number of replicates) degrees of freedom. The relative standard deviation is expressed as RSD = SD/Mean Value. Hence, it is possible to determine the IDL in units of the amount of the standard by applying the following formula (Kafeenah et al., 2018; Wells et al., 2011):

$$IDL = t \times (RSD/100\%) \times \text{amount measured} \quad (3.3)$$

3.3.1.3 Precision

Precision is a measure of the degree of variation in analytical measurement under a normal operation and usually expressed as the per cent relative standard deviation (RSD%). Repeatability refers to the variability results in measurement over a short period

under the same conditions (intra-assay precision). In order to address the precision of a method, a minimum of 10 replicates are required. In this study, the inter-day precision was estimated based on 10 runs per-day (10 replicates) for four days.

3.3.2 Method Validation

The matrix-matched calibration curves were constructed with matrix spikes. Limit of detection (LOD) and limit of quantification (LOQ) was determined as signal to noise (S/N) = 3 and S/N = 10. The accuracy of the method was studied using the standard addition method with the three concentration levels (1, 10 and 20 µg/kg) for AFB1, AFG1, OTA and 0.3, 3 and 6 µg/kg for the other mycotoxins (AFB2 and AFG2). The precision tests were performed by employing the method of standard addition with the intermediate spiked concentration of 10 µg/kg for AFB1, AFG1, OTA and 3µg/kg for AFB2 and AFG2. The RSD value of the same day was used for evaluation of the intra-day precision (n = 40), while RSD value in three consecutive days were used for inter-day precision (n = 10).

The recovery was obtained according to equation 3.4:

$$RR=\frac{C}{B} \times 100 \ldots\ldots\ldots (3.4)$$

where B is the average peak area obtained after the extraction from a spiked standard and, C is the average peak area obtained from a spiked standard prior to the extraction. (Gjelstad & Pedersen-Bjergaard, 2013)

The enrichment factor (EF) was determined by equation(3.5):

$$EF = \frac{Vs*RR}{100*Va} \ldots\ldots\ldots\ldots\ldots (3.5)$$(Gjelstad & Pedersen-Bjergaard, 2013)

where RR is the recovery of the method, Vs is the sample volume, and Va is the acceptor solution volume.

The effect of sample matrices on ionization efficiency was computed using equation 3.6 (Hall et al., 2012),and indicated as ion suppression when the value is negative, or positive value for ion enhancement. If the average matrix effect is greater than 20%, it can be considered to have a significant effect.

$$ME = \frac{B-A}{A} \times 100 \quad \text{(3.6)}$$ (Hall et al., 2012; Pizzutti et al., 2014)

ME; Matrix effect, A; Average peak area obtained from the neat standard.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 LC-ESI-QQQ-MS/MS Method Development

Liquid chromatography–mass spectrometry (LC–MS) has become the preferred analytical technique for many challenging assays based on its selectivity, sensitivity, and broad applicability to compounds of varying polarities. Despite the advantages of the technique, the complexity of LC–MS systems often leaves analysts struggling to meet method detection limits. Several strategies are applied to improve method sensitivity by the careful selection of LC conditions, and the optimization of MS interface settings.

4.1.1 Total Ion Scan

In tandem mass spectrometry, precursor or parent ions selected in the 1st quadrupole were fragmented in a collision cell (2nd quadrupole) to produce an analyte-specific product or daughter ions and subsequently monitored under multiple reaction monitoring (MRM) data acquisition mode in the 3rd quadrupole (Kotretsou & Koutsodimou, 2006). The full-scan mode was applied in a range from 100 to 500 m/z for the preliminary screening of mycotoxins. The results revealed that all mycotoxins could produce parent ion $[M+H]^+$ under ESI-positive mode. The selection of parent and product ions was summarized in Table 4.1.

4.1.2 MS Transitions (Product Ion Scan) and Ion Ratio

The EU regulations required the selection of two product or daughter ions for quantitative and qualitative confirmation purpose (Directive, 2002). The selection of product ions was carried out by varying the collision energy to yield the best intensity. The two highest abundance fragments were used for the subsequent experiments. Agilent mass hunter qualitative software was used in this work, where it included the information of relative ion ratios for further verification. Figure 4.1 shows the observed ion ratios and MRM transitions.

Table 4.1: Mycotoxins MS database and product ions.

Compound name	MF	Mass	MW	PI	MI	Product Ion (*m/z*)		CE
Aflatoxin (B1)	$C_{17}H_{12}O_6$	312.063388	312.27358	313.1	$[M+H]^+$	Quantifier	241.1	41
						Qualifier	285.2	25
Aflatoxin(B2)	$C_{17}H_{14}O_6$	314.079038	314.28946	315.1	$[M+H]^+$	Qualifier	259.1	29
						Quantifier	287.2	29
Aflatoxin(G1)	$C_{17}H_{12}O_7$	328.058303	328.27298	329.1	$[M+H]^+$	Quantifier	243.1	29
						Qualifier	215.2	37
Aflatoxin (G2)	$C_{17}H_{14}O_7$	330.073953	330.28886	331.1	$[M+H]^+$	Quantifier	313.1	25
						Qualifier	245.0	29
Ochratoxin A (OTA)	$C_{20}H_{18}ClNO_6$	403.082265	403.8130	404.1	$[M+H]^+$	Qualifier	221.0	38
						Quantifier	239.0	26

CE: Collision energy; MF: Molecular formula; MI: Molecular ion; MW: Molecular weight; PI: Precursor ion

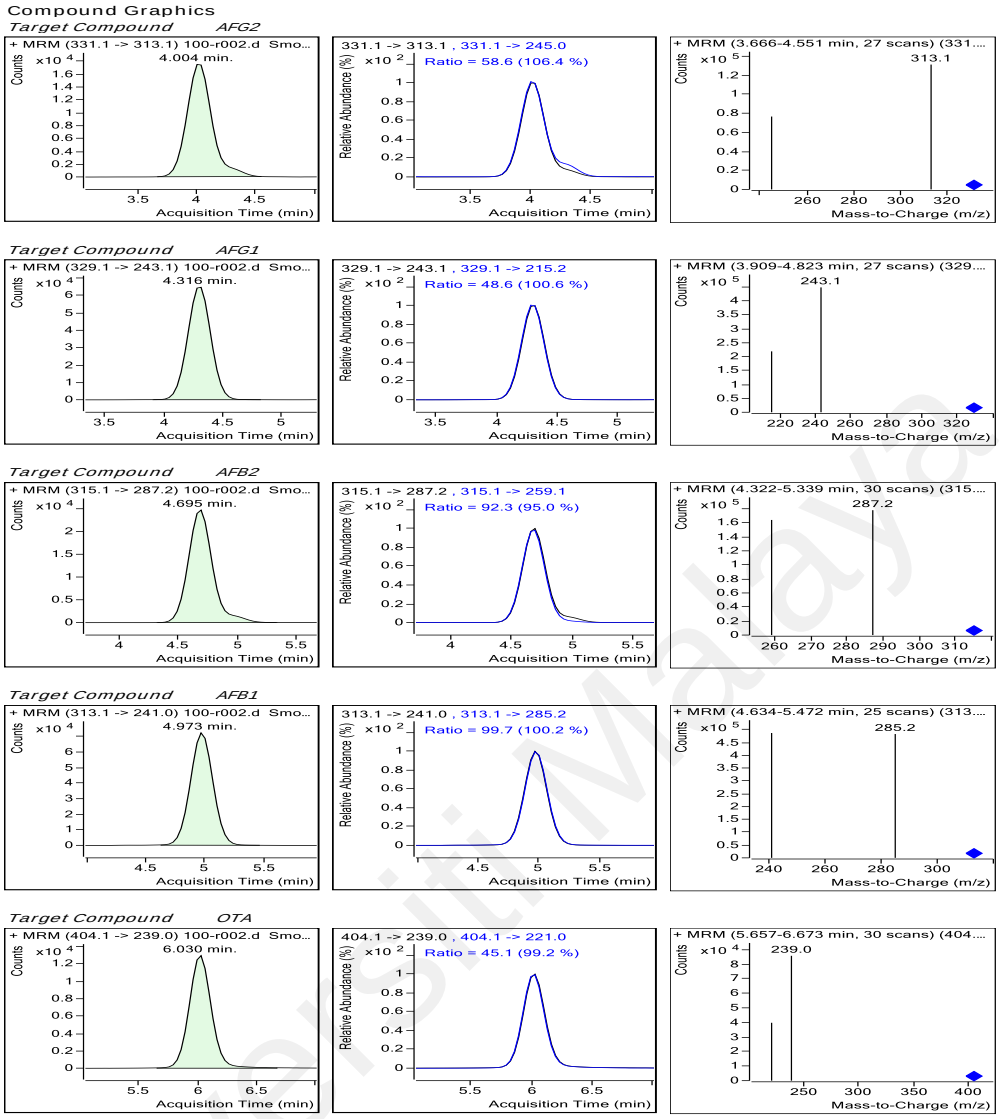

Figure 4.1: MRM transitions of target analyte

4.1.3 Univariate Optimization

4.1.3.1 Mobile Phase Additives

MeOH and acetonitrile are routinely used in LC analysis, including the separation of mycotoxins (B1, B2, G1, G2, and OTA). Previous studies indicated that addition of additives such as formic acid, acetic acid, ammonium acetate,and ammonium formate improved the ionization efficiency and produce better chromatography spectrum in the

analysis of mycotoxins (Sulyok et al., 2006).A combination of ammonium formate and formic acid in the mobile phase gave the best chromatographic results due of the increase in polarity (Liao et al., 2011) and the ionization mode (positive or negative) is taken intop consideration in the optimization process. In the present study, a combination of formic acid and the ammonium formate was selected as an additive for the mobile phase A (H_2O) (Fig. 4.2). Our results indicated that the combination of formic acid and the ammonium format gave the best response in the positive mode in contrast with previous report on the determination of 24 mycotoxins using LC-MS/MS which indicated the best response in the negative mode ionization(Lauwers et al., 2019).

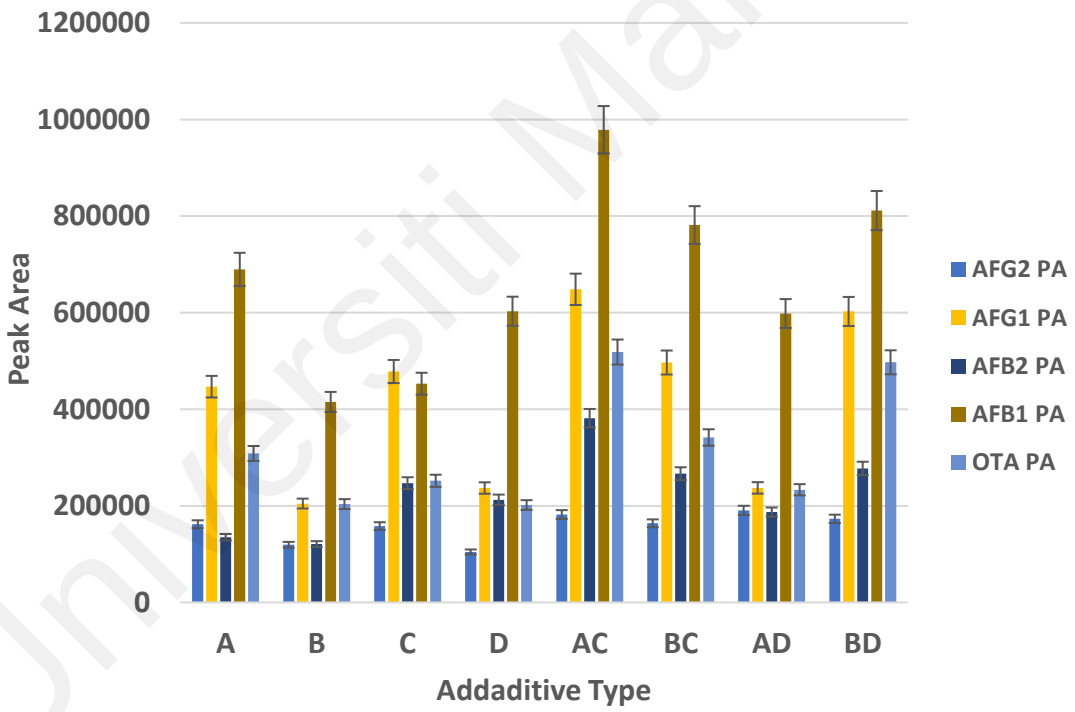

Figure 4.2: Effect of additives on mycotoxins peak area . A: 5 mM ammonium formate; B: 5 mM ammonium acetate; C: 0.05% formic acid; D: 0.05% acetic acid; AC: 5 mM ammonium formate + 0.05% formic acid; BC: 5 mM ammonium acetate + 0.05% formic acid; AD: 5 mM ammonium formate + 0.05% acetic acid; BD: 5 mM ammonium acetate + 0.05% acetic acid.

4.1.3.2 Mobile Phase pH

The pH of the mobile phase has a significant effect on the chromatographic quality, i.e. selectivity, peak shape, and retention time. The acceptable pH range for the mobile phase used is dependent on the stationary. For example, the separation of analytes does not use mobile phases with pH smaller than 2 or greater than 8. Most LC columns with silica-based stationary phase are compatible in this pH range. The columns that are stable in a wider pH range are preferred. The problem with the need for a wide pH range is encountered in particular for basic compounds that can be partially ionized in the pH range of 4 to 8. Some components of the analyte interact with the silanol groups (free or ionized) of the stationary phase, producing an undesirable peak shape (Moldoveanu & David, 2012).

In reversed-phase chromatography, the pH and ionic strength of the aqueous mobile phases are important parameters in method development since altering the pH of the mobile phase using volatile buffers makes the analytes into an unionized form(Sargent, 2013). The pH was varied by adjusting the ammonium formate and formic acid in the mobile phase A, which aimed to investigate its influence on the peak area and their retention time. pH range of 2.5-6 was investigated in this study and the results indicated that the optimum pH value is 3.00 (Fig. 4.3 and4.4). Reducing the mobile phase pH also have effects on enhancing the ionization process, leading to improved peak shapes, and therefore increased LODs (Osteresch et al., 2017).

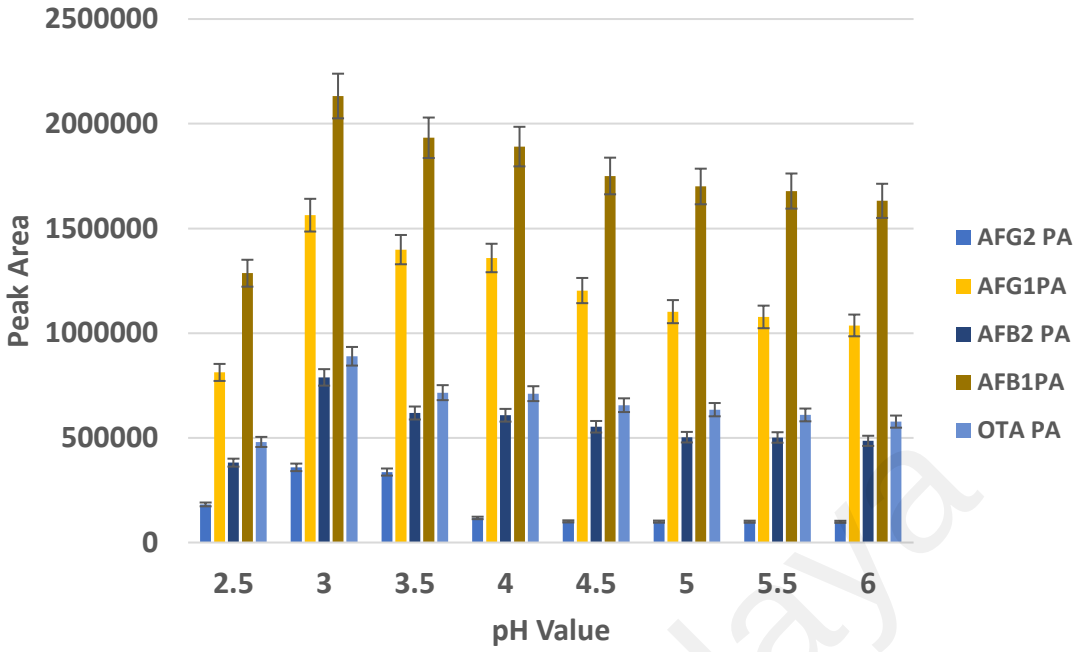

Figure 4.3: Mobile phase pH effects on mycotoxins peak area .

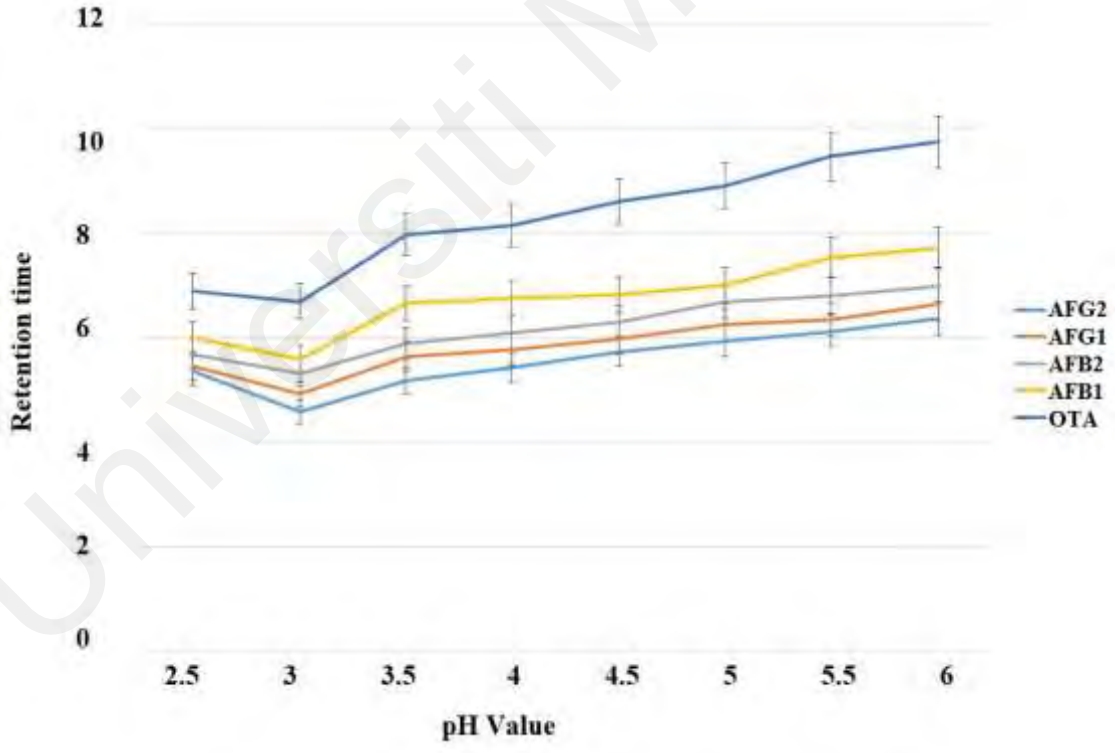

Figure 4.4: The effect of mobile phase pH on mycotoxins retention time.

4.1.3.3 Organic Solvent Percentage

The percentage of organic solvent in reversed-phase LC gradient mode plays an important role in the separation of the compounds. Gradient separation in LC-MS/ MS offers three distinct advantages, i.e. 1) sharper chromatographic peaks with lesser tailing and fronting; 2) shorter elution time; and 3) gradient separation as basic sample clean-up procedure. The initial high percentage of organic solvent enhanced the ionization and elution rates of the polar mycotoxins, which leads to improved sensitivity and shorter retention time, respectively (Frenich et al., 2009). The results indicated the percentage organic solvent that gave the best optimum peak area and retention time response is between 50-60% (Fig. 4.5 and 4.6) and thus 55% was selected for the subsequent experiment.

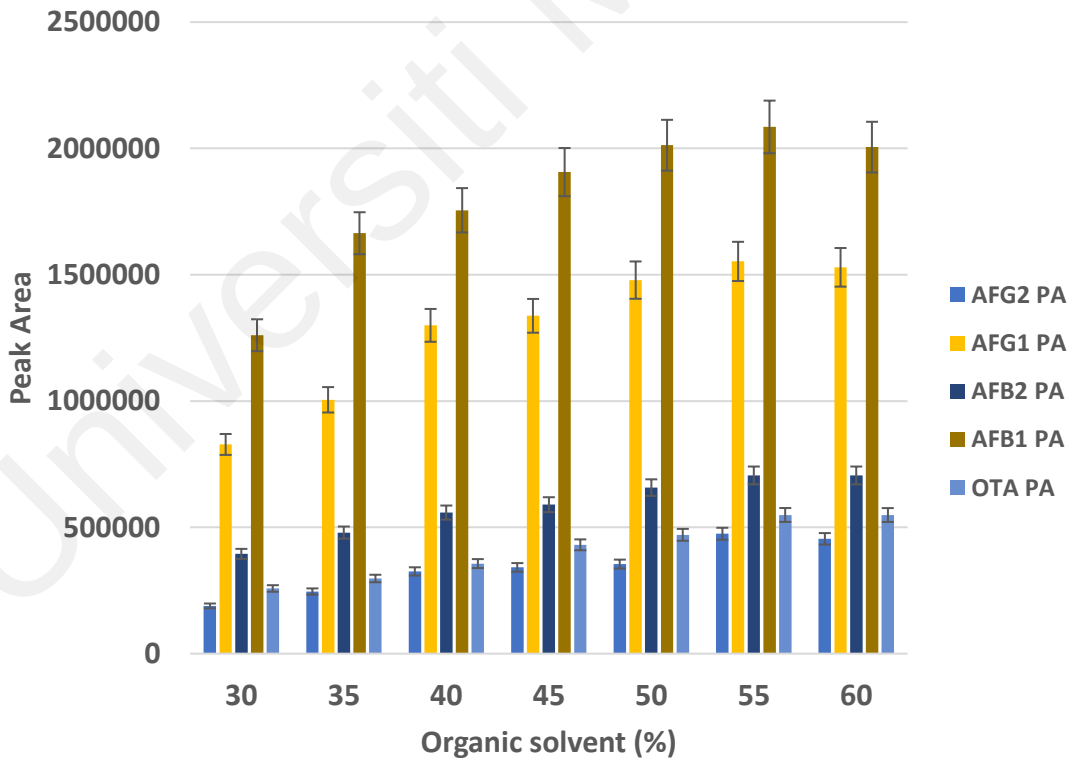

Figure 4.5:The effect of organic solvent percentage on mycotoxins peak areas .

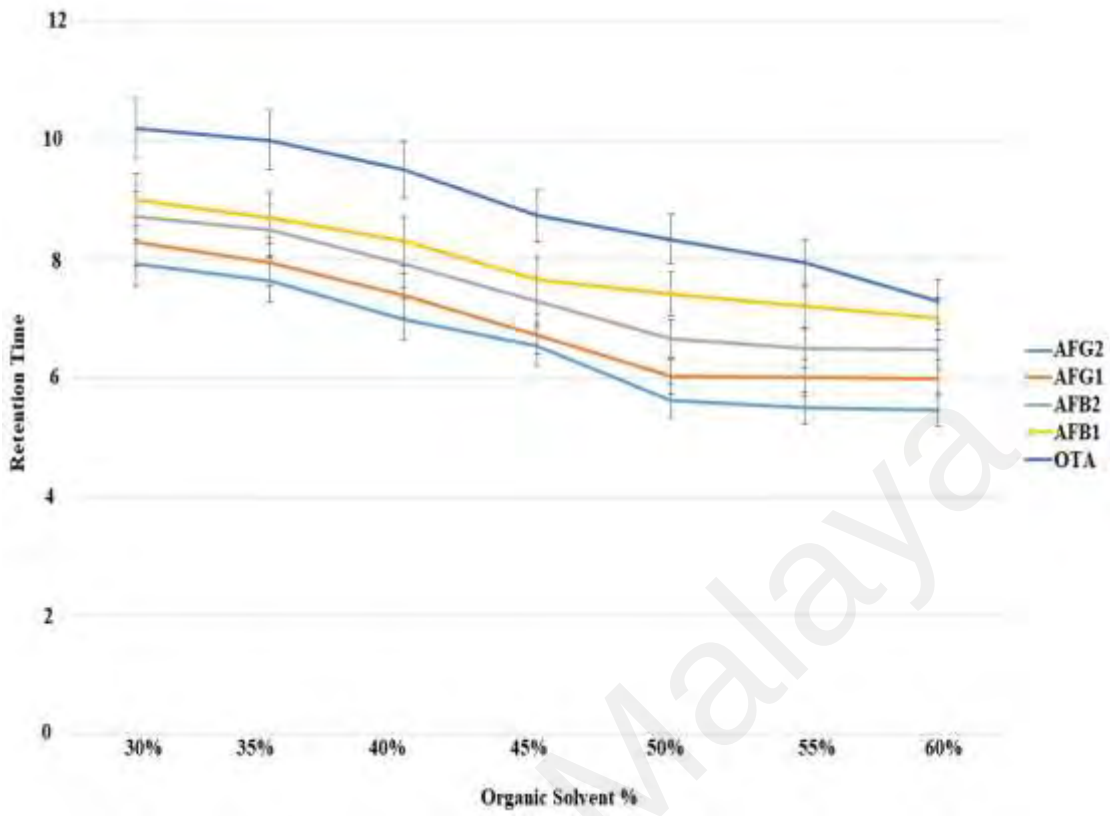

Figure 4.6: Effect of organic solvent percentage on mycotoxins retention time.

4.1.3.4 Mobile Phase Flow Rate

The optimum flow rate is dependent on the characteristics of the mobile phase such as viscosity, diffusion speed and mass transfer factors. The flow rate in ESI mode control the transition of charged droplets to gas-phase ions and hence directly affects the ionization efficiency (Venn, 2008). The effect of the mobile phase flow rate in the range of 0.10-0.20 mL/min on the peak area and retention time response was presented in Figure 4.7 & 4.8. The results were satisfactory within the study region of 0.15 and 0.20 mL/min. Even though a flow rate of 0.20 mL/min led to the shortest retention time, the peaks of AFG2 and AFG1 were overlapping. Therefore, 0.150 mL/min was selected.

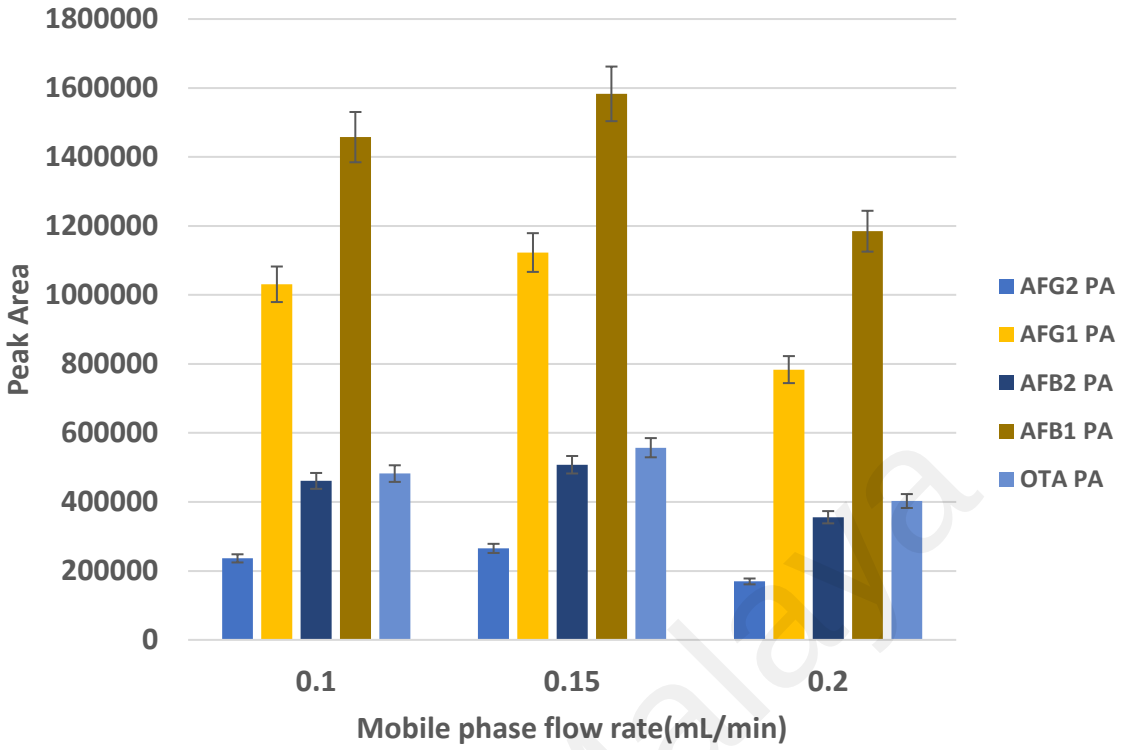

Figure 4.7: Effects of flow rate on mycotoxins peak area

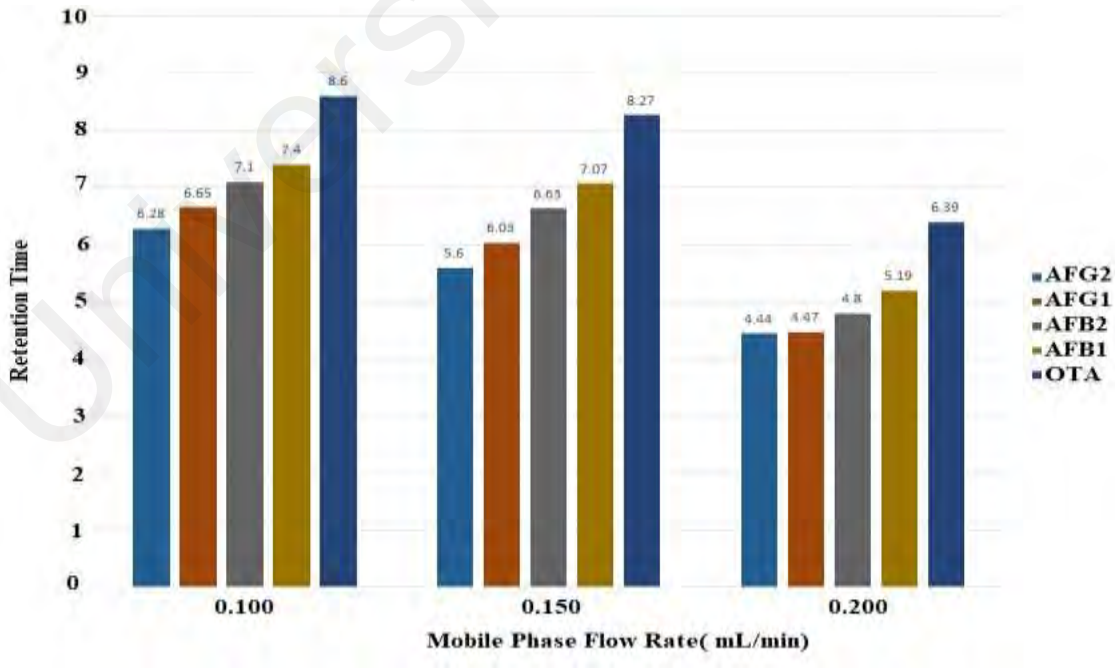

Figure 4.8: Effect of mobile phase flow rate on mycotoxins retention time.

4.1.3.5 Column Temperature

The column temperature is a critical LC parameter which affects the retention time, peak shape and separation selectivity. Solute pka values are affected by the column temperature (Ball, 2013), therefore, it is a good practice to set the column temperature above the ambient temperature by column heating oven to achieve good chromatographic results (Dolan, 2002). Column temperatures in the range of 25-40 °C were evaluated in this study. The results indicated that 30 °C gave the best performance in peak area, while the shortest retention time was observed for 35 and 40 °C (Fig. 4.9 and 4.10). Column temperature of 30 °C (best RSD) was selected for subsequent study as the peak area response has a higher importance than retention time.

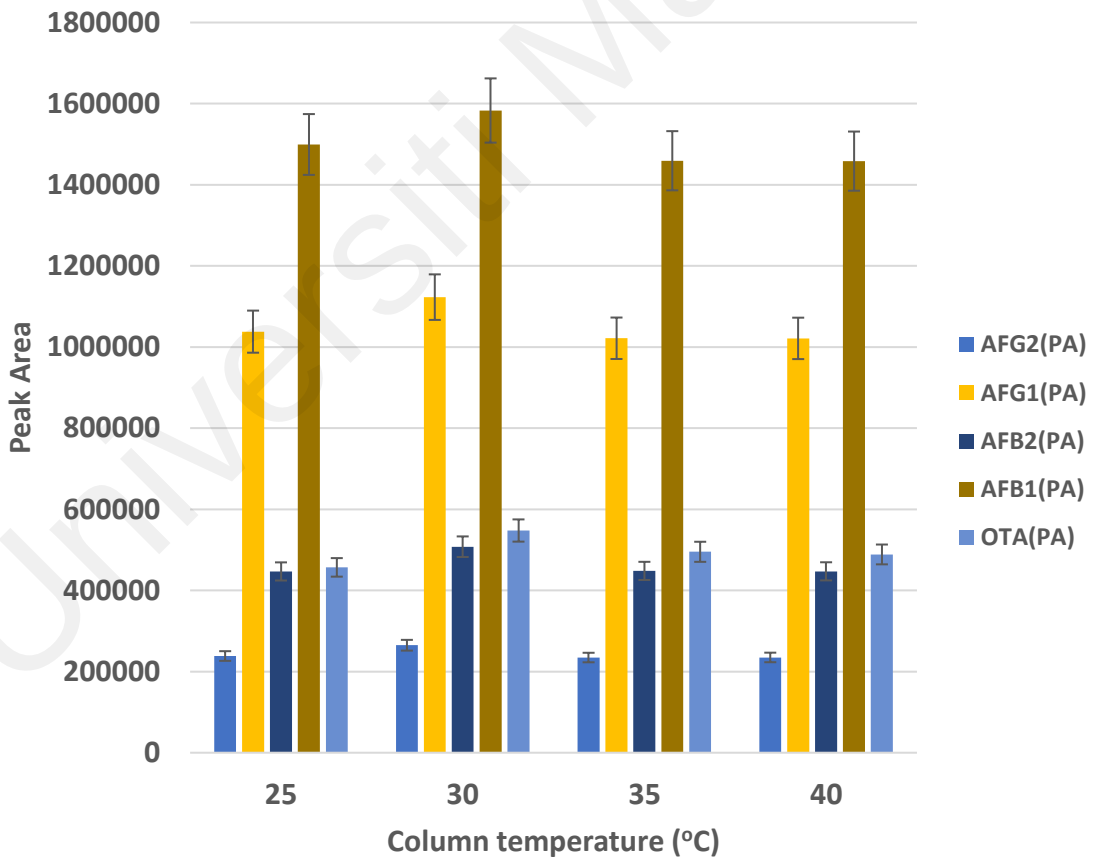

Figure 4.9: Column temperature effects of mycotoxins peak Area response

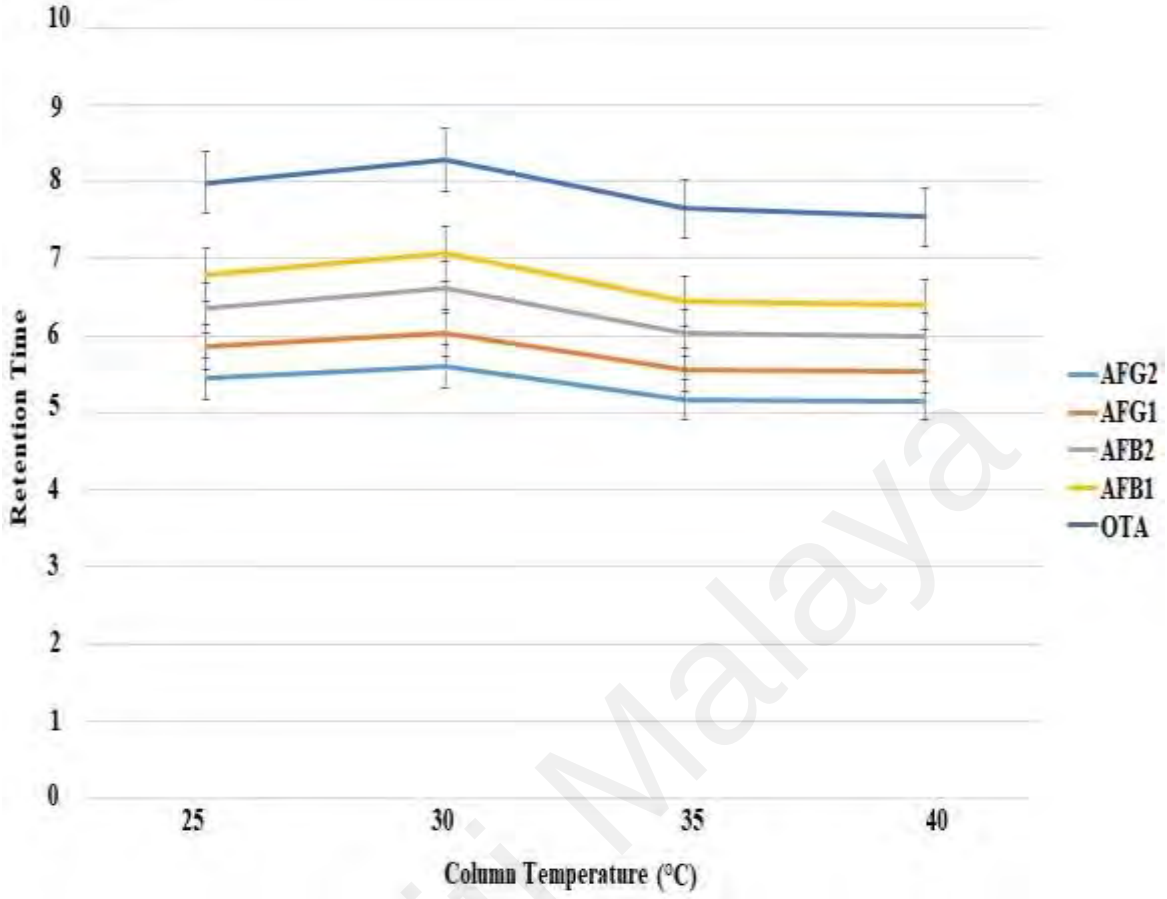

Figure 4.10: Effect of column temperature on mycotoxins retention time.

4.1.3.6 Injection Volume

Injection volume determined the analyte amount per solvent volume and hence affected the ionization efficiency of ESI processes. Improper injection volume may cause broadening, fronting, and tailing of peaks in the chromatogram. The injection volume of 2 to 5µL was evaluated in this study (Fig. 4.11). The results indicated that peak areas increased when injection volume was increased from 2 to 4 µL. At an injection volume of 5 µL, the peak area did not show significant changes. Hence, the injection volume of 4 µLwas selected for subsequent study.

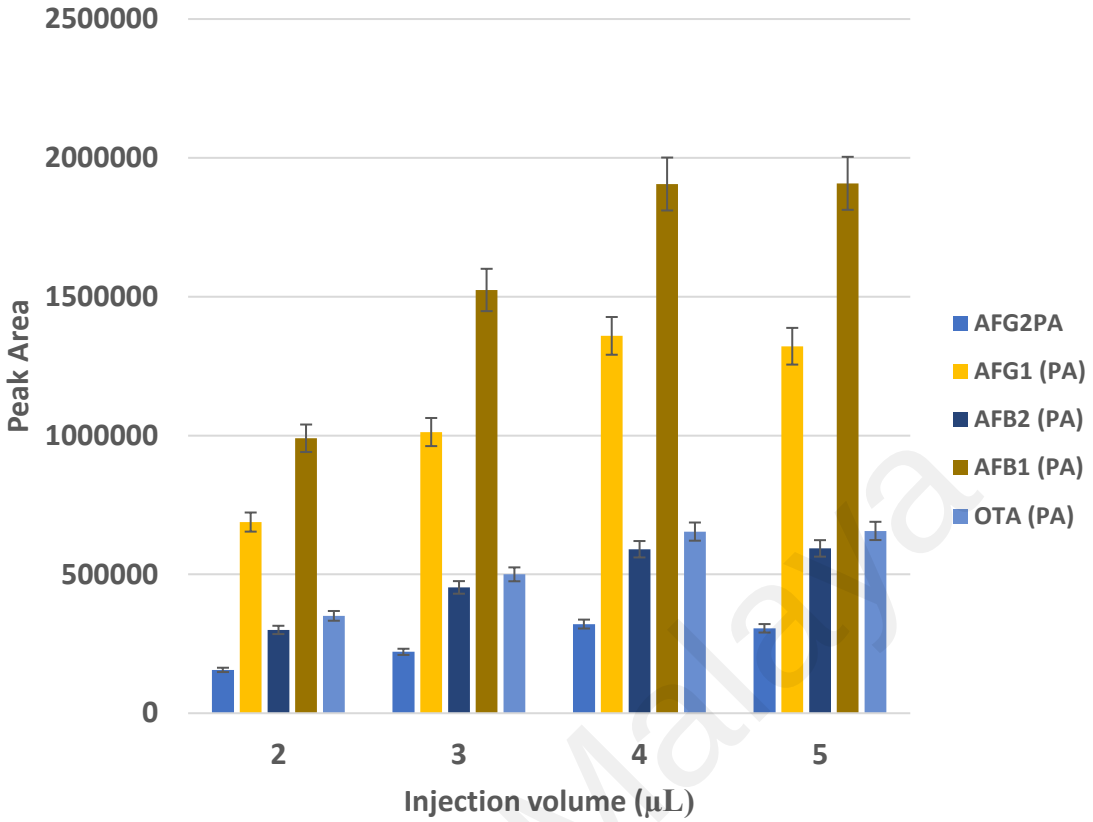

Figure 4.11: Effect of injection volume (IV) on mycotoxins peak area.

4.1.3.7 Sheath Gas Flow and Temperature

Sheath gas was used to confine the nebulizer spray and enhance solvent evaporation of the analyte droplet ions. Typical sheath gas operation temperature and flow rate should be studied in the device limit)150-300 °C and 9-12 L/min) respectively. The results indicated the highest peak area at 300-250 °C, while the RSD value was the best at 250 °C (Fig. 4.12). Therefore, 250 °C was selected as the optimum sheath gas temperature as a result of the ability of this degree to give the optimum evaporation of the solvent. The difference between 11 and 12 L/min results was insignificant (Fig. 4.13) and 11 L/min was finally selected for subsequent experiments to restrict the nebulizer spray.

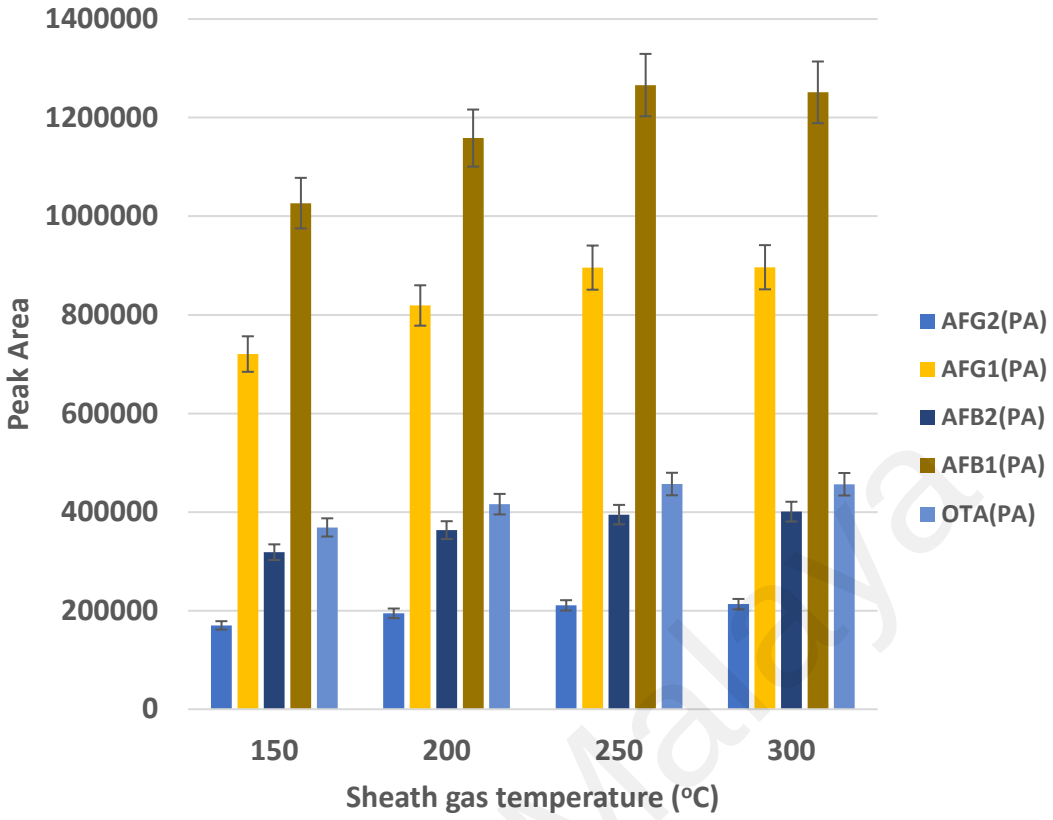

Figure 4.12: Effect of sheath gas temperature on mycotoxins peak area .

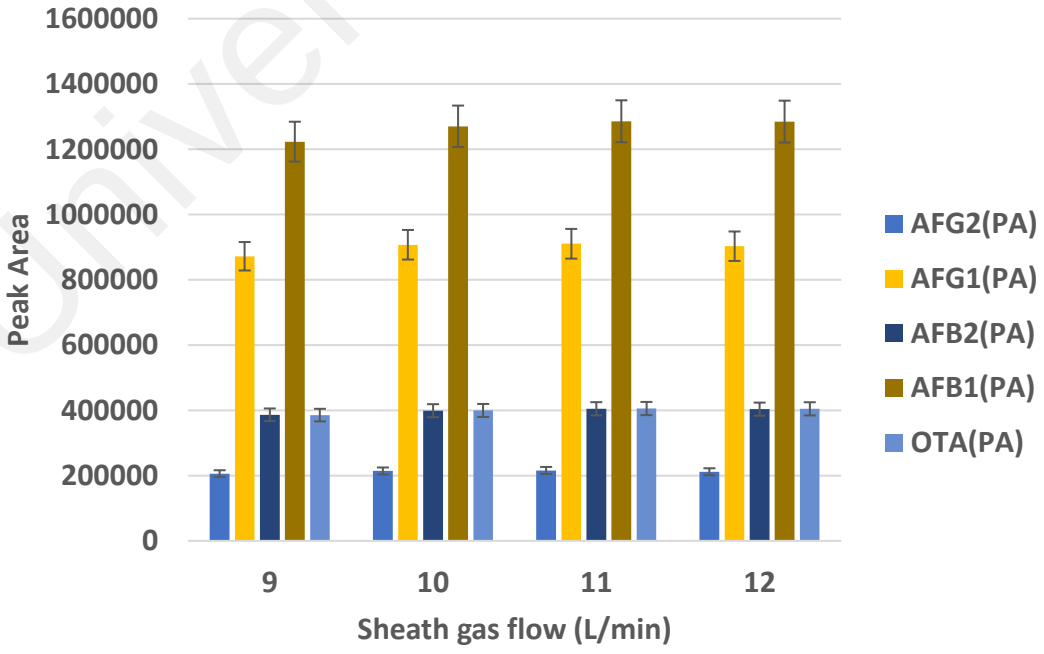

Figure 4.13: Effect of sheath gas flow rate on mycotoxins peak area.

4.1.3.8 Drying Gas Flow and Temperature

The result suggests that drying gas temperature and flow rate of 200 °C and 12 L/min respectively gave the best peak area as shown in the Figures 4.14 & 4.15. Higher drying gas and flow rates prevent fewer mobile ions from reaching the capillary. On the contrary, low gas flow rates adversely affect sensitivity due to excessive ion salvation. Similarly, the optimum temperature can influence the mass spectral quality (higher S/N) by minimizing the response of solvent clusters which are more volatile than the analyte molecules

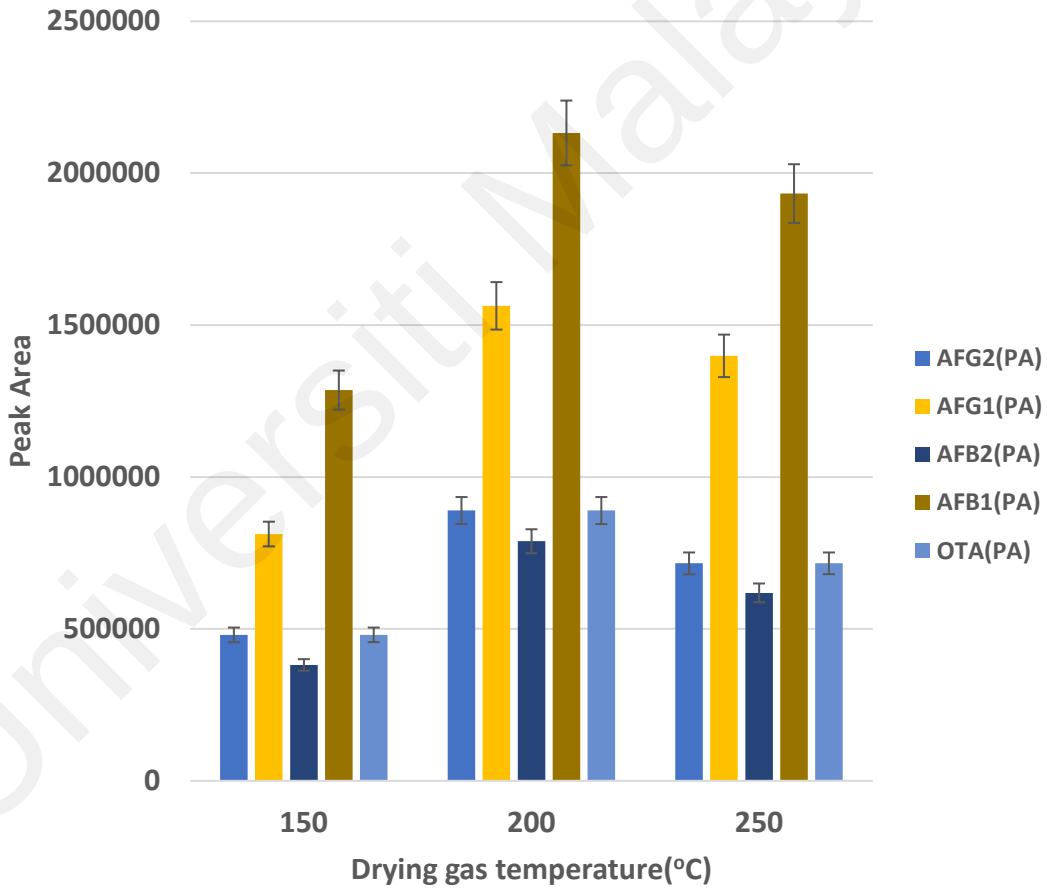

Figure 4.14: Effect of drying gas temperature on mycotoxins peak area response

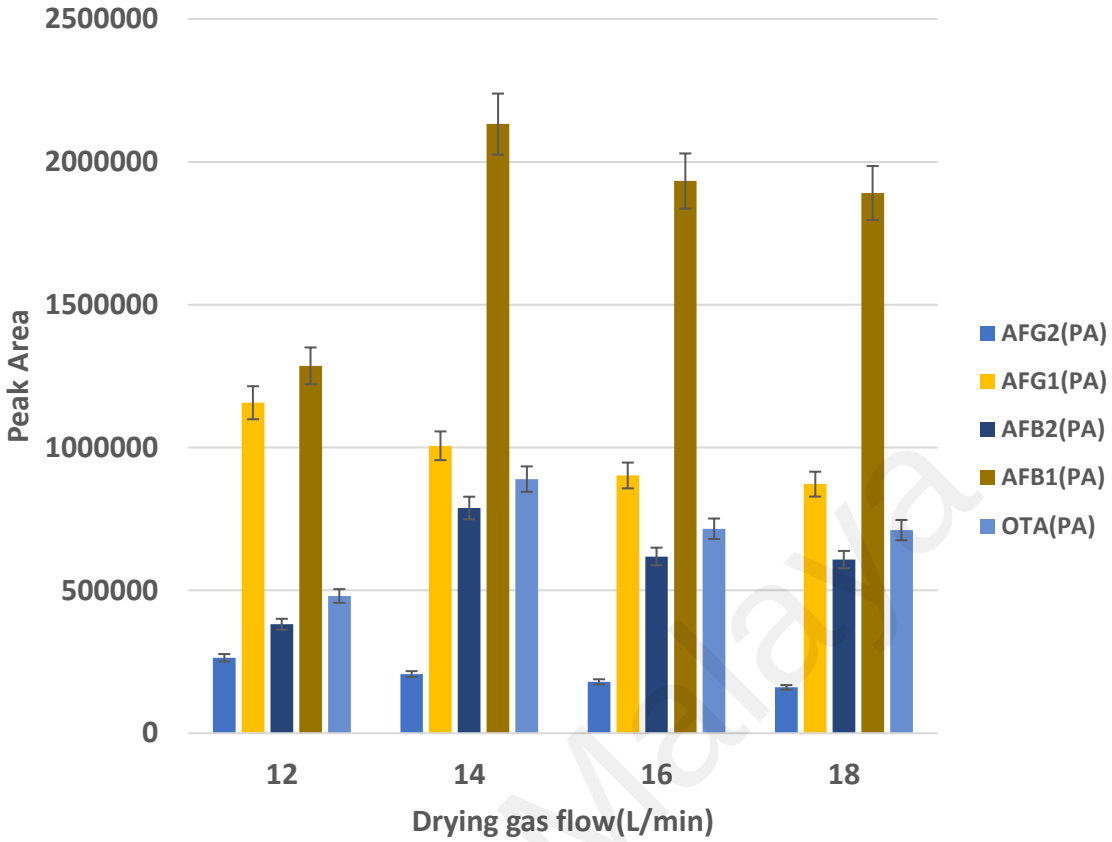

Figure 4.15: Effect of drying gas flow on mycotoxins peak area response

4.1.3.9 Nebulizer Gas Pressure

Nebulizer gas pressure plays an important role in converting the mobile phase solvent into droplets and aerosols during the transition toward the MS inlet. The nebulizer pressure was evaluated in the range from 20 to 35 psi and the observed peak area decreased gradually as the pressure increased higher than 25 psi (Fig. 4.16). This may be attributed to irregular fragmentation of the droplets at high pressure and subsequent transformation into smaller ionized aerosols. Hence, 25 psi was selected for subsequent experiments.

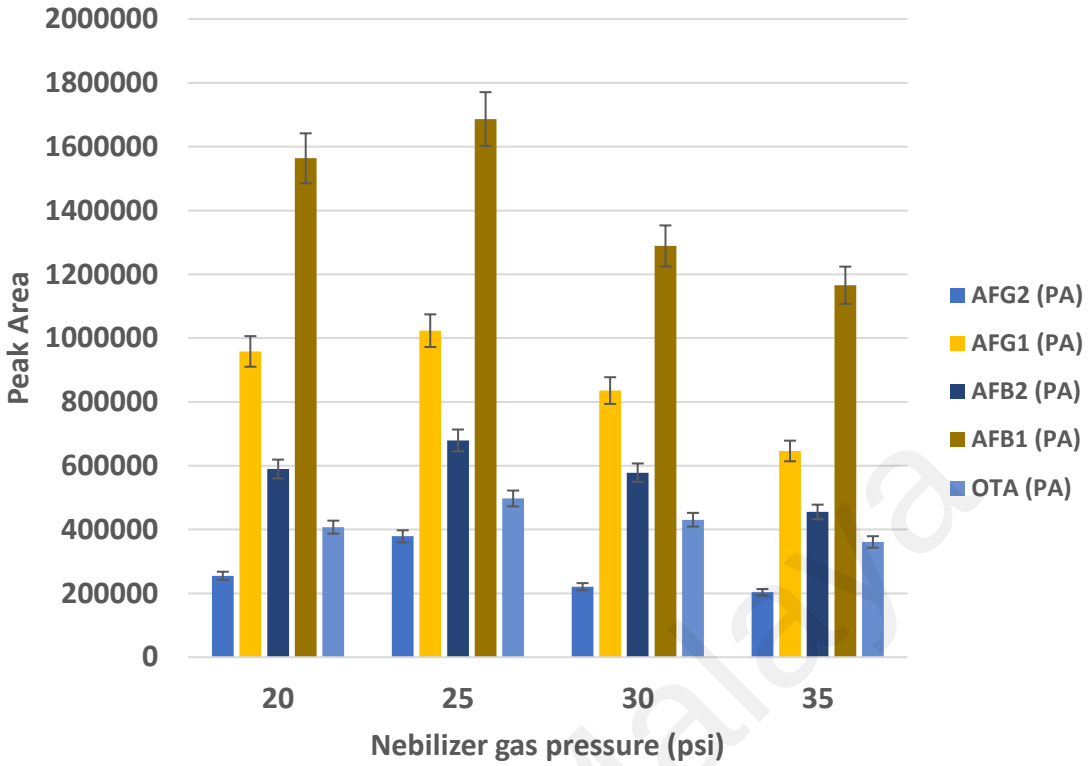

Figure 4.16: The effect of nebulizer pressure (NP) on mycotoxins peak area.

4.1.3.10 Summary of Univariate Optimized Parameters

The univariate optimized of LC-MS/MS parameters are summarized in Table 4.2

Table 4.2: LC-MS/MS optimized parameters from univariate method.

Factor	Optimized value
Mobile phase additives	Ammonium formate / formic acid
Mobile phase pH	pH 3
Organic solvent	50 %
Flow rate	0.15 mL/min
Column temperature	30 °C
Injection volume	3 µl
Sheath gas temperature	250 °C
Sheath gas flow rate	11 mL/min
Drying gas temperature	200 °C
Drying gas flow rate	12 mL/min
Nebulizer gas pressure	25 psi

4.1.4 Multivariate Optimization

4.1.4.1 Plackett-Burman Design (PBD)

The distribution of measurement deviation is shown in the residual plot (Fig. 4.17). The random pattern plot indicated a good fit for a linear model for both (TCPA & MRT). In this study, experimental and adjusted R^2 were determined as 90.33% and 85.17%, respectively for TCPA, and 95.03% and 90.47% respectively for MRT.

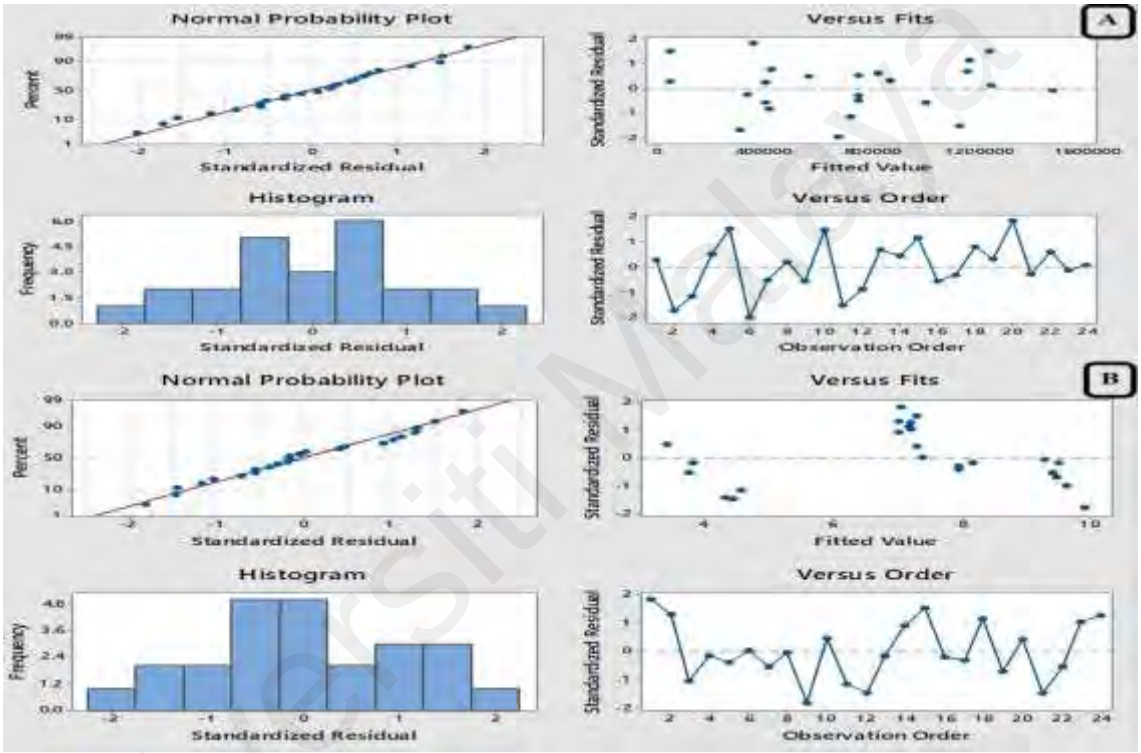

Figure 4.17: Residual plot for (A) TCPA and (B) MRT.

The results from ANOVA (Table 4.3) analysis revealed that the significant factors with $P < 0.05$ are injection volume (IV), gas flow (GF), and gas temperature (GT) for TCPA, while the flow rate (FR) and organic solvent percentage (OS) are the significant factors in effecting MRT. PBD is useful just for screening the design space to detect large main effects. Also, it is the less precise computation of the main and some interaction effects (Cavazzuti, 2012). Therefore, non-significant parameters were not used in the next optimization step by BBD, and the value was kept according to the univariate method.

Table 4.3: ANOVA for the screening model

Source	DF	TCPA		MRT	
		F-Value	P-Value	F-Value	P-Value
Model	11	7.35	0.001	17.29	0.000
pH	1	2.82	0.119	0.01	0.912
OS	1	0.24	0.630	87.66	0.000
FR	1	4.23	0.062	92.31	0.000
CT	1	1.10	0.315	0.40	0.537
IV	1	53.50	0.000	0.02	0.899
SGT	1	2.14	0.169	2.02	0.181
SGF	1	0.66	0.434	2.48	0.142
GF	1	5.54	0.036	2.27	0.096
GT	1	4.96	0.046	0.07	0.793
NP	1	1.18	0.300	0.01	0.915
CE	1	4.51	0.055	1.89	0.194
Residual Error	12	-	-	-	-
Total	23	-	-	-	-

CE: collision energy , CT: column temperature, DF: Degree of freedom, FR: Flow rate, F-Value: Fisher test value, GF: Gas flow, GT: Gas temperature, IV: Injection volume, OS: Organic solvent percentage, MRT:Mean retention time, NP: Nebulizer pressure, P-Value: Probability value, SGF: Sheath gas flow, SGT: Sheath gas temperature, TCPA: Total chromatogram peak area.

The standardized Pareto bar charts (Fig. 4.18) shows the influence of each variable in the model. The confidence level of 0.05 is represented by the vertical red line and variables exceeding the line are hence significant. These variables were selected for further optimization process.

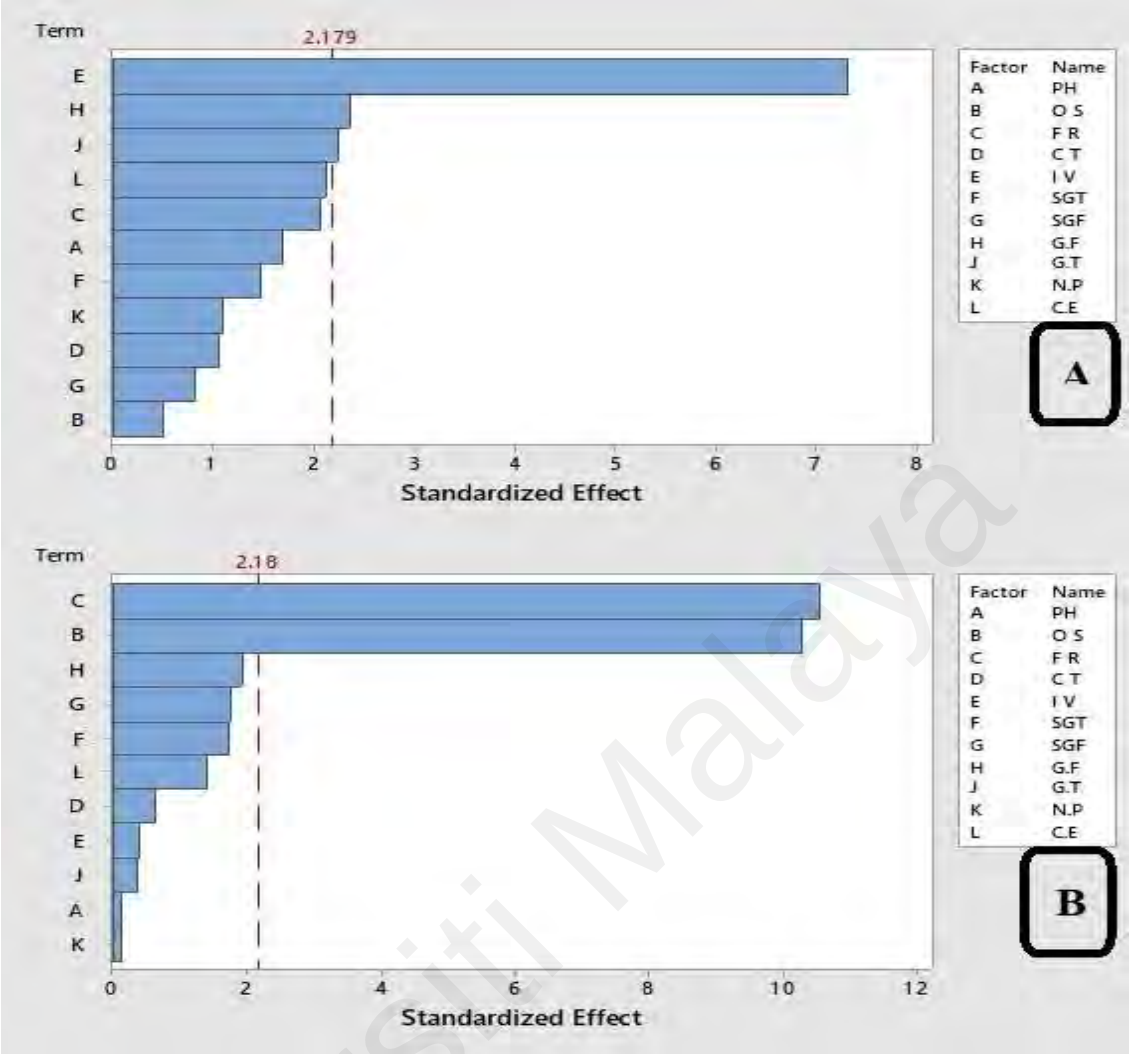

Figure 4.18: Standardize pareto chart for (A) TCPA and (B) MRT.

Normal probability plot highlighted whether the significance effect is positive or negative. (Fig. 4.19). It indicates the change in response if a given factor changes from the low to the high level, where the variables that do not fall close to the normal probability line is deemed significant (Bordagaray et al., 2011). As shown in Figure 4.18, injection volume (IV), gas flow (GF), and gas temperature (GT) have positive effects on TCPA , where as mobile phase flow rate (FR) and organic solvent percentage (OS) have negative effects on MRT. These five significant factors were included in the optimization proccess using a Box-Behnken design.

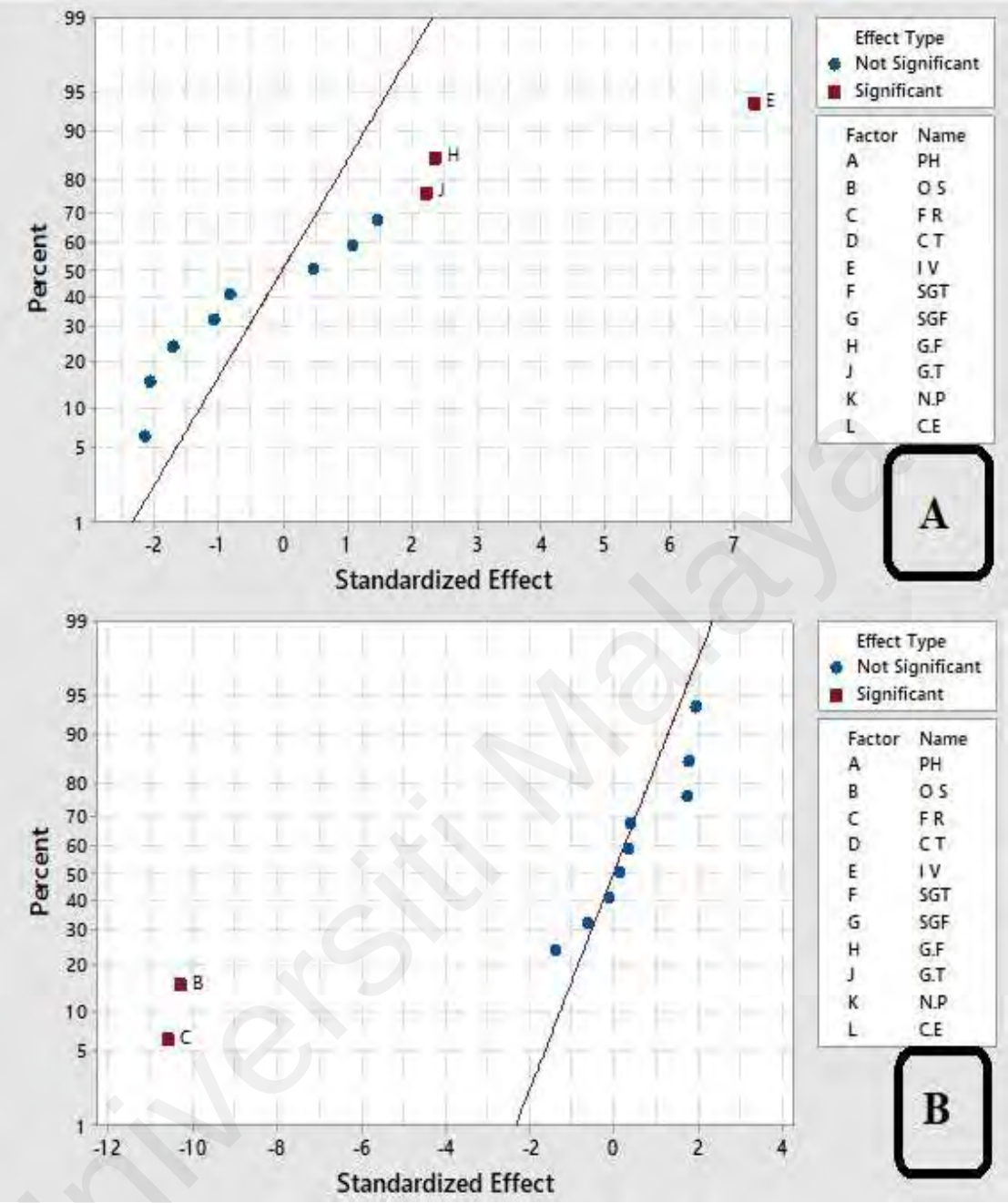

Figure 4.19: Normal probability plot of standardize effect for (A) TCPA and (B) MRT.

Main effects plot was used to examine the effect of study factors. Figure 4.20 showed the degree of increase in TCPA when the injection volume, gas flow, and gas temperature increase. Meanwhile, MRT decreased as the mobile phase flow rate and organic solvent percentage increased. Other factors did not have significant effects on TCPA or MRT.

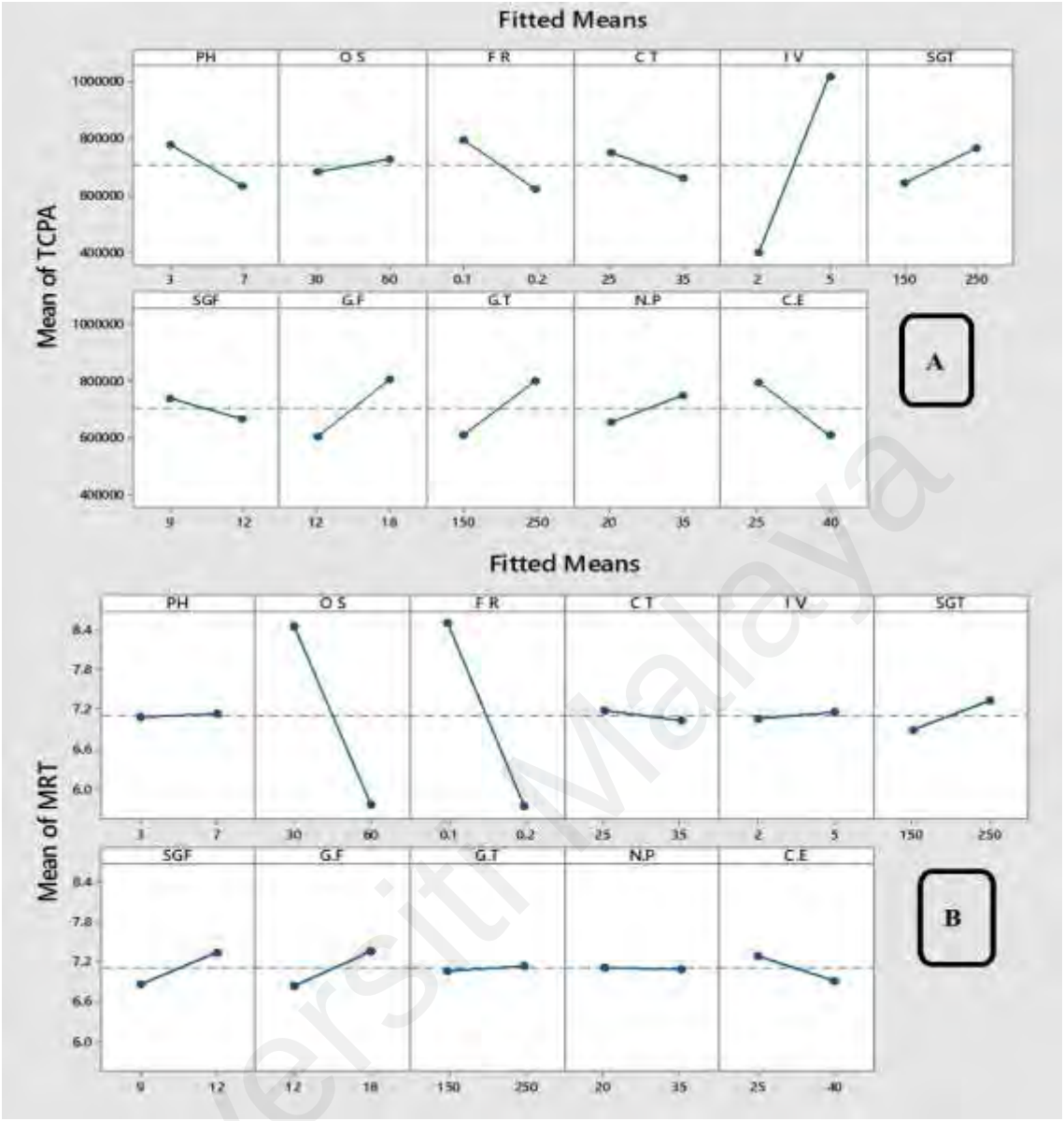

Figure 4.20: Main effects plots for (A) TCPA and (B) MRT.

Figure 4.21 shows the predicted responses under the optimized parameters suggested by PBD. Table 4.4 shows the comparison of optimized parameters derived from univariate and PBD methods. The results obtained from both optimization strategies were comparable, but the amount of time required for PBD was significantly lesser than the univariate method, highlighting the advantages of the PBD method.

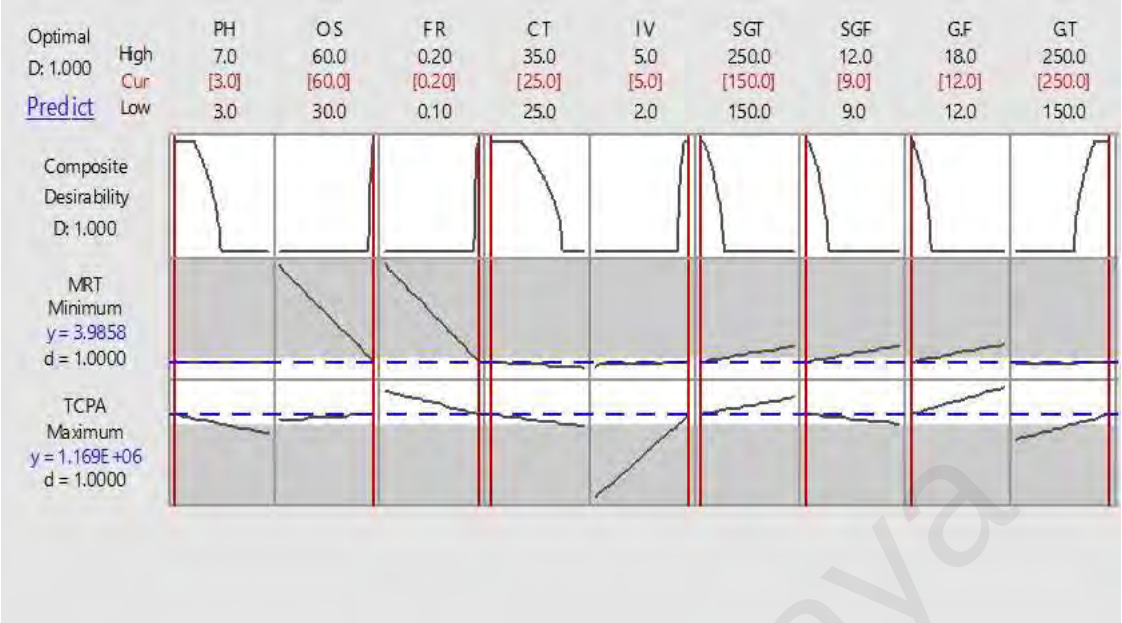

Figure 4.21: Optimized parameters displayed in response optimizer.

Table 4.4: Univariate and PBD optimized paramaters

Factors	Univariate	PBD
pH	3	3
OS (%)	55	60
FR (mL/min)	0.15	0.15
CT (ºC)	30	35
IV (µL)	3	5
SGT (ºC)	250	150
SGF (L/min)	11	9
GF (L/min)	12	17.5
GT (ºC)	200	250
Number of experiments	44	24
No. injections per experiments	3	3
Total of injections	132	72
Time of each run	10 min	10 min
Total time spend	1320min	720 min

CT: column temperature, FR: flow rate, GF: gas flow, GT: gas temperature, IV: injection volume, OS: organic solvent percentage, PBD: Plackett-Burman design, SGF: sheath gas flow, SGT: sheath gas temperature.

4.1.4.2 Box-Behnken Design (BBD)

The five most significant factors involved via PBD optimization, i.e. i) percentage of organic solvent; (ii) flow rate; (iii) injection volume; (iv) gas flow and (v) gas

temperature, were subjected for further optimization process using a BBD, while other parameters were kept constant (Low et al., 2012) i.e. pH, column temperature, sheath gas flow, sheath gas temperature, and nebulizer pressure were set at pH 3, 30 ºC, 11 L/min, 250 ºC, 20 psi, and the collision energies were summarized in Table 4.1. The random distribution of measurement deviation around the mean is shown in the residual plots (Fig. 4.22**).**

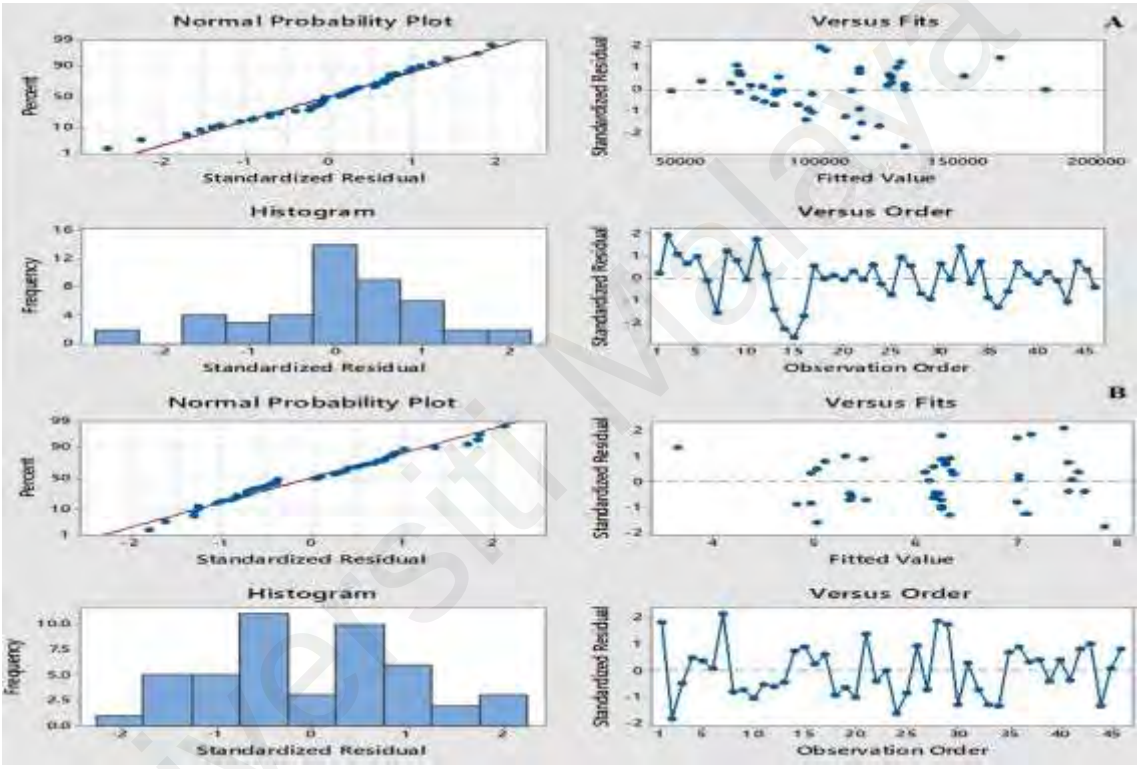

Figure 4.22: BBD residual plot for (A) TCPA and (B) MRT.

Table 4.5 shows the ANOVA results for the quadratic model. The lack-of-fit *P*-values for TCPA and MRT were determined as 0.593 and 0.825 indicating good-fit of the quadratic model. The polynomial fit quality was expressed by the determination coefficient (R^2) (Saguy & Graf, 1990) where the experimental and adjusted R^2 values were 98.92% and 97.83%, respectively for TCPA; and 99.15% and 98.41%, respectively for MRT. High R^2 values indicated a goodness-of-fit and good correlation between the observed and predicted values(Paíga et al., 2016). The 2-way interaction effects were not significant for TCPA. However, a significant interaction was observed between flow rate

and organic solvent percentage on the MRT. The relationships between the responses (TCPA and MRT) and the instrumental parameters are shown in the response plot (Fig. 4.23) and main effect plot (Fig 4.24).

Table 4.5: ANOVA results of BBD model

Source	DF	TCPA		MRT	
		F-Value	P-Value	F-Value	P-Value
Model	20	2.53	0.015	133.30	0.000
Blocks	1	4.62	0.042	7.31	0.012
Linear	5	4.18	0.007	547.40	0.000
OS	1	0.91	0.350	1927.53	0.000
FR	1	1.10	0.305	807.80	0.000
IV	1	17.33	0.000	1.60	0.219
GT	1	0.87	0.361	0.06	0.806
GF	1	0.70	0.412	0.01	0.908
Square	5	3.61	0.014	0.82	0.550
O S*O S	1	1.85	0.186	0.44	0.513
F R*F R	1	0.07	0.798	3.02	0.095
I V*I V	1	0.04	0.848	0.19	0.664
G T*G T	1	5.20	0.032	1.18	0.287
G F*G F	1	11.66	0.002	0.02	0.889
2-Way Interaction	10	0.96	0.497	5.10	0.001
O S*F R	1	0.35	0.560	45.54	0.000
O S*I V	1	4.62	0.042	0.95	0.338
O S*G T	1	0.02	0.882	0.43	0.517
O S*G F	1	0.08	0.781	1.26	0.273
F R*I V	1	1.00	0.326	0.75	0.395
F R*G T	1	0.00	0.998	1.72	0.202
F R*G F	1	2.39	0.135	0.00	0.983
I V*G T	1	0.30	0.588	0.12	0.737
I V*G F	1	0.27	0.606	0.01	0.910
G T*G F	1	0.60	0.445	0.21	0.648
Lack-of-Fit	20	0.95	0.593	0.57	0.825

DF: Degree of freedom, FR: flow rate, F-Value: Fisher test value, GF: gas flow, GT: gas temperature, IV: injection volume, MRT: Mean retention time, OS: organic solvent, P-Value: Probability value, TCPA: Total chromatogram peak area.

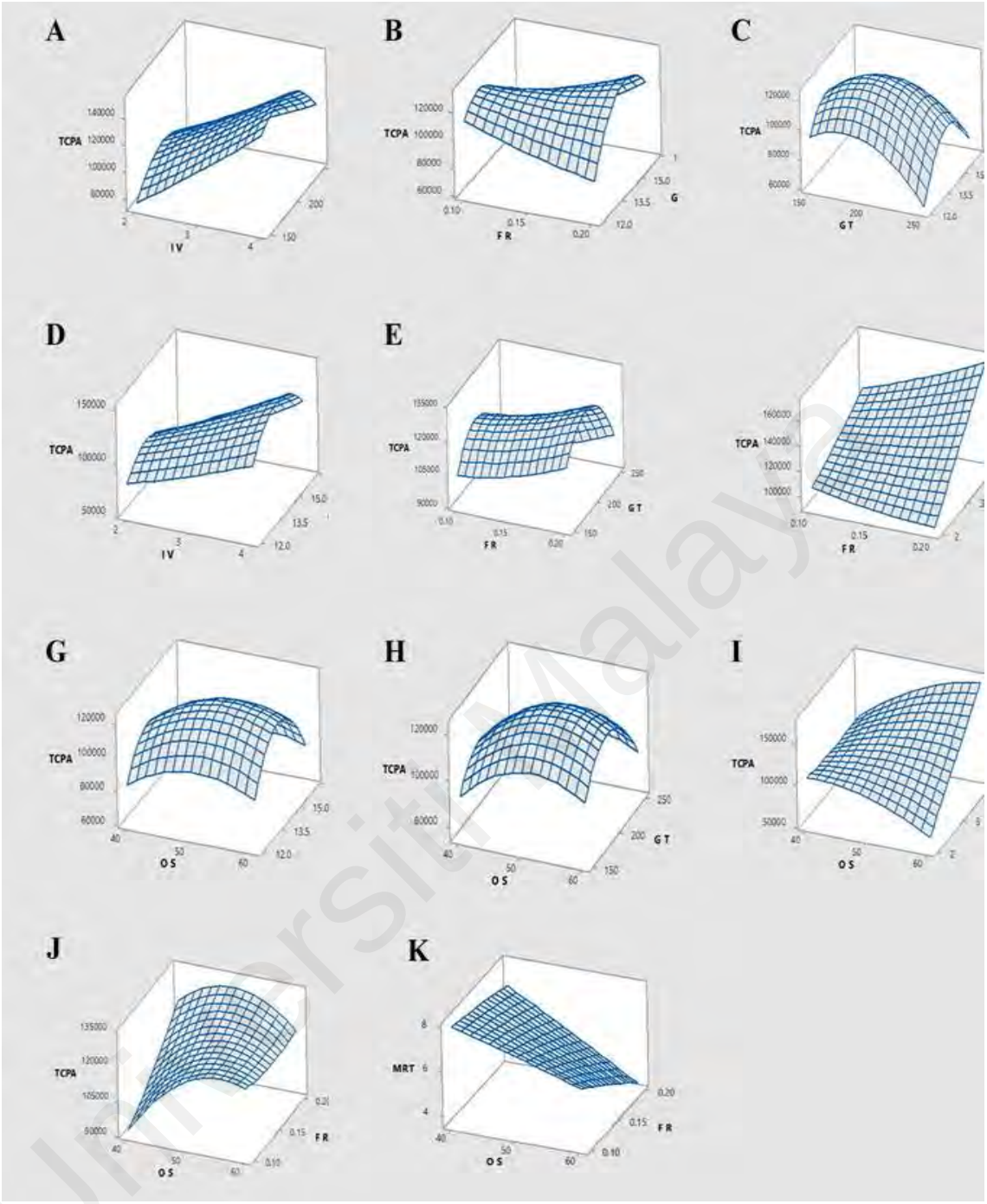

Figure 4.23: Response surface plot for TCPA (A) Injection volume vs. Gas temperature; (B) Flow rate vs. Gas flow; (C) Gas temperature vs. Gas flow; (D) Injection volume vs. Gas flow; (E) Flow rate vs. Gas temperature; (F) Flow rate vs. Injection volume; (G) Organic solvent vs. Gas temperature; (H) Organic solvent vs. Gas temperature; (I) Organic solvent vs. Injection volume; (J) Organic solvent vs. Flow rate; and MRT response surface plot for (K) Organic solvent vs. Flow rate.

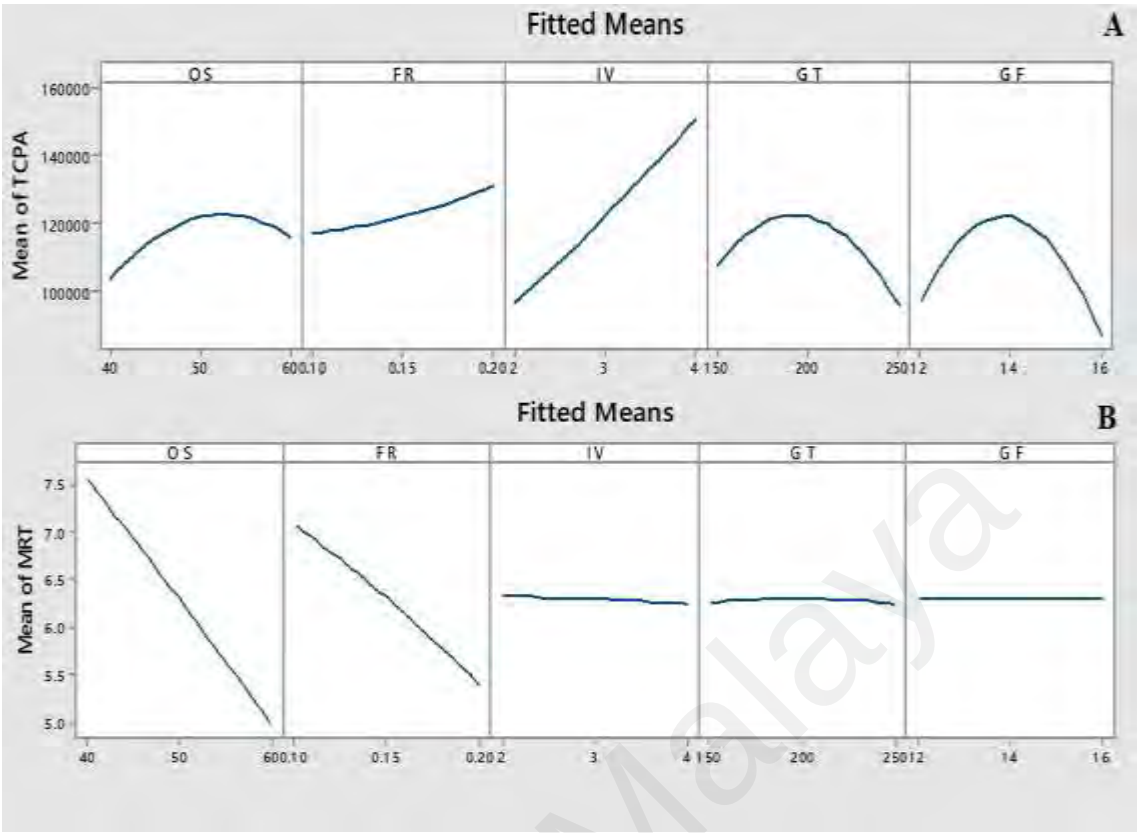

Figure 4.24: BBD main effects plots for (A) TCPA and (B) MRT.

The main effect plot (Fig. 4.24A) shows that injection volume and TCPA increased hand-in-hand. The analyte concentration in the initial spray solution is an important factor that significantly affected the ESI-MS signal intensity. For multiple charged analyte, ions of discrete charge state exhibited a linear response of the ion signal with the analyte concentration in the solution until a certain concentration limit was exceeded. This concentration limit is dependent on the nature of the analyte. Beyond that concentration, the signal intensity lost that linear response and may even decrease at extremely high concentration (Banerjee & Mazumdar, 2012). Moreover, drying gas temperature and flow rate plays the leading role in the quality and quantity of the ions produced in the source. Therefore, increasing gas flow (GF) and temperature (GT) will cause TCPA to increase to a certain extent and subsequently decrease with an increment of GF and GT. In the case of MRT response (Fig. 4.24B), increasing the organic solvent percentage (OS)

and flow rate (FR) of mobile phase caused reduction of retention time as a result of mobile phase viscosity reduction. Mobile phases used in LC-M/MS consisted of MeOH and/or MeCN, and a volatile buffer such as ammonium formate. MeCN and MeOH have low viscosity (Zhou 2005). Furthermore, the increase of mobile phase flow rate decreased the analytes' retention time, while other factors such as injection volume (IV), gas flow (GF) and temperature (GT) had no significant effect on MRT. The individual (d) and composite (D) desirability values were used for assessing the impact of multiple variables on TCPA and MRT responses. The desirability value ranged from 0 to1, with 1 representing the ideal case, 0 indicating responses unacceptable limits, 0.5 for medium desirability, and 1 for very desirable. The goal was to maximize the TCPA and minimize the MRT. Figure 4.25 shows the response optimizer plot which indicated an optimized composite desirability (D) value of 1.00 for both TCPA and MRT, suggesting the suitability of the proposed model. With the value of very desirable composite desirability (1.00), the final optimized parameters from BBD study were organic solvent = 60%, flow rate = 0.200 mL/min, injection volume = 4 µl, gas flow = 13.65 L/min, and gas temperature = 170.20 ^{o}C. For the validation of the predicted response, the experimental instrument response for TCPA and MRT was compared. The experimental TCPA and the MRT responses were illustrated in Table 4.6. Both responses were smaller than predicted response. However, when both of these responses (TCPA & MRT) from multivariate optimization were compared to the univariate optimization, it was revealed that multivariate optimization gave better results. Figures 4.26 and 4.27 show the LC-MS/MS total ion chromatogram (TIC) of the mycotoxin's mixture obtained under univariate and multivariate optimized parameters. The difference in the response can be attributed to the interaction effect among the factors

Table 4.6:Comparison the predicted, experimental multivariate and univariate study

LC-MS/MS Parameters		Predicted Multivariate	Observed Multivariate	Observed Univariate
pH		3	3	3
OS (%)		60	60	55
FR (mL/min)		0.2	0.2	0.150
CT (ºC)		30	30	30
IV (µL)		4	4	3
SGT (ºC)		250	250	250
SGF (L/min)		11	11	11
GF (L/min)		13.65	14.00	12
GT (ºC)		170.20	170.00	200
NP (psi)		20	20	20
Response	TCPA	182555	173679	127682
	MRT	3.5	4.7	5.40

CT: column temperature, FR: flow rate, GF: gas flow, GT: gas temperature, IV: injection volume, NP: nebulizer pressure, OS: organic solvent percentage, PBD: Plackett-Burman design, SGF: sheath gas flow, SGT: sheath gas temperature.

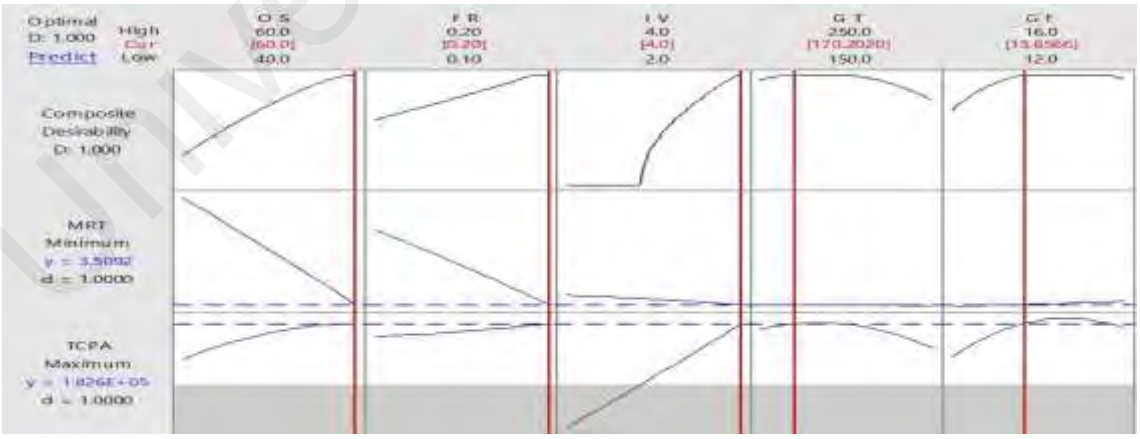

Figure 4.25: BBD optimized parameters displayed in response optimizer.

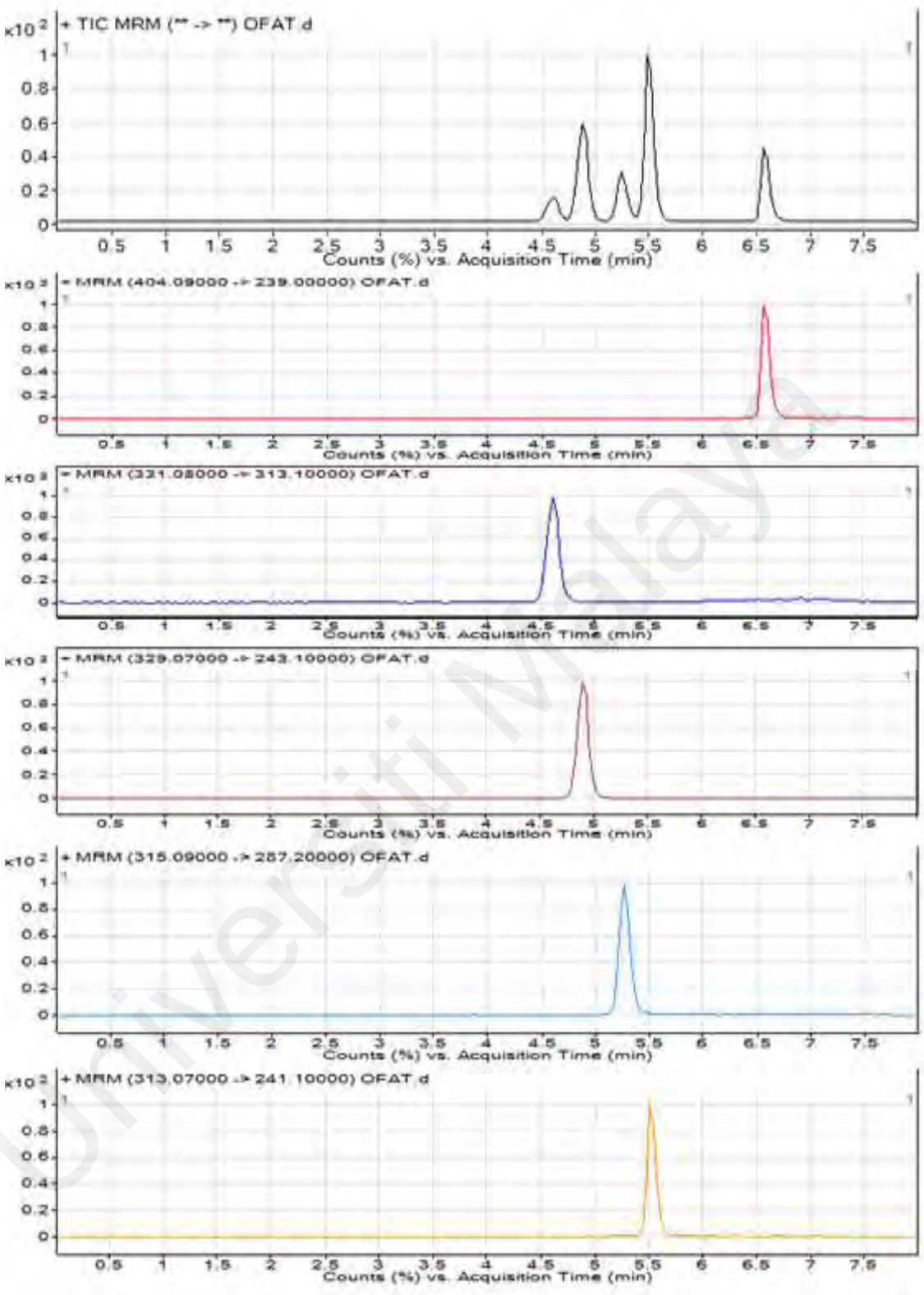

Figure 4.26: Total ion chromatogram (TIC) and MRM chromatogram of mycotoxins standard from univariate study

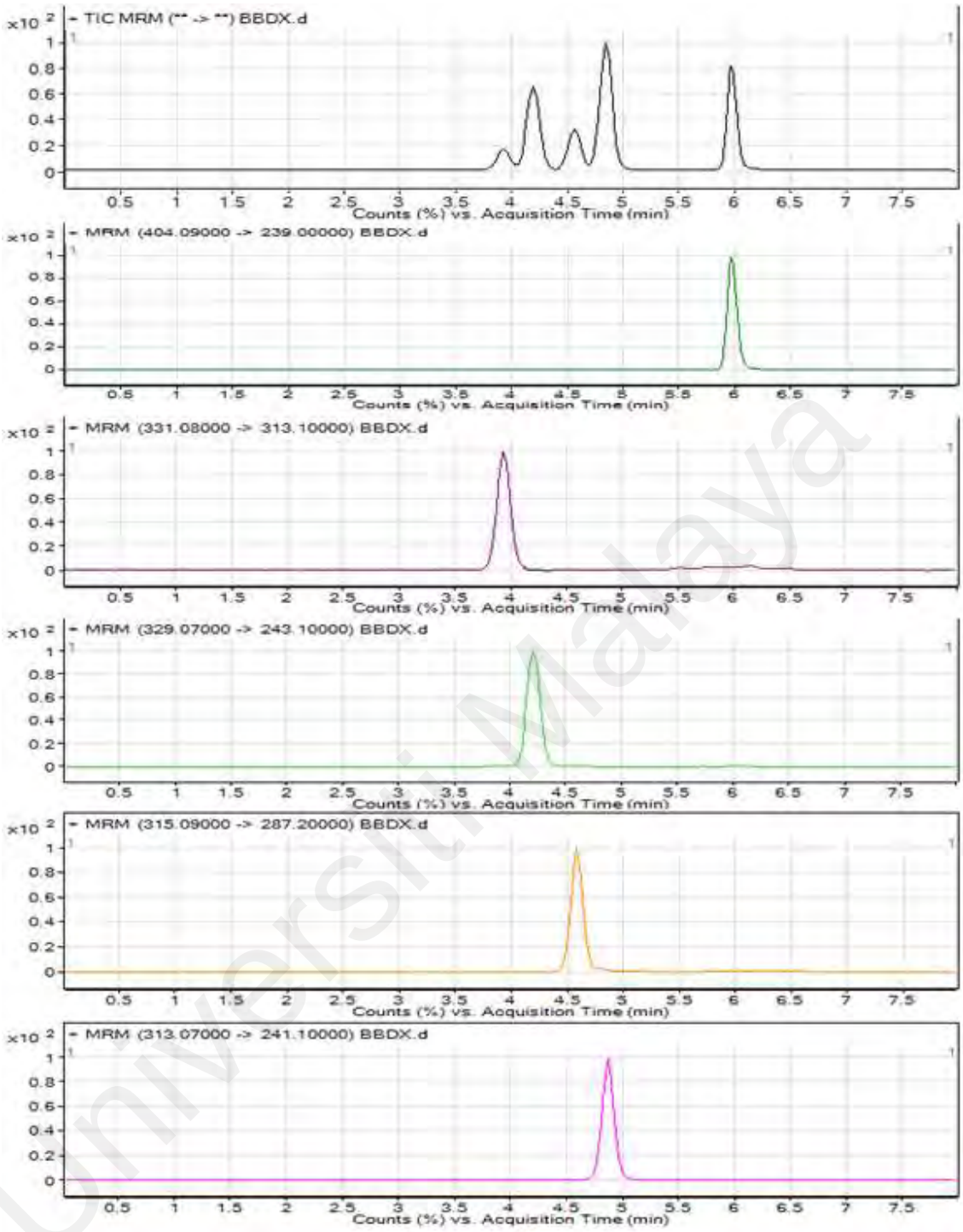

Figure 4.27: Total ion chromatogram (TIC) and MRM chromatogram of mycotoxins standard from BBD study

4.1.5 Method Performance

The optimized conditions for the simultaneous determination of five mycotoxins derived from RSM were subjected to method validation. The validation was performed using mycotoxins standard in methanol and spiked mycotoxins in free peanut QuEChERS extract. All experiments were carried out in triplicates. A blank sample was run in between samples to avoid carryover and contamination. The linearity (regression equation, correlation of determination R^2), precision as intra-day (RSD), inter-day (RSD), and instrument detection limit (IDL) parameters are summarized in Table 4.7. Good linearity values were obtained for all five mycotoxins in both MeOH and matrix matched samples. The R^2 was determined as greater than 0.9992 with the concentration ranges of 0.018- 50 µg/L. Satisfactory precision was obtained with RSD lower than 20% in both MeOH and matrix matched for all mycotoxins. The experiments provided acceptable repeatability results with RSD < 4%. To evaluate the IDL, the mycotoxins mixture was analyzed in eight replicates, and the results indicated the IDL values in the range of 1.41 -3.61 ng.

4.1.6 Analysis of Mycotoxins in Food Sample

4.1.6.1 Method Performance

Method validation was carried out using QuEChERS-LC-ESI-QQQ-MS/MS in optimum conditions. The method linearity was estimated from the matrix-matched calibration curve, with each calibration curve constructed using individual mycotoxin average peak area versus corresponding concentration. The calibration curves were linear over the tested concentration range. The correlation coefficients (R^2) of the calibration curves were greater than 0.9967 for all studied mycotoxins. The method demonstrated good sensitivity with LOD ranging from 0.05 to 0.1 µg/L or µg/Kg, and LOQ ranging from 0.08 to 0.3 µg/L or µg/Kg. The details are summarized in Table 4.8.

Table 4.7: LC-MS/MS validation data

Mycotoxins	AFB1		AFB2		AFG1		AFG2		OTA	
Linearity Range (µg/L)	0.02-50		0.012-15		0.02-50		0.012-15		0.02-50	
	Pure[a]	Post[b]	Pure	Post	Pure	Post	Pure	Post	Pure	Post
R^2	0.9999	0.9998	0.9994	0.9992	0.9997	0.9995	0.9999	0.9998	0.9998	0.9993
IDL (ng)	1.75	2.35	2.93	3.01	2.60	3.22	3.12	3.61	1.41	2.48
Intra-day Precision (RSD%)	1.32	1.67	0.64	1.84	0.81	1.23	1.17	1.25	2.75	3.67
Inter-day Precision (RSD%)	2.78	2.95	1.63	1.70	0.97	1.80	3.6	3.11	3.3	3.89

AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; OTA: Ochratoxin A; R2: regression coefficient; IDL: instrumental detection limit; RSD: relative standard deviation; a: standard in methanol; b: standard spiked in peanut extract.

Table 4.8: Method performance of QuEChERS-LC-MS/MS

Samples Matrix		Fruit juice	Dried fruits	Flour	Nut	Spice
AFB1	Linearity Range	1-30	1-30	1 -30	1-30	1-30
	R2	0.9991	0.9994	0.9993	0.9991	0.9989
	LOD	0.05	0.06	0.05	0.08	0.08
	LOQ	0.08	0.08	0.08	0.13	0.13
AFB2	Linearity Range	0.3-10	0.3-10	0.3-10	0.3-10	0.3-10
	R2	0.9990	0.9989	0.9990	0.9988	0.9987
	LOD	0.06	0.05	0.05	0.08	0.08
	LOQ	0.09	0.09	0.08	0.1	0.1
AFG1	Linearity Range	1-30	1-30	1-30	1-30	1-30
	R2	0.9992	0.9992	0,9991	0,9990	0.9989
	LOD	0.08	0.075	0.08	0.08	0.08
	LOQ	0.13	0.13	0.13	0.13	0.13
AFG2	Linearity Range	0.3-10	0.3-10	0.3-10	0.3-10	0.3-10
	R2	0.9989	0.9986	0.9987	0.9986	0.9984
	LOD	0.06	0.05	0.05	0.08	0.08
	LOQ	0.09	0.09	0.08	0.1	0.1
OTA	Linearity Range	1-30	1-30	1-30	0.1-30	1-30
	R2	0.9991	0.9991	0.9989	0.9968	0.9967
	LOD	0.07	0.07	0.08	0.09	0.1
	LOQ	0.1	0.1	0.2	0.2	0.3

AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; LOD: Limit of detection; LOQ: Limit of quantification; OTA Ochratoxin A; R2: Regression coefficient; RSD: Relative standard deviation.

The results (Table 4.9) indicated good intra-day and inter-day precision below the acceptance limit of the method (<20%), with intra-day and inter-day precision ranging from 0.12 to 7.25% and from 0.23 to 10.28%, respectively. The overall method of performance satisfied the requirements established by the European Union (EU) legislation (European Commission 2006). The trueness as recovery was determined at different concentrations spiked in selected food matrices (SANTE-11813, 2017) and the results were summarized in Table 4.9. The recovery of the target analytes ranged from 81.94 to 101.67% in different food sample matrices and consistent with the recommended validation method (European Commission 2006). The performance results revealed the suitability of the validated method for the determination of trace concentration of mycotoxins (aflatoxins and ochratoxins).

Table 4.9: Recovery and precision for QuEChERS-LC-MS/MS method

Mycotoxin	Con[a]	Fruit juic[e]			Dried fruit[f]			Flour[g]			Nut[h]			Spice[i]		
		RE[b]	Intra[c]	Inter[d]	RE	Intra	Inter	RE	Intra	Inter	RE	Intra	Inter	RE	Intra	Inter
AFB1	5	97.40	1.50	2.12	100.15	0.69	0.82	99.62	3.00	2.49	98.92	0.61	1.11	93.80	1.65	3.37
	10	99.92	1.38	4.01	99.45	1.16	1.28	100.70	0.46	0.43	99.88	1.06	1.93	97.81	2.33	1.63
	30	99.98	0.21	0.23	99.64	3.43	4.86	99.80	0.54	2.30	98.88	0.60	3.29	99.73	0.89	5.55
AFB2	1.5	100.72	0.96	3.38	99.60	1.34	1.10	97.33	1.22	2.48	92.58	0.80	4.24	90.43	3.16	7.20
	3	99.19	1.06	1.33	100.11	2.30	3.57	99.53	1.09	1.16	96.31	1.70	2.30	96.81	2.33	6.81
	10	100.27	1.94	3.27	99.28	1.99	3.31	98.40	1.30	2.57	99.93	0.54	2.65	98.54	6.29	4.20
AFG1	5	96.75	1.17	2.25	98.80	1.61	1.01	99.07	1.17	3.46	95.06	5.48	2.28	99.82	1.17	1.63
	10	100.87	1.75	4.21	99.45	1.07	5.60	100.1	0.65	6.62	101.67	1.06	10.10	97.81	2.33	8.04
	30	99.66	2.84	1.55	98.78	2.68	2.98	101.45	2.82	2.30	97.60	1.85	6.27	99.83	0.61	3.34
AFG2	1.5	96.75	1.18	1.90	98.8	1.60	5.47	88.80	3.01	6.18	88.03	1.17	7.10	84.52	1.18	7.73
	3	97.45	1.10	1.77	99.45	1.06	1.30	96.63	0.65	3.65	87.78	1.05	6.30	84.22	2.33	9.02
	10	99.09	0.70	0.75	97.73	0.60	2.94	97.83	4.04	2.77	94.78	0.54	8.55	84.10	3.56	10.28
OTA	5	96.75	1.17	1.92	90.70	1.61	1.01	94.29	6.37	4.04	91.62	1.17	4.51	85.99	7.25	8.17
	10	99.56	0.48	0.54	97.21	1.03	1.40	100.7	0.66	6.48	91.97	0.19	4.67	81.94	2.33	9.49
	30	98.04	0.55	1.35	98.28	0.12	0.88	97.93	0.55	1.35	94.90	0.56	6.36	87.40	0.55	8.71

a: Mycotoxins concentration (µg/L for fruit juice&µg/Kg for other samples), b: Recovery(%), c: Intra-day precision, d:Inter-day precision, e: Apple juice, f: Raisin, g: Wheat flour, h: Peanut, i: Mixture spice

4.1.6.2 Comparison of the Developed Method with other Methods

To our knowledge, there are no reports on the comparison of LC-MS/MS instrument for mycotoxins separation. Therefore, the LC-MS/MS methods developed in this study was subjected to comparison with other methods. As shown in Table 4.10, the present validated method revealed a distinguished result for the extraction of the studied mycotoxins (AFB1, AFB2, AFG1, AFG2, and OTA). The LOQs of the present method are lower than other methods. Moreover, recovery and precision are comparable with those of other methods. All these results indicate that the method can be used for the studied mycotoxins (AFB1, AFB2, AFG1, AFG2, and OTA) from various food samples.

4.1.6.1 Occurrence of Mycotoxins in Food Samples

The aflatoxins were detected in 19 food samples as indicated in the Table 4.11. Aflatoxins were detected in two dried raisins, four pistachios, two peanut, three cereal product, four spices, and four chilli samples, with concentrations ranging from 1.50-16.93 μg/kg. The quantities of detected aflatoxins were in the range of 0.76-10.23 μg/Kg in the peanut samples and 0.81-10.15 μg/Kg in pistachio samples. It is common to detect aflatoxins above the European legal maximum limit in raisin, non-roasted peanut, wheat flour, chili, and spices. In some samples, the RSD was high as a result of heterogeneity of the mycotoxins in the sample. In addition to aflatoxins, wheat flour and peanut samples showed the presence of trace amount of ochratoxin A (OTA). The concentration of OTA in wheat flour and peanut sample were 1.2 and 1.20-3.53 μg/Kg, respectively. The detected concentration of OTA is below the European legal maximum limit for OTA(European Commission, 2006) and international limits for OTA in cereal products (AC04318739, 2004). Mycotoxins were not detected in the fruit juice and fig samples.

Table 4.10: Comparison of the developed method with other methods

Method	Matrix	Mycotoxins	R2	LOQ	RSD	RE (Average)	Ref.
QuEChERS-LC-MS/MS	High oil content (almonds, peanuts, walnuts, hazelnuts, pecan nuts, cashews)	AFG2	>0.9942	1.25	20<	73.66	Cunha et al., 2018
		AFG1	>0.9857	1.25	19<	78.00	
		AFB2	>0.9938	1.25	14<	80.00	
		AFB1	>0.9787	1.25	19<	68.33	
		OTA	>0.9939	5	17<	76.00	
QuEChERS-LC-MS/MS	High oil content (sesame butter)	AFG2	0.9987	0.21	6<	93.0	Liu et al., 2014
		AFG1	0.9979	0.21	3<	95..0	
		AFB2	0.9983	0.21	5<	97.0	
		AFB1	0.9991	0.21	5<	99.9	
		OTA	0.9987	0.74	-	-	
QuEChERS-LC-MS/MS	High-sugar and high-water content (Grapes and Wines)	AFG2	0.9988	0.18	18<	94.39	Zhang et al., 2018
		AFG1	0.9988	0.75	16<	87.95	
		AFB2	0.9993	0.39	8<	94.41	
		AFB1	0.9990	0.75	11<	100.29	
		OTA	0.9998	0.3	17<	96.06	
QuEChERS-LC-MS/MS	Food containing complex components (Different species and medicinal herbs)	AFG2	>0.9996	0.25	10<	76.19	Cho et al., 2019
		AFG1	>0.9947	1	9<	82.58	
		AFB2	>0.9968	0.25	7<	87.94	
		AFB1	>0.9933	1	10<	84.39	
		OTA	>0.9996	0.5	16<	66.5	
QuEChERS-LC-MS/MS	Different food matrices	AFG2	>0.9984	0.08-0.1	11<	93.06	Present work
		AFG1	>0.9989	0.13	11<	99.11	
		AFB2	>0.9987	0.08-0.1	7<	97.94	
		AFB1	>0.9989	0.08-0.13	6<	99.04	
		OTA	>0.9967	0.1-0.3	10<	93.82	

Table 4.11: Occurrence of mycotoxins in food samples

Sample	T.S	Positive (%)		Concentration*(RSD)				
		AFB1	OTA	AFB1	FB2	AFG1	AFG2	OTA
Apple juice	10	0	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Grape juice	10	0	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Orange juice	10	0	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Pomegranate juice	10	0	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Raisin	10	20	0	2.73(9.49), 5.67(15.76)	0.84(14.33), 1.33(18.36)	1.5(8.81), 2.51(0.83)	1.47(8.5)	n.d
Fig	10	0	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Wheat flour	10	30	10	1.50(10.01), 7.33(18.1), 10.12(6.81)	0.45(8.45), 2.70(9.81)	1.80(18.71), 2.61(12.72)	n.d	1.2(10.37)
Barley flour	10	0	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Peanut	10	20	20	5.36(3.69), 10.23(1.9)	1.45(12.91), 2.22(13.30)	2.00(5.03), 4.35(6.20)	0.76(7.9), 0.82(17.4)	1.20(8.54), 3.53 (13.72)
Pistachio	10	40	10	5.3(13.20), 5.48(3.35), 7.48(25.85), 10.15(1.5)	1.46(15.1), 1.60(15.7), 3.47(17.9)	1.90(4.2), 2.1(14.07), 2.5(15.26), 3.31(8.88)	0.81(14.20), 0.90(10.51)	<LOQ
Chili	10	40	0	4.90(11.45), 5.26(3.4), 8.70(4.22), 16.93(15.16)	1.45(2.58), 4.69(11.43), 8.11(13.11)	1.76(8.31), 1.89(20.75), 2.10(19.25), 6.96(10.70)	0.71(15.68), 0.96(17.05)	<LOQ
Spice	10	40	0	4.70(5.68), 7.41(1.6), 10.69(12.50), 14.36(8.15)	1.52(8.14), 2.26(10.90), 3.43(18.10), 4.13(8.4)	1.55(9.7), 1.79(18.68), 7.74(3.01)	<LOQ	<LOQ

AFB1:Aflatoxin B1,AFB2: Aflatoxin B2 ,AFG1: Aflatoxin G1,AFG2: Aflatoxin G2,OTA:Ochratoxin A, Positive(%) : Percent of positive samples, T.S: Total samples. *µg/L for liquid samples & µg/Kg for non-liquid samples. <LOQ: Lower than quantification limit

4.2 Determination of Aflatoxins and Ochratoxin A using Hollow Fiber Liquid-Phase Microextraction (HF-LPME)

4.2.1 Univariate Optimization

4.2.1.1 Organic Solvent

The results are shown in Figures 4.28 & 4.29. The results indicated that 1-octanol gave the best extraction efficiency for AFTs and OTA. The low water solubility, vapor pressure properties, and presence of long hydrocarbon chain and hydroxyl group in 1-octanol made it the most suitable for extraction of polar molecules 1-octanol revealed the best response for all studied mycotoxins. Therefore, it was selected for further experiments.

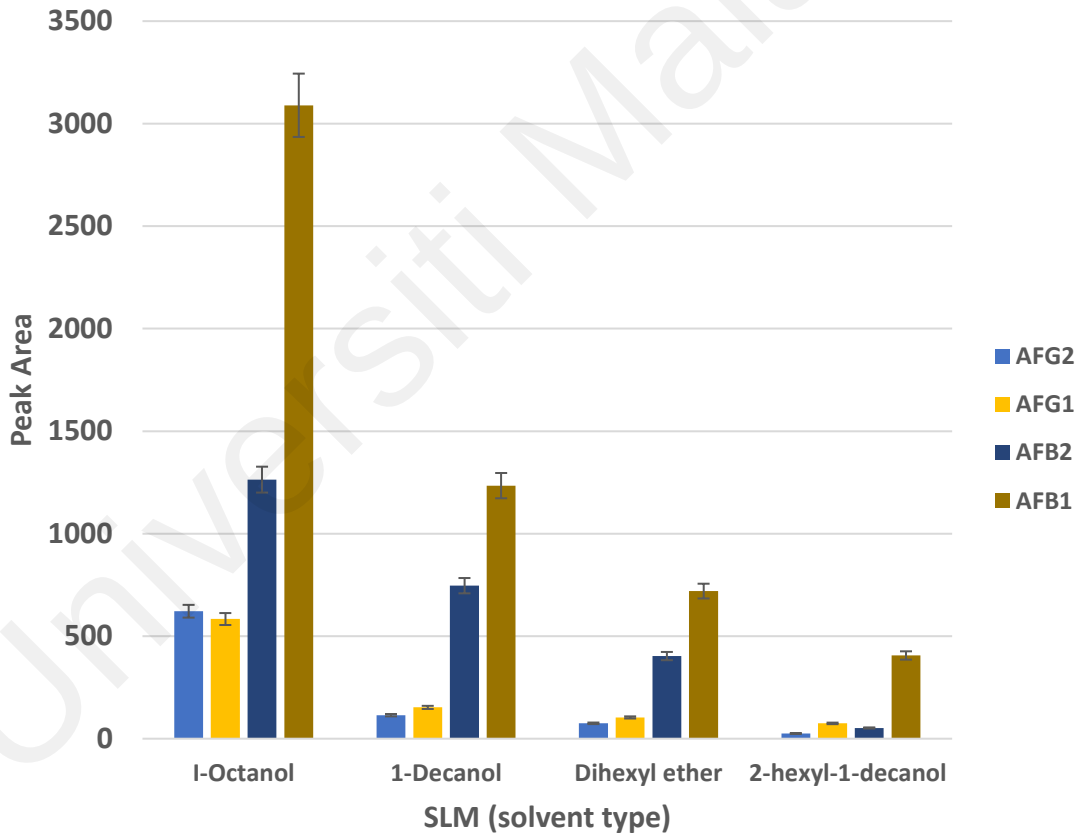

Figure 4.28: Effect of SLM solvent in aflatoxins extraction.

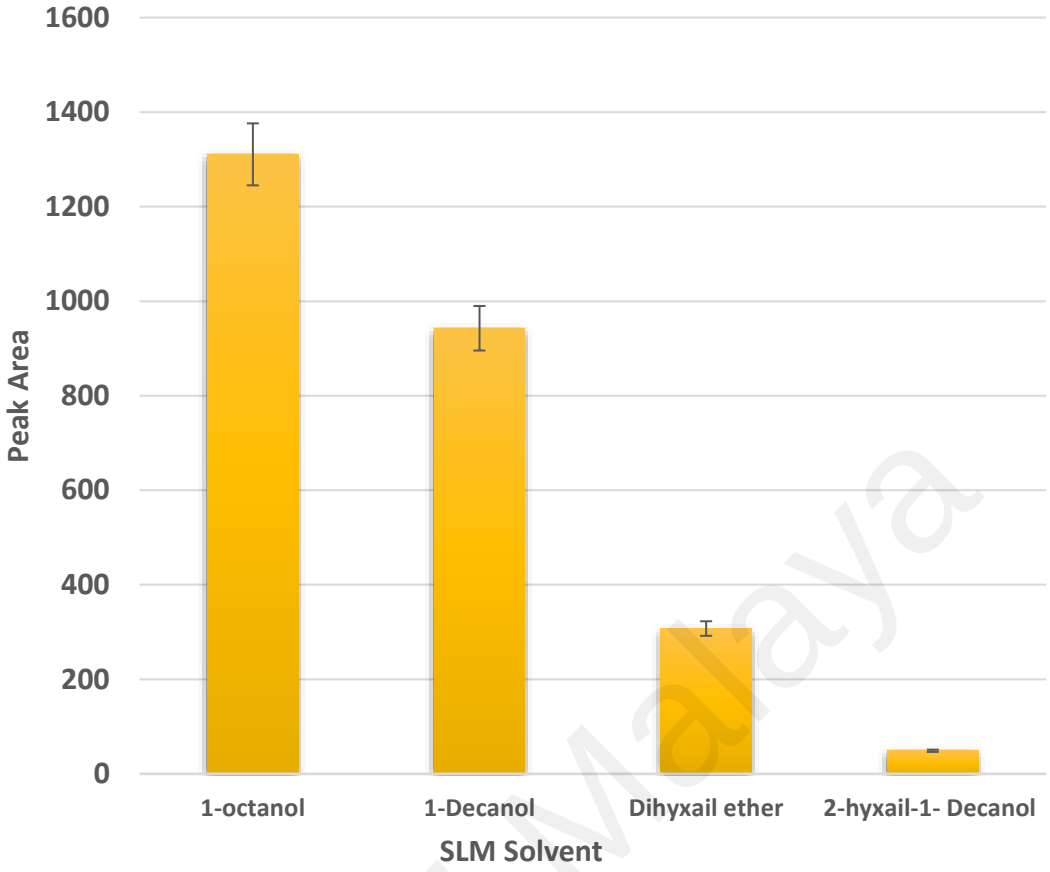

Figure 4.29: Effect of SLM solvent in ochratoxin A extraction.

4.2.1.2 Hollow Fiber Configuration and Length

HF-LPME configuration is an important factor in the improvement of method efficiency. Two configurations, i.e. rod-like and U-shaped were evaluated with aflatoxin only. The U-shaped configuring produced better results (greater peak area) than the rod-like configuration in the extraction of aflatoxins due to large interaction area between the hollow fiber and the sample solution (Ghambarian et al., 2012) (Fig. 4.30). Consequently, the U-shaped hollow fiber was selected for subsequent studies.

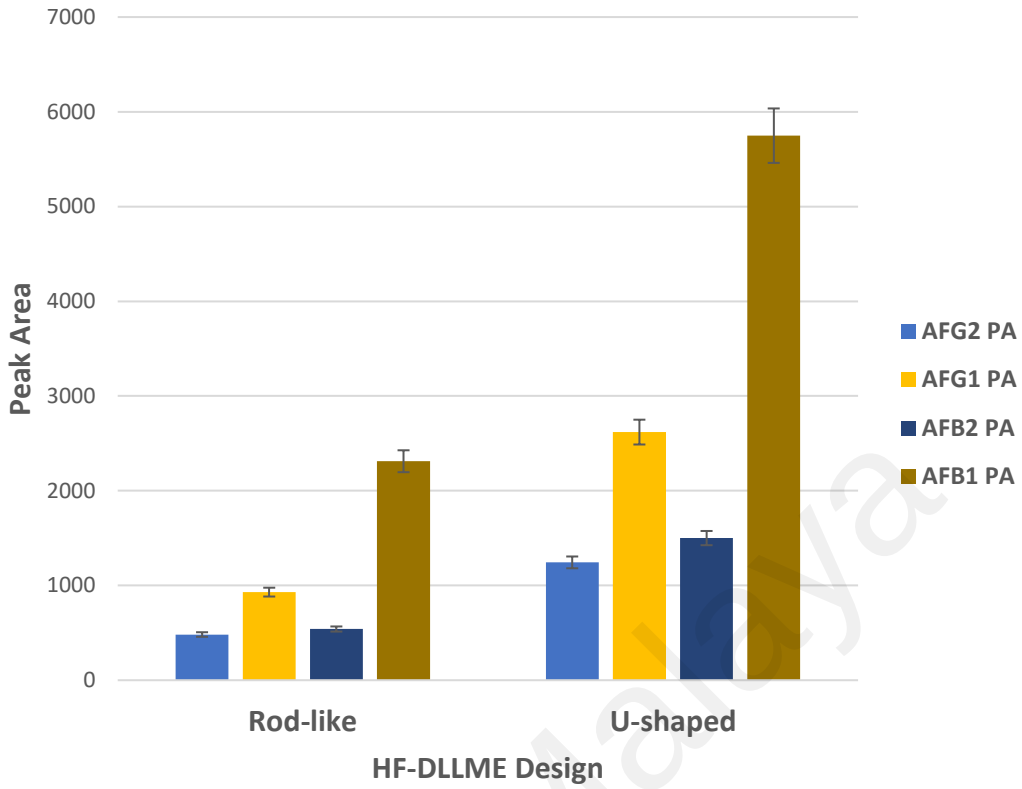

Figure 4.30: Effect of hollow fiber configuration in aflatoxins extraction.

Another variable studied was the length of the fiber. The acceptor phase volume should be large enough to promote analyte transport to the acceptor phase (Nazaripour et al., 2016). Therefore, four different lengths (4, 5, 6, and 7 cm) of hollow fiber were investigated in the study. The results indicated that the best extraction was achieved using 6 cm hollow fiber and held a suitable acceptor phase volume ;thus,it was selected for further experiments(Fig. 4.31 and 4.32).

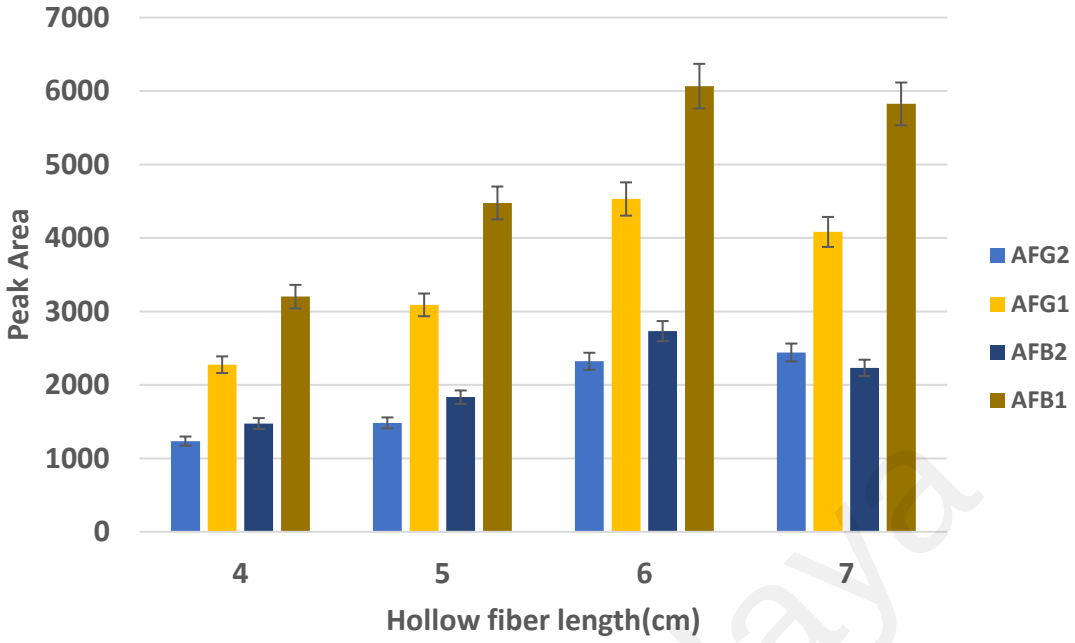

Figure 4.31: Effect of hollow fiber length in aflatoxins extraction.

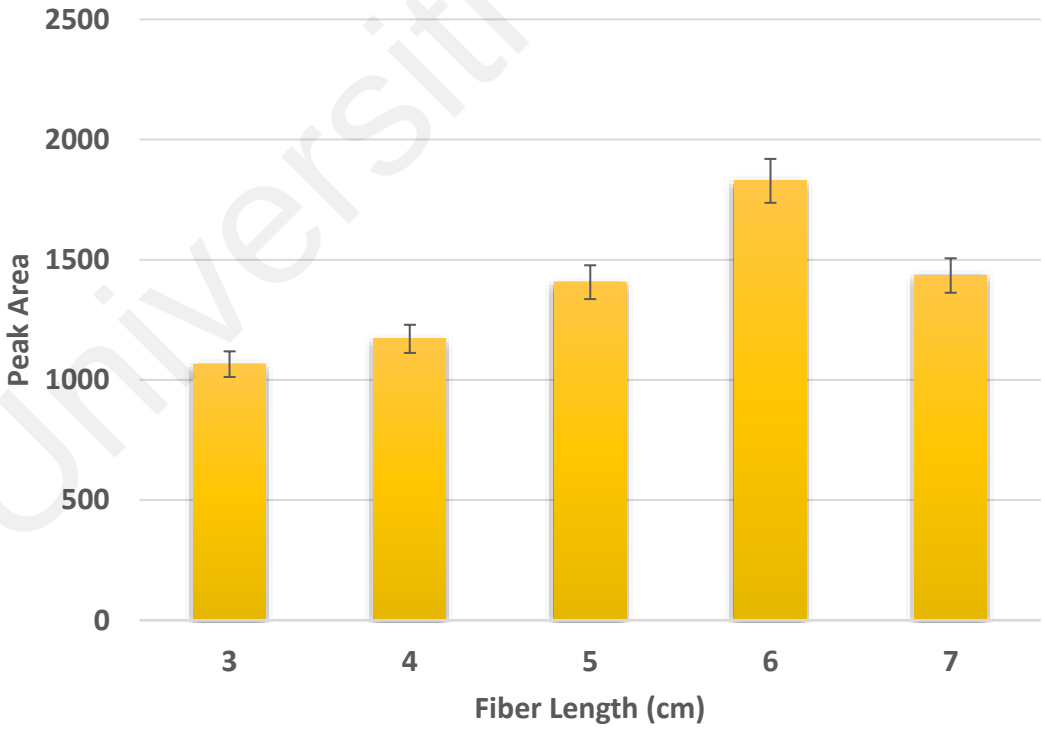

Figure 4.32: Effect of hollow fiber length in OTA extraction.

4.2.1.3 Stirring Rate

The stirring of the sample solution facilitate mass-transfer and improves extraction efficiency as a result of decreased thickness of the diffusion layer(Alsharif et al., 2017). The effects of stirring rates at 350, 700,1000 and 1200 rpm were evaluated in the study. The results showed that the best stirring speed for the extraction aflatoxins (AFB1, AFB2, AFG1, and AFG2) was at 700 rpm (Fig. 4.33). Stirring below 700 rpm may be insufficient to reduce the thickness of diffusion layer effectively, while stirring above 700 rpm may lead to vibration of the hollow fiber and result in loss of solvent in the hollow fiber pores. Thus, a stirring rate of 700rpm was selected for subsequent experiments.

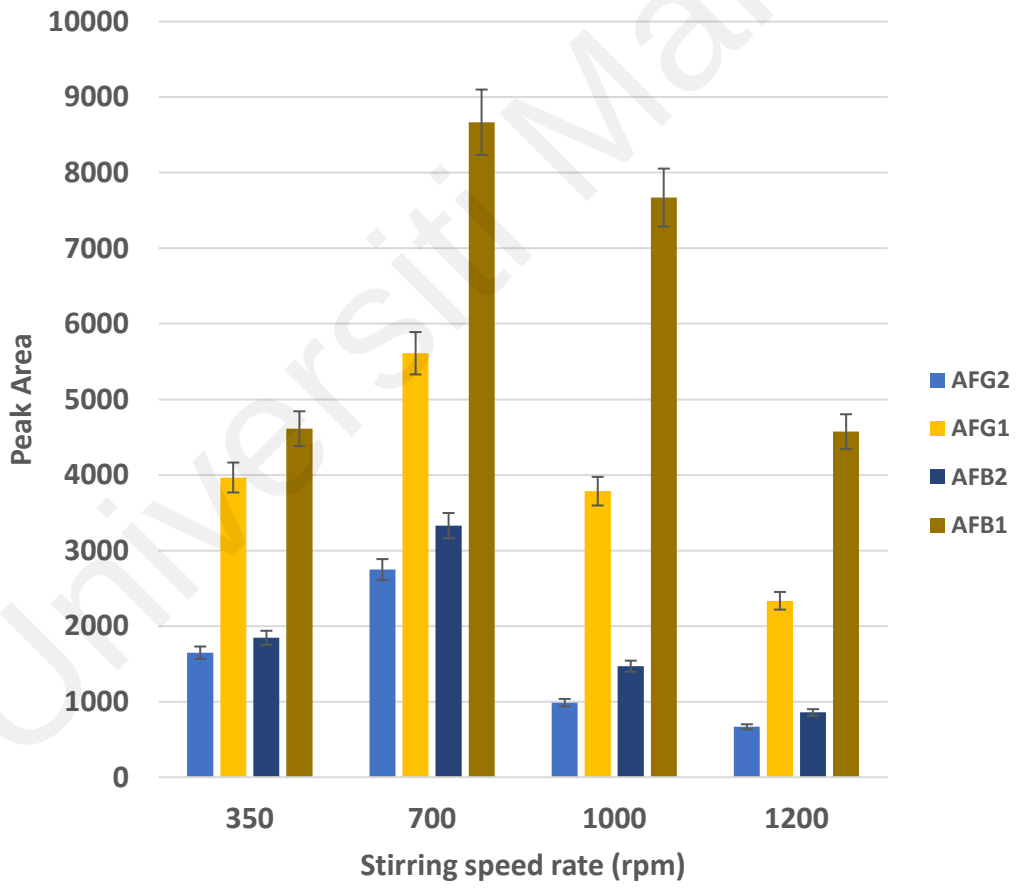

Figure 4.33: Effect of the stirring speed in aflatoxins extraction.

4.2.1.4 Extraction Time

Extraction time is an important parameter because the mass transfer is a time-dependent process. The extraction time profile was evaluated at various times in the range of 30 to 120 minutes (Fig. 4.34). The results suggested that the best extraction time was 50 min and the extraction efficiency droped from 60 min onwards. Therefore extraction time of 50 min was selected for the subsequent procedures.

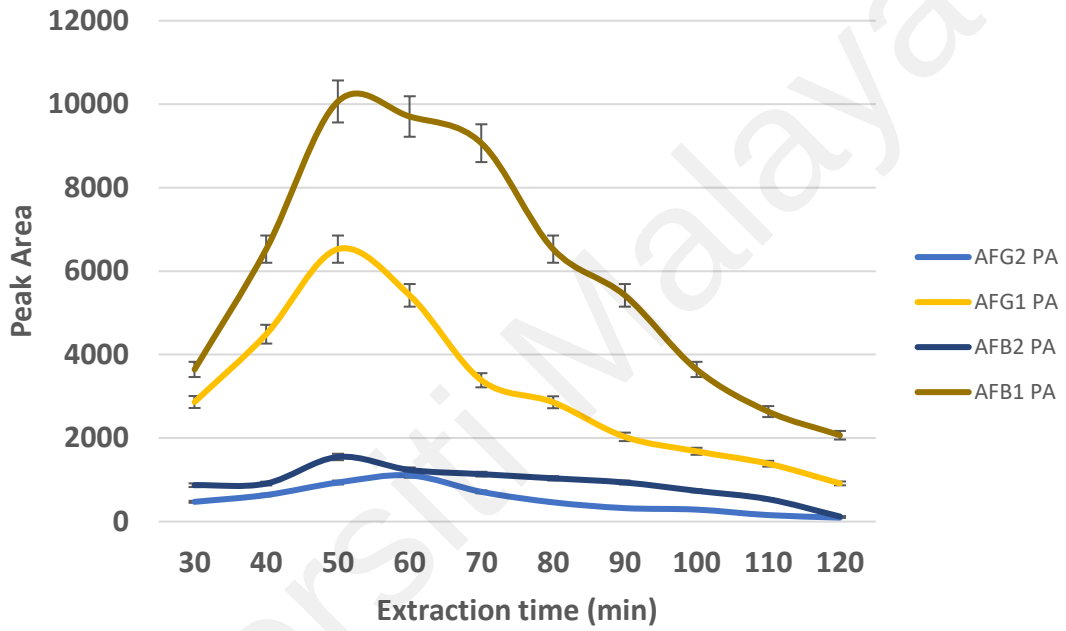

Figure 4.34:Effect of the extraction time in aflatoxins extraction.

4.2.1.5 Donor Phase pH

The pH value of the donor phase (sample aqueous solution) is another important parameter, which in a great extent affects the solubilization characteristics of the analyte as it can change the charge distribution in target analyte molecule to its neutral form and decrease its solubility in the donor phase (Banforuzi & Hadjmohammadi, 2017). Therefore, pH range of 3–9 (adjusted with hydrochloric acid and sodium hydroxide) was investigated in this study for the extraction of aflatoxins. The results indicated the best donor phase pH was 8 (Fig. 4.35).

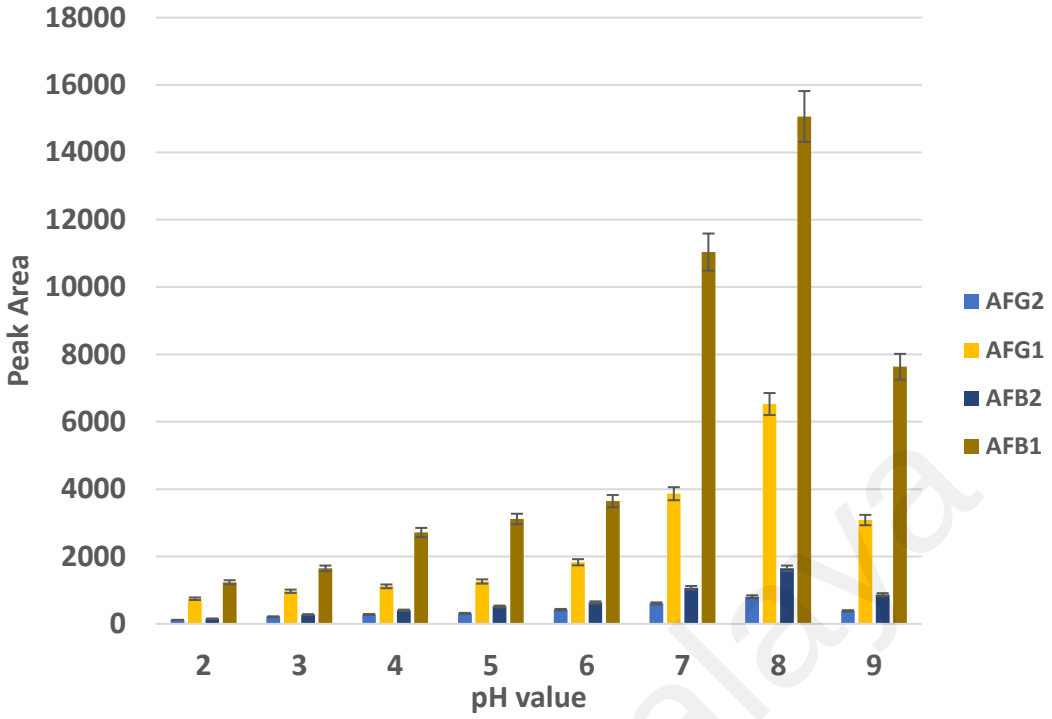

Figure 4.35: Effect of the donor phase pH in aflatoxins extraction.

4.2.1.6 Salting-Out Effect

The salting-out effect was investigated by adding different amounts of sodium chloride to the aqueous solution. It is well-known that the addition of salt may cause a decrease of organic compounds solubility in aqueous solution due to the increase of ionic strength and partition coefficients in organic phase, and hence, increase the extraction efficiency of target analytes from aqueous to the organic phase. In this study, 2% was the best of seven different percentages of NaCl (0-6%) added to the sample. The results indicated (Fig. 4.36) that increasing the percentage of salt from 0 to 2% caused increment of efficiency, and when the salt concentration was higher than 2%, the efficiency reduced. Therefore, the addition of 2% g of NaCl was selected for further experiments.

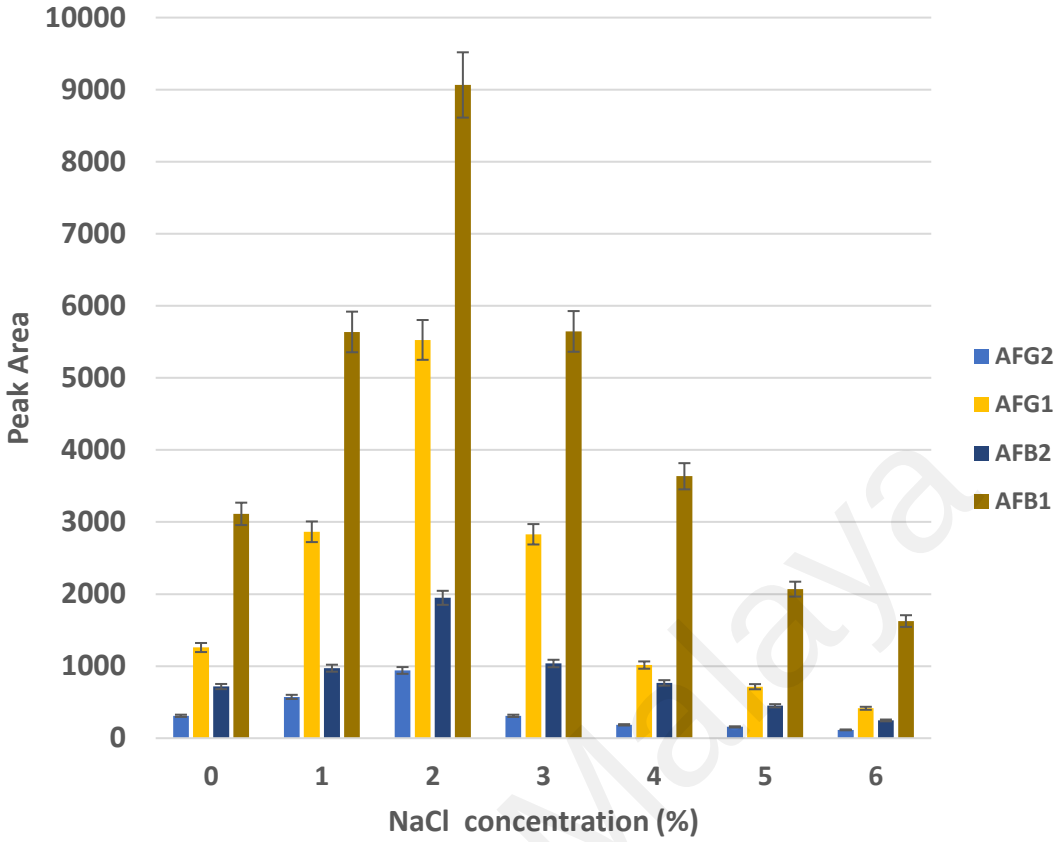

Figure 4.36: Effect of NaCl % in aflatoxins extraction.

4.2.1.7 Extraction/Dispersion Solvent Ratio and Volume

The optimum ratio and volume of chloroform/acetonitrile in providing maximum peak area for each aflatoxin and OTA were optimized. The results (Fig. 4.37 & 4.38) indicated that the best extraction/dispersion ratio is 1:4 (chloroform:acetonitrile) displaying the highest peak area response for both AFTs and OTA. Figures 4.39 & 4.40 indicated the optimum volume that provided the best peak area (150 µL for AFTs and 200 µL for OTA). Therefore, 150 µL and 200 µL were selected for the AFTs and OTA experiments, respectively.

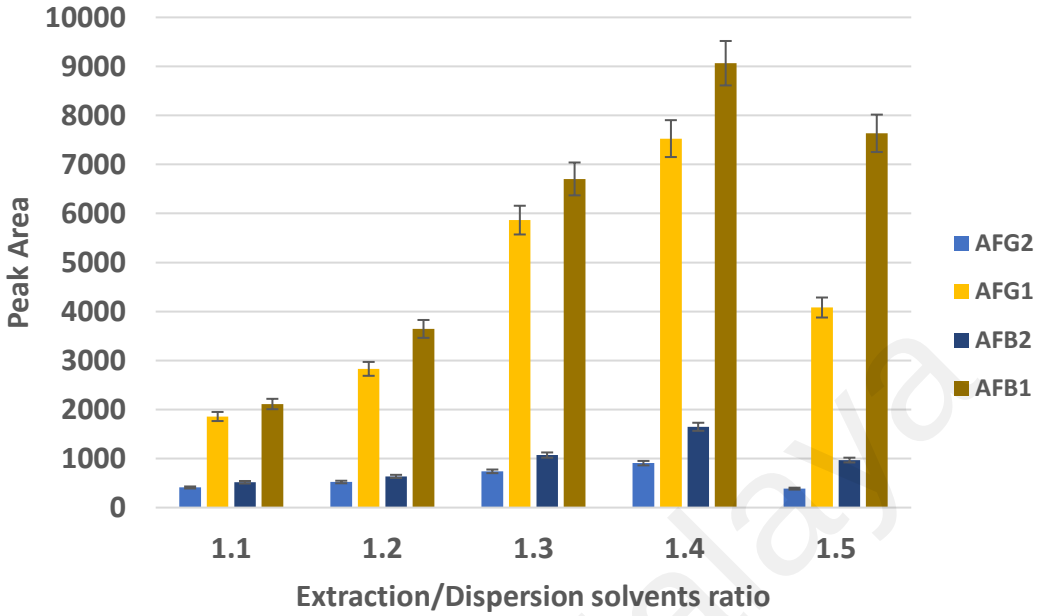

Figure 4.37: Effect of extraction/dispersion solvents ratio in aflatoxins extraction.

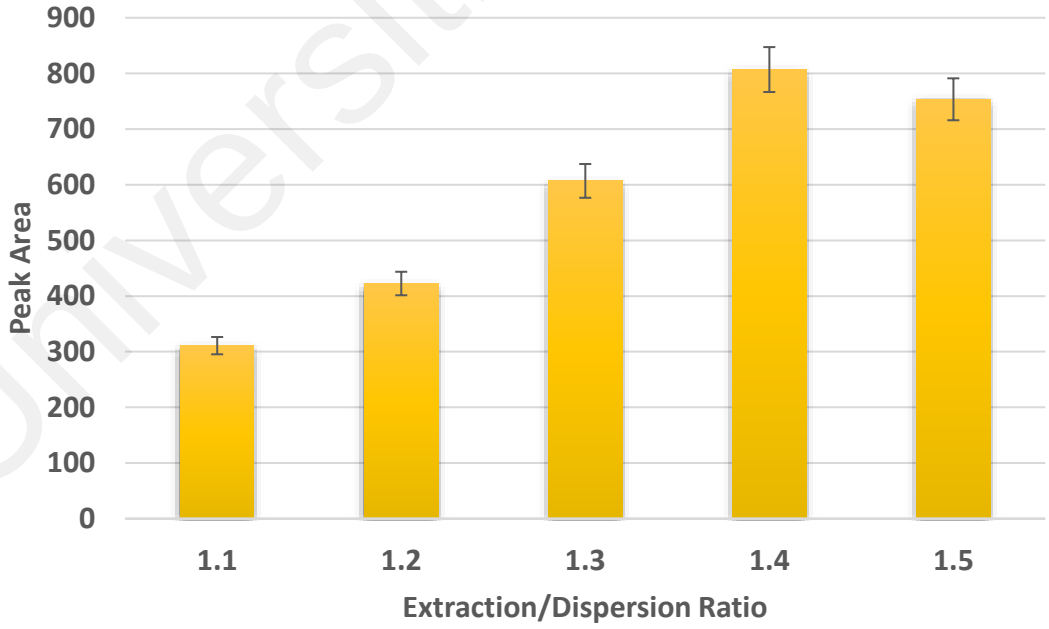

Figure 4.38: Effect of extraction/dispersion solvents ratio in ochratoxin A extraction.

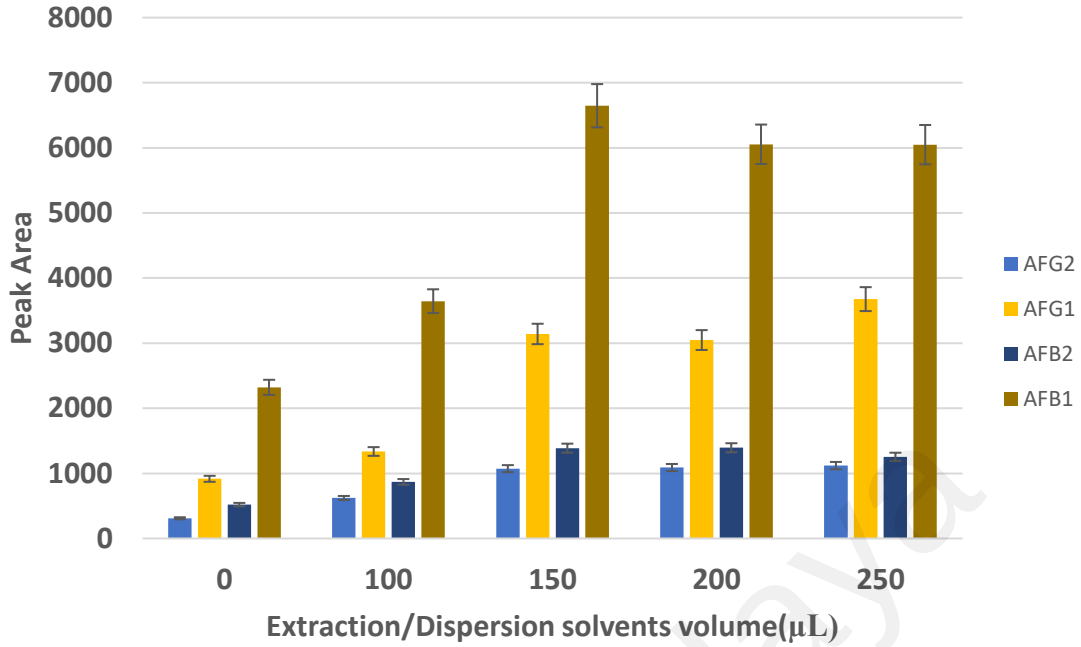

Figure 4.39: Effect of extraction/dispersion solvents volume in aflatoxins extraction.

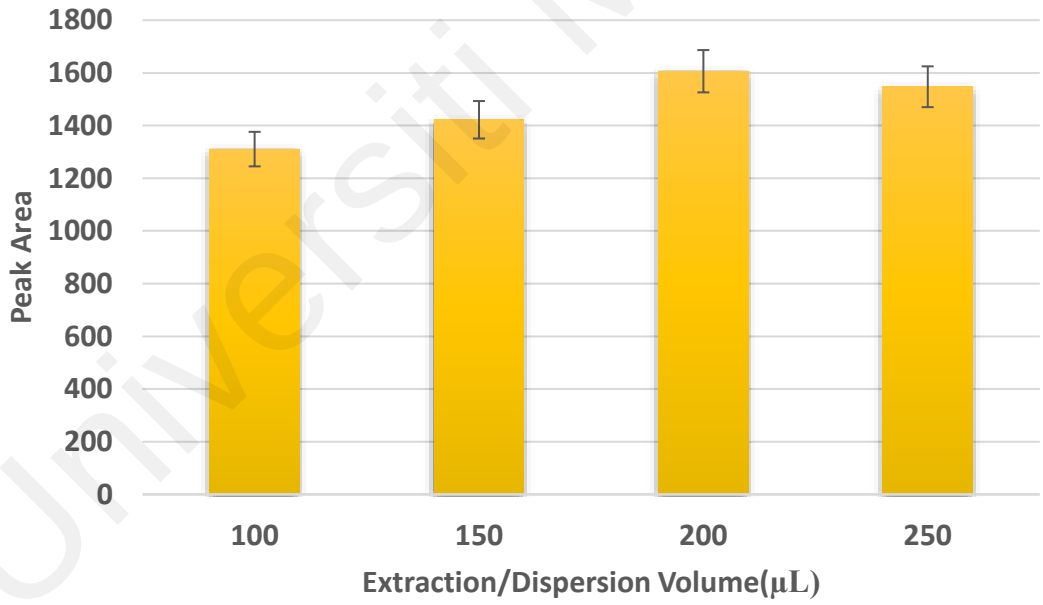

Figure 4.40: Effect of extraction/dispersion solvents volume in ochratoxin A extraction.

4.2.1.8 Desorption Time

The effect of desorption time needed to transfer mycotoxins from the membrane into solvent was investigated in the range of 1–25 min using an ultrasonic cleaner bath at 30

°C. Figures 4.41 and 4.42 showed that the desorption was after 15 min for AFTs and 20 min for OTA. Hence, 15- and 20-min desorption time conditions were chosen for the analysis of AFTs and OTA, respectively.

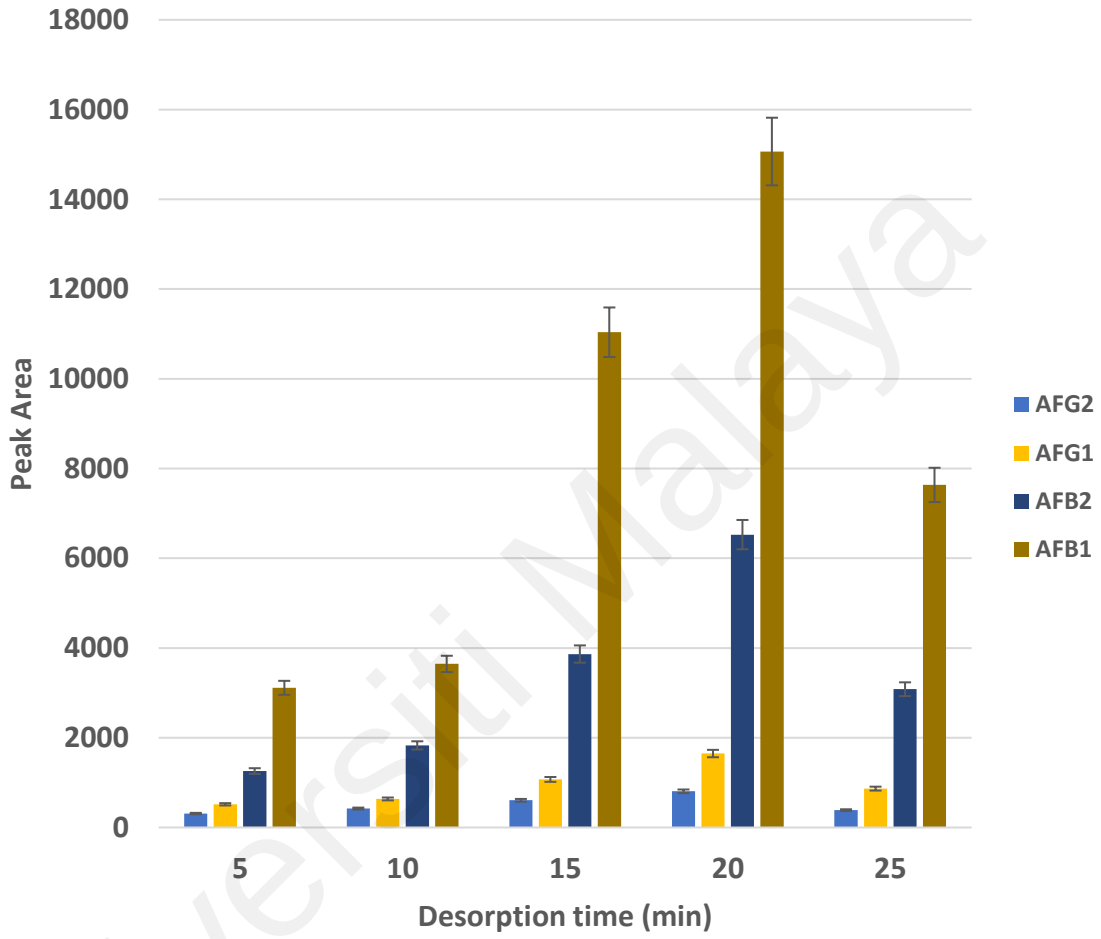

Figure 4.41: Effect of desorption time in aflatoxins extractions.

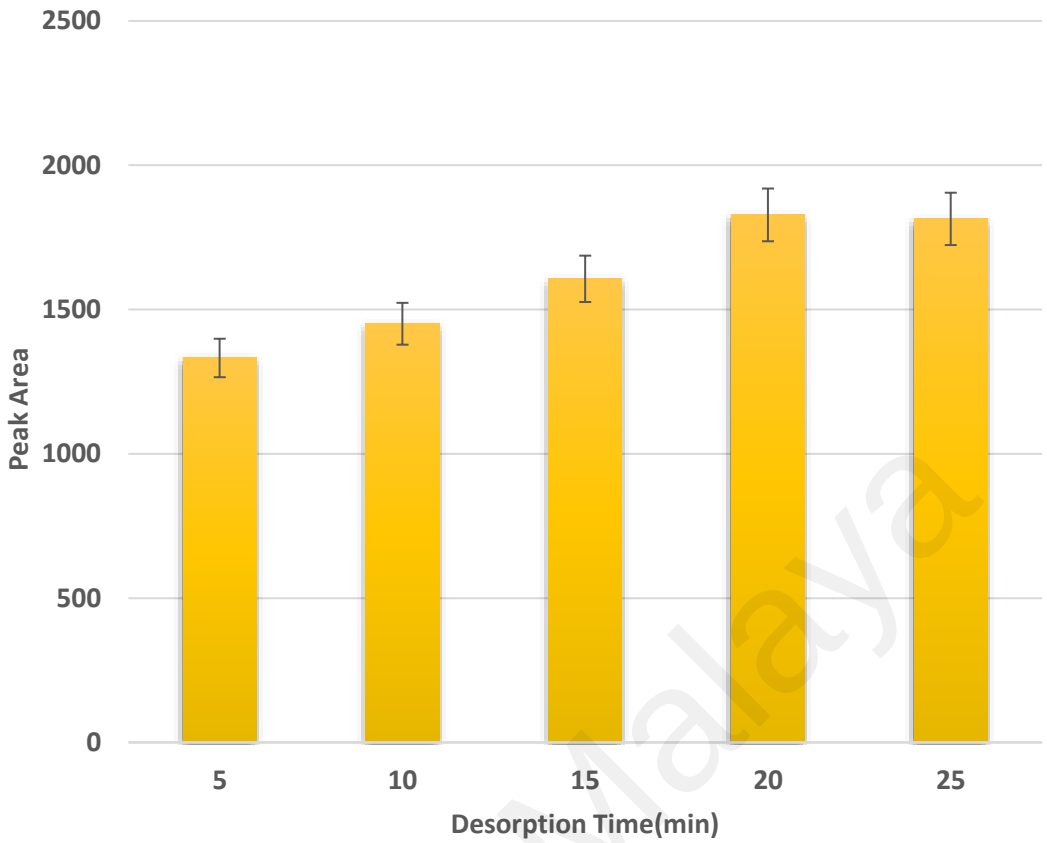

Figure 4.42: Effect of desorption time in Ochratoxin A extraction.

4.2.2 Multivariate Optimization for OTA Analysis.

In the present study, the ANOVA results (Table 4.12) revealed $R^2 = 0.93$ with $p<0.05$ for linear and square terms, and no significant interacting effects among the investigated factors. Equation 4.1 illustrates the relationship between the four variables (*i.e.,* pH of the donor (A), NaCl % (B), extraction time (C), and stirring rate (D)) and the OTA peak area (OTAPA):

$$-OTA(PA)=-0.1126+5.57x10^{-3}(A)+0.006.5x10^{-3}(B)+0.0002.02x10^{-4}(C)+8.4x10^{-5}(D)- 2.269x10^{-3}(A2)-0.0007.40x10^{-4}(B2)0.7x10^{-4}(C2)-0.1x10^{-4}(D2)+0.0002.88x10^{-4}(A*B)+0.12x10^{-4}(A*C)+0.1x10^{-5}(A*D)+6.3x10^{-5}(B*C)-1x10^{-5}(B*D)+0.1x10^{-5}\ (C*D) \quad (4.1)$$

Table 4.12: Central composite design(CCD) ANOVA analysis.

Source	DF	Seq SS	Contribution	Adj SS	AdjMS	F-V	P-V
Model	15	0.047732	93.42%	0.047732	0.003182	13.24	0.000
Blocks	1	0.001192	2.33%	0.001192	0.001192	4.96	0.043
Linear	4	0.039308	76.93%	0.039308	0.009827	40.89	0.000
PH	1	0.038096	74.56%	0.038096	0.038096	158.52	0.000
NaCl	1	0.000966	1.89%	0.000966	0.000966	4.02	0.065
EX.T	1	0.000242	0.47%	0.000242	0.000242	1.01	0.332
St.S	1	0.000004	0.01%	0.000004	0.000004	0.02	0.902
Square	4	0.006751	13.21%	0.006751	0.001688	7.02	0.003
PH*PH	1	0.004357	8.53%	0.005516	0.005516	22.95	0.000
NaCl*NaCl	1	0.000042	0.08%	0.000241	0.000241	1.00	0.334
EX.T(min)*EX.T(min)	1	0.000849	1.66%	0.001182	0.001182	4.92	0.044
St.S(rpm)*St.S(rpm)	1	0.001502	2.94%	0.001502	0.001502	6.25	0.025
Interaction	6	0.000481	0.94%	0.000481	0.000481	0.33	0.908
PH*NaCl	1	0.000033	0.06%	0.000033	0.000033	0.14	0.716
PH*EX.T(min)	1	0.000013	0.03%	0.000013	0.000013	0.06	0.816
PH*St.S(rpm)	1	0.000014	0.035	0.000014	0.000014	0.06	0.813
NaCl*EX.T(min)	1	0.000228	0.45%	0.000228	0.000228	0.95	0.346
NaCl*St.S(rpm)	1	0.000003	0.005	0.000003	0.000003	0.01	0.920
EX.T(min)*St.S(rpm)	1	0.000190	0.37%	0.000190	0.000190	0.79	0.389
Error	14	0.003364	6.58%	0.003364	0.000240		
Lack-of-Fit	10	0.002650	5.19%	0.002650	0.000265	1.48	0.375
Pure Error	4	0.000714	1.40%	0.000714	0.000179		
Total	29	0.051096	100.00%				

Adj MS: Adjusted mean squares; Adj SS: Adjusted sum squares; DF: Freedom degree; EX.T: Extraction time; St.S: Magnetic stirrer speed; Seq SS: the sequential sum of squares.

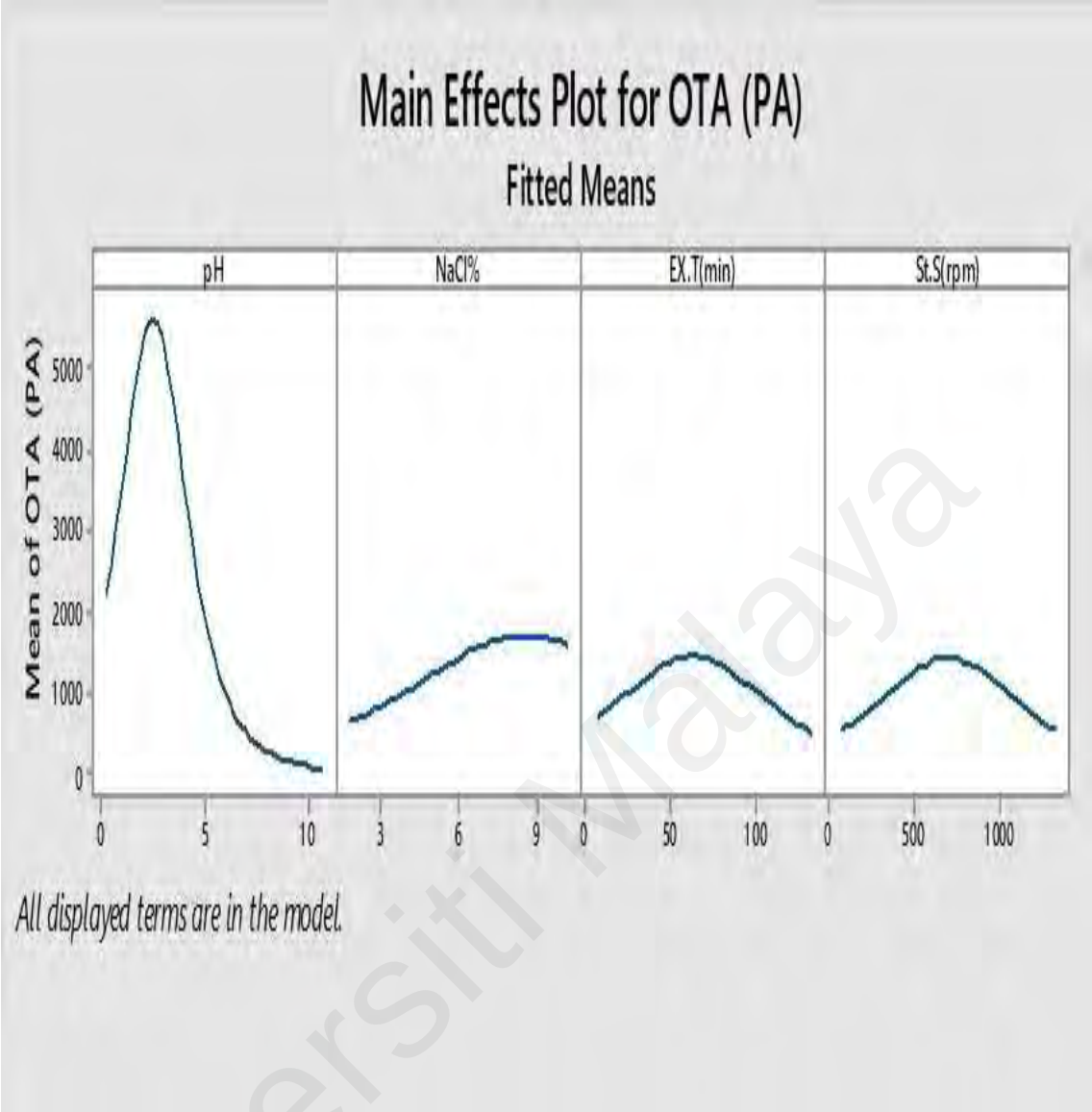

Figure 4.43: The main effect plot for OTA peak area response.

Figure 4.43 shows the effect of each factor on the extraction efficiency. The pH of the donor phase played a key role in the extraction of the analyte. OTA is a weak acid with two pKa values, *i.e.,* pH 4.4 for the carboxyl group of the phenylalanine and pH 7.05-7.3 of the phenolic hydroxyl group. Since OTA is a weak acid, the donor phase should be sufficiently acidic to change the analyte to its neutral form and decrease its solubility in this phase with the pH lower than the pKa of the target analyte. Figure 4.43 shows the highest peak area was obtained with pH of donor phase very close to pH 2 when other

parameters held at mean suggested that at this pH, the donor phase had a significant influence on the mass transfer. The extraction efficiency was increased by increasing the stirring rate, which was attributed to convection enhancement. However, application of higher stirring speed may cause a decrease of the contact area between the aqueous donor phase and hollow fiber as a result of the formation of fine bubbles on the surface of the hollow fiber (Zargar et al., 2015). A stirring speed of 700 rpm was found to give the best response. The extraction efficiency of OTA was enhanced by the addition of a NaCl salt as a result of the salting-out effect. It was observed that increasing the NaCl concentration up to 7% w/v improved the extraction efficiency of OTA. NaCl levels higher or lower than 7% will negatively affect the analyte partitioning between the phases (Alsharif et al., 2017). Lastly, the optimized extraction time was 70 min. The response surface plot in Figure 4.44(A-F) shows the effect of pH and other factors on OTA peak area.

The optimized conditions in the present study for OTA in liquid food samples can be summarized as follows: A) univariate optimization: SLM = 1-octanol, hollow membrane length = 6 cm; B) The response surface optimization graph (Fig. 4.45) showed optimum conditions (0.9725 optimal desirability): (1) the pH of donor phase = 2.6; (2) extraction time = 70 min; (3) stirring rate = 700 rpm; (4) 7.1 % w/v of NaCl in the donor phase; and (5) desorption time = 20 min.

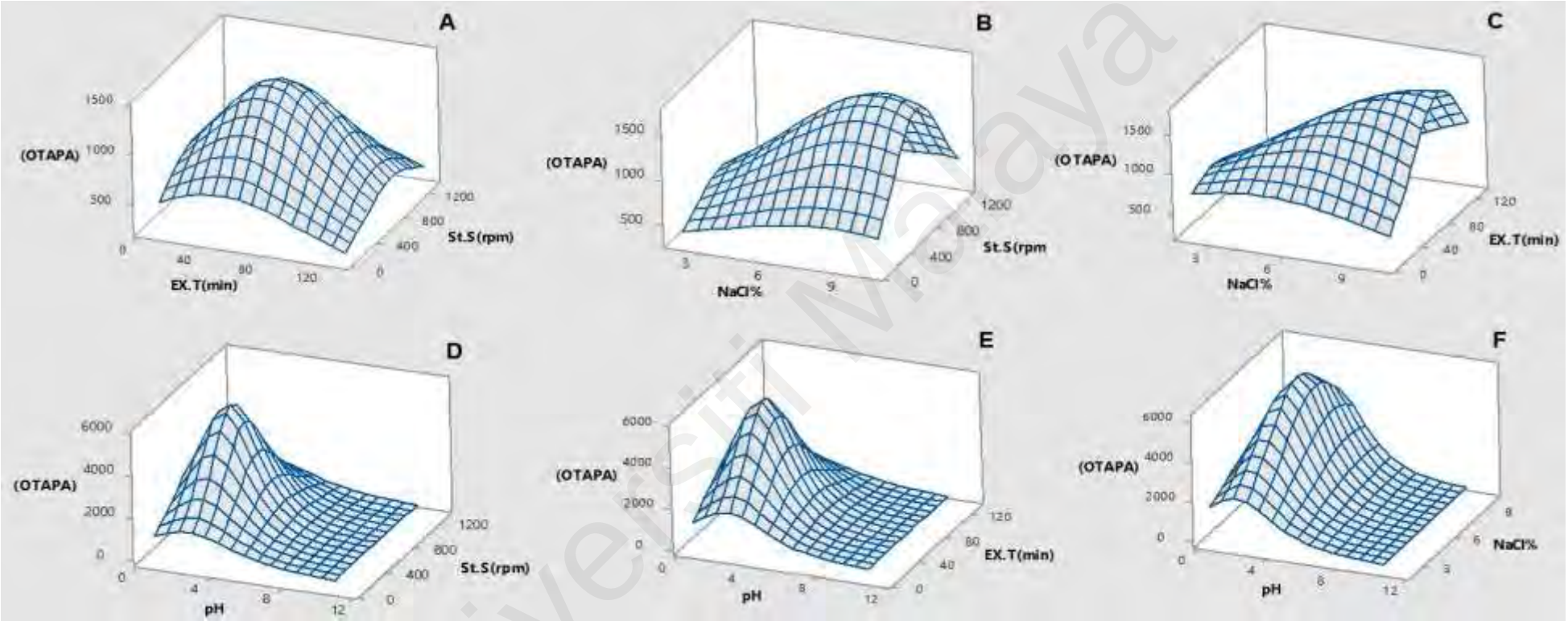

Figure 4.44: Response surface plot of OTA peak area response (A) Ext.T (min) vs. St.S (rpm); (B) NaCl% vs. St.S(rpm); (C) NaCl% vs. Ext.T (min); (D) Stirring speed vs. pH; (E) Ext.T (min) vs. pH; (F) NaCl vs. pH

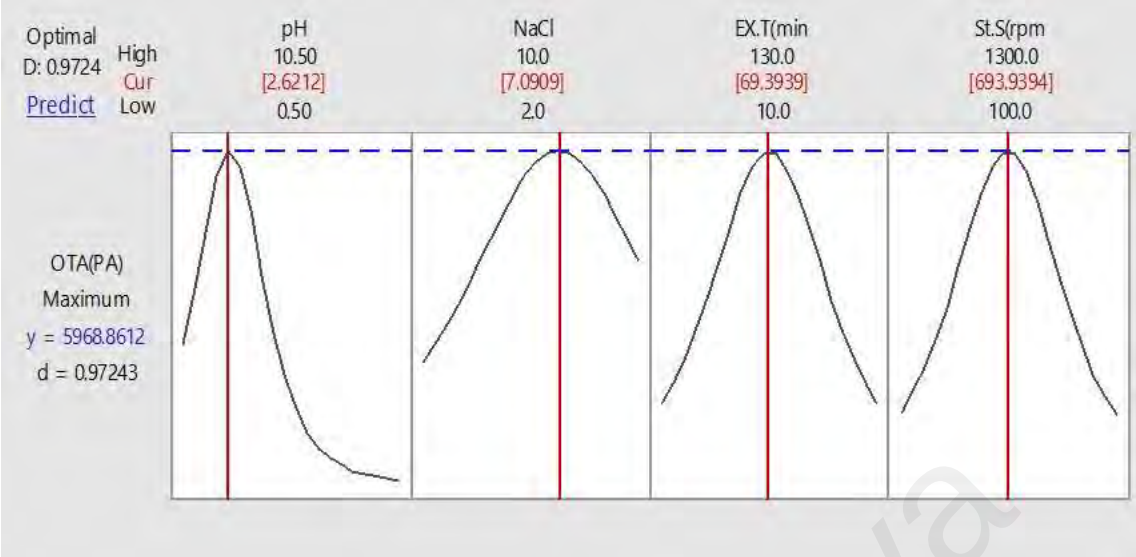

Figure 4.45: Response optimizer plot for optimized parameters

4.2.3 Summary of U-Shaped-HF-LPME Optimized Conditions

Optimized conditions in the present study for aflatoxins in liquid food samples can be summarized in Table 4.13.

Table 4.13: U-Shaped-HF-DLLME optimized conditions

Factor	Aflatoxins	OTA
SLM(solvent type)	1-octanol	1-octanol
Fiber length(cm)	6	6
pH	8	2.6
Salt(%)	2	7.1
Extraction time(min)	50	60
Agitation speed(rpm)	700	700
Extraction/dispersion ratio	1:4	1:4
Extraction/dispersion volume(µL)	150	200
Desorption time(min)	15	20

4.2.4 Analysis of Mycotoxins in Solid Food Sample

4.2.4.1 QuEChERS U-Shaped HF-LPME

Optimization of the QuEChERS extraction solvent composition was investigated by applying the simple lattice design. The simple lattice design is an alternative to simplex centroid designs where reducing the number of experiments is one of the objectives. It is

represented as a triangle in which each corner represents one of the main component and the center of each side be regarded as a mixture of the ingredients at the corners connected by the side (Brereton, 2007).

Table 4.14: Simple lattice design ANOVA analysis

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	8	8674.71	8674.71	1084.34	476.33	0.000
Linear	2	8221.06	534.66	267.33	117.43	0.000
Quadratic	3	406.43	43.20	14.40	6.33	0.009
MeOH*MeCN	1	10.62	11.45	11.45	5.03	0.046
MeOH*H2O	1	125.74	12.71	12.71	5.58	0.038
MeCN*H2O	1	270.06	41.41	41.41	18.19	0.001
Inverse	3	47.23	47.23	15.74	6.92	0.007
1/MeOH	1	8.00	7.38	7.38	3.24	0.099
1/MeCN	1	28.58	27.96	27.96	12.28	0.005
1/H2O	1	10.65	10.65	10.65	4.68	0.053
Residual Error	11	25.04	25.04	2.28		
Lack-of-Fit	1	5.54	5.541	5.541	2.84	0.123
Pure Error	10	19.50	19.500	1.950		
Total	19	8699.75				

Adj MS: Adjusted mean squares; Adj SS: Adjusted sum squares; DF: Freedom degree; Seq SS: the sequential sum of squares.

ANOVA analysis was performed (Table 4.14) and the mixture surface and contour plots were constructed for the OTA recovery (Fig. 4.46 & 4.47). ANOVA was applied to the linear and quadratic models and presented a *p*-value of 0.009, which indicates that the model is very suitable. The Lack-of-Fit was insignificant, due to high *p*-value (0.123) and lower *F*-value (2.84) and it endorsed the suitability of the model for an experimental run. Among the studied solvents, their interactions were significant and the model showed

that the values of the experimental, predicted, and adjusted R^2 were 99.71, 99.06, and 99.50, respectively. Figure 4.48 shows the optimization result with a higher than 92.14% OTA recovery. Figures 4.46 and 4.47 showed the proportional increase of water and methanol will leads to the decrease of OTA recovery. In contrast, the recovery of OTA was increased with the increase of acetonitrile.

It is notable from the plots (Fig. 4.48) that the maximum recovery consisted of a high percentage of acetonitrile with a lower percentage of the methanol and water. The optimal result with the desirability of 97% was MeOH:ACN:H_2O (10:70:20).

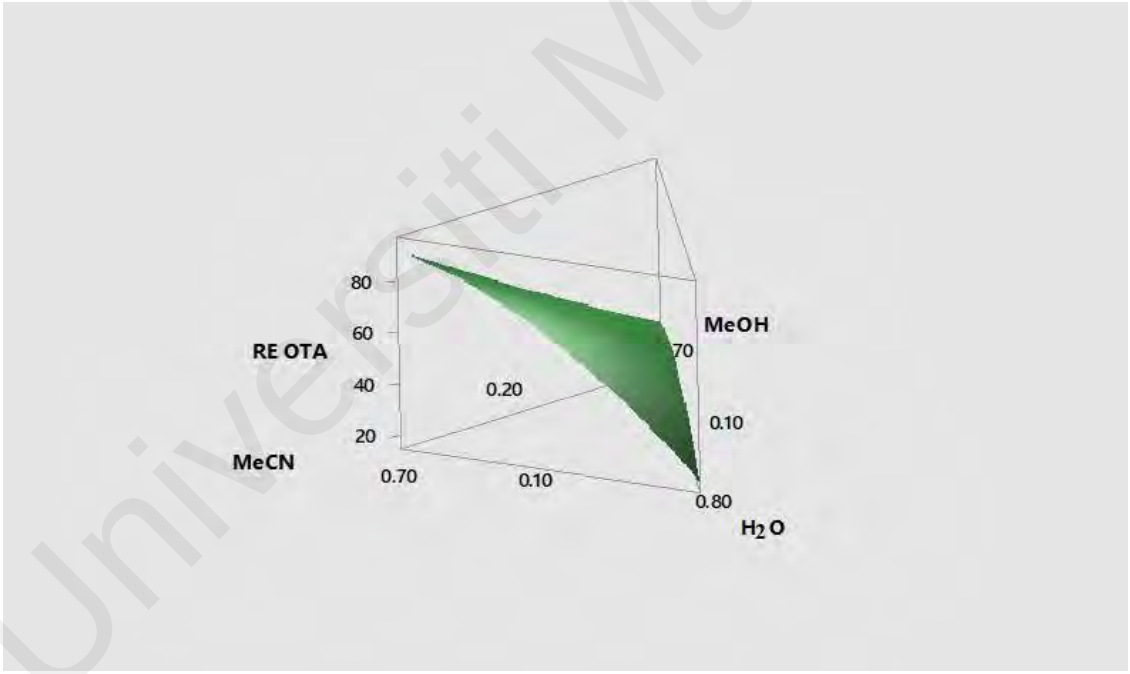

Figure 4.46: Mixture surface plot for OTA recovery.

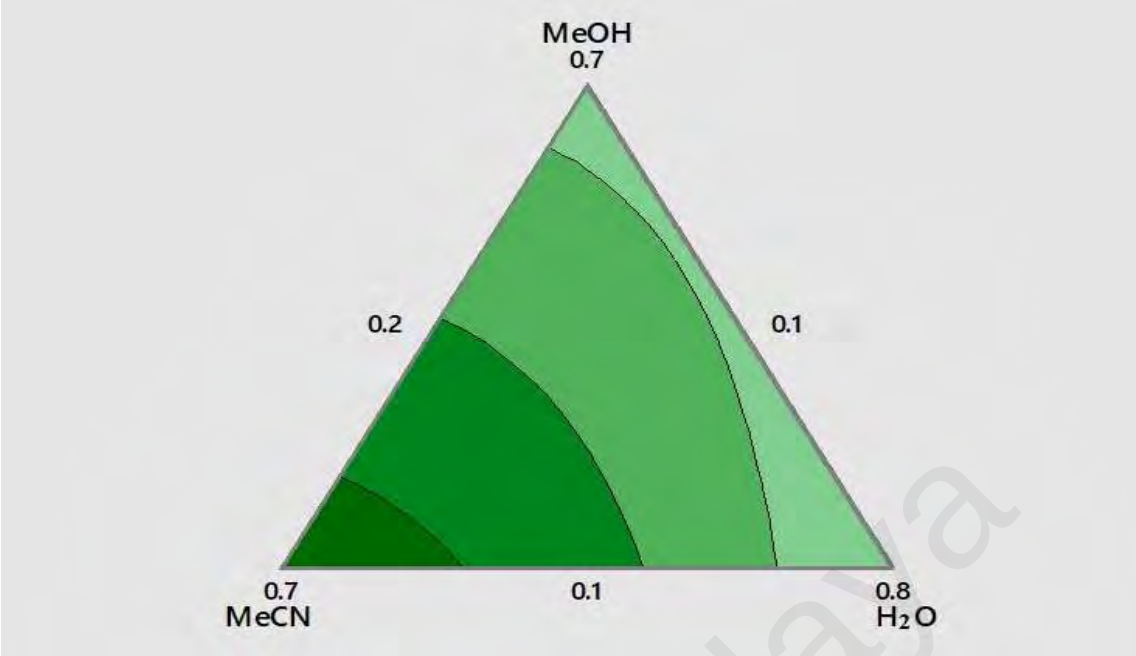

Figure 4.47: Mixture contour plot for OTA recovery

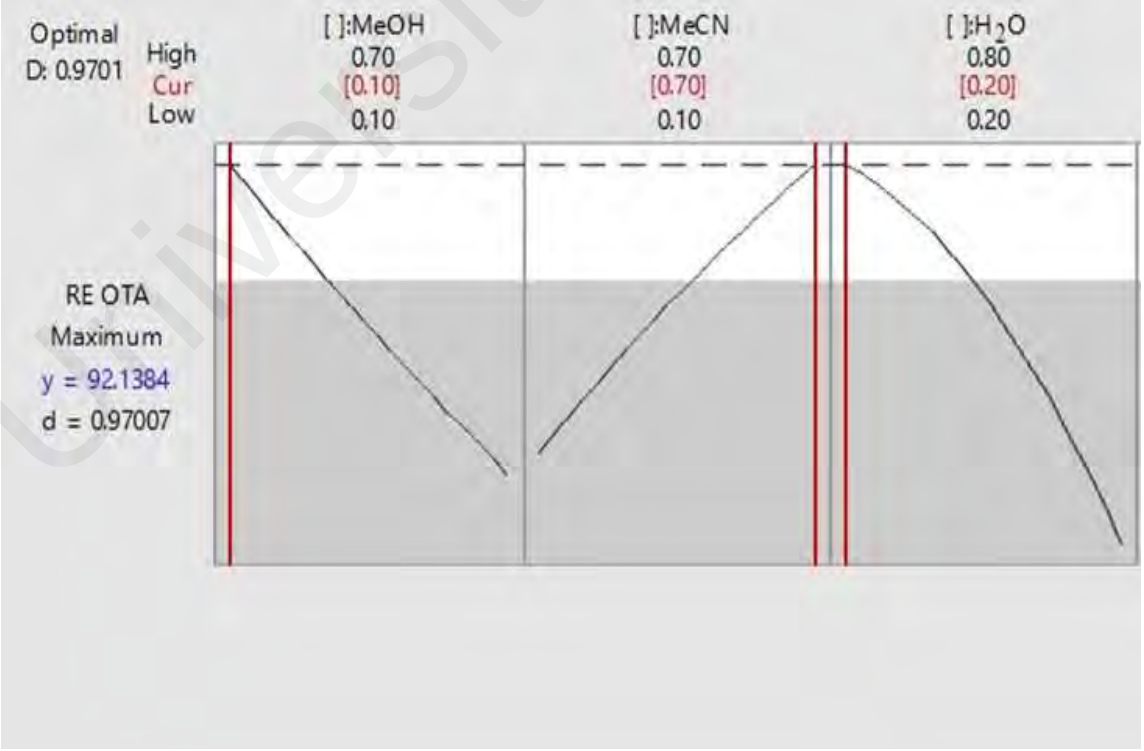

Figure 4.48: Response optimizer for maximum component desirability.

4.2.4.2 Ultrasound-Assisted Solid-Liquid Extraction (UASLE) Combined with U-Shaped HF-LPME.

(a) Extraction Solvent Composition

ANOVA analysis was performed (Table 4.15) and the mixture surface and contour plot were constructed for the average AFG2 peak area (Fig. 4.49 and 4.50). From Table 4.15, experimental, adjusted and predicted R^2 values are 99.88 %, 99.80%, and 99.56%, respectively. The R^2 values revealed the fitting quality of the polynomial model (Paíga et al., 2016) and exceeded the lowest proposed limit of 0.80 (Saguy & Graf, 1990).

Table 4.15: Simple lattice design ANOVA analysis

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	8	17359633	17359633	21699633	1184.76	0.000
Linear	2	12968239	415970	207985	113.56	0.000
Quadratic	3	4388590	1354902	451634	246.58	0.000
MeOH*MeCN	1	2927850	438929	438929	239.65	0.000
MeOH*H2O	1	3410	86	86	0.05	0.832
MeCN*H2O	1	1457330	196445	196445	107.26	0.000
Inverse	3	2804	2804	935	0.51	0.683
1/MeOH	1	259	239	239	0.13	0.725
1/MeCN	1	2489	2475	2475	1.35	0.270
1/H2O	1	56	56	56	0.03	0.865
Residual Error	11	20147	20147	1832	-	-
Lack-of-Fit	1	47	47	47	0.02	0.881
Pure Error	10	20100	20100	2010	-	-
Total	19	17379780	-	-	-	-

Adj MS: Adjusted mean squares; Adj SS: Adjusted sum squares; DF: Freedom degree; Seq SS: the sequential sum of squares.

The contour plot (Fig. 4.50) indicates that the higher average AFG2 peak area (AFG2PA) was found between a mixture of the three solvents; methanol, acetonitrile, and water containing 1% acetic acid. Response optimizer (Fig. 4.51) was used to determine the maximum desirability of the AFG2PA. The component mixture with the optimal desirability of 1.00 consisted of 10 % of methanol, 63.33 % of acetonitrile and 26.67% of water containing 1% acetic acid.

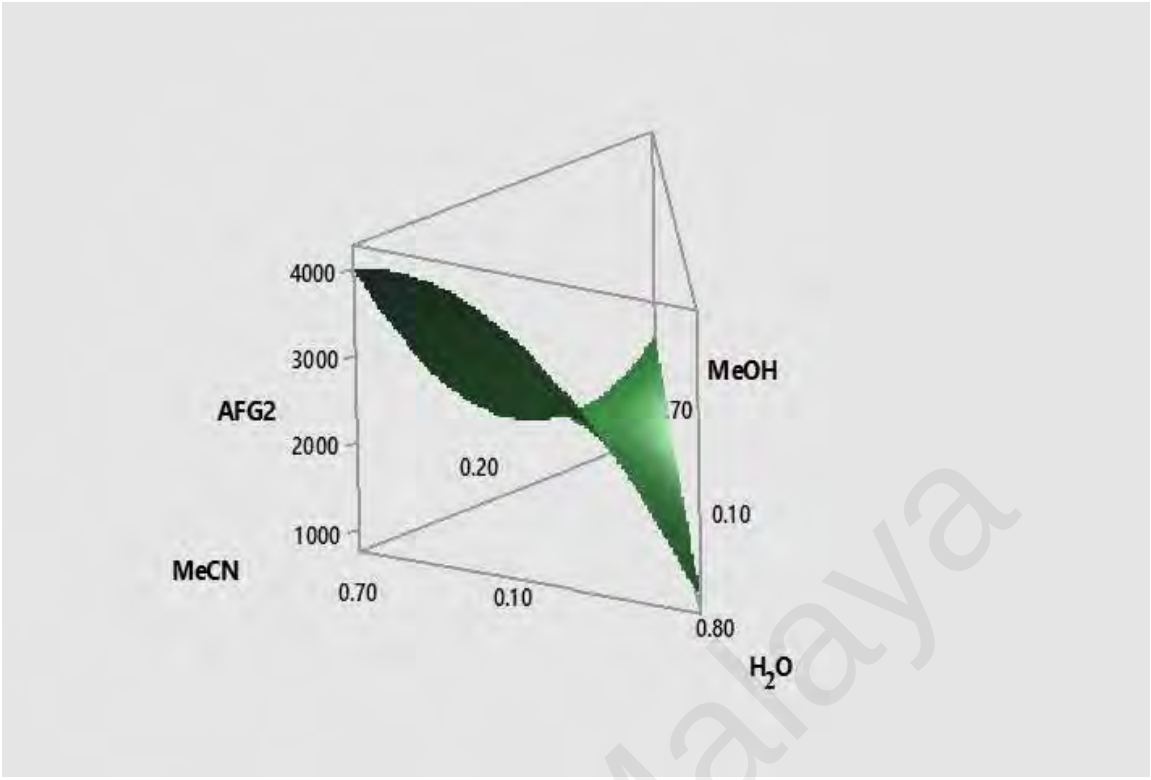

Figure 4.49: Mixture surface plot for AFG2 peak area response

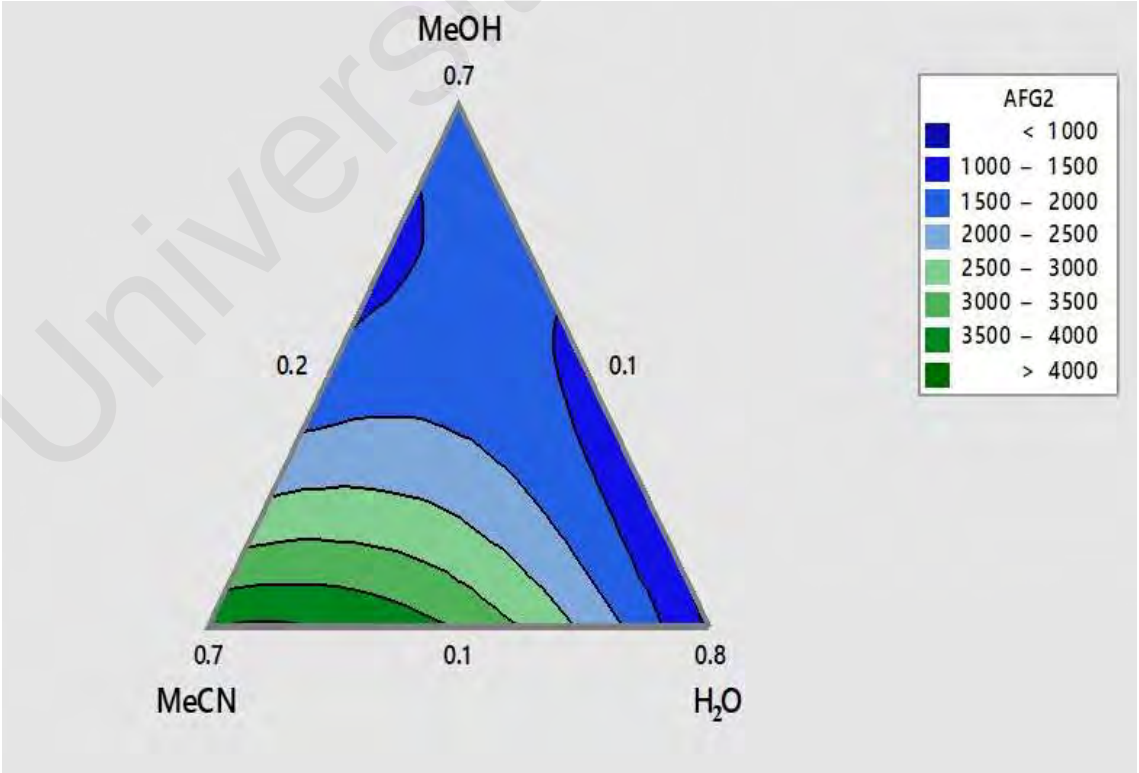

Figure 4.50:Mixture contour plot for AFG2 peak area response.

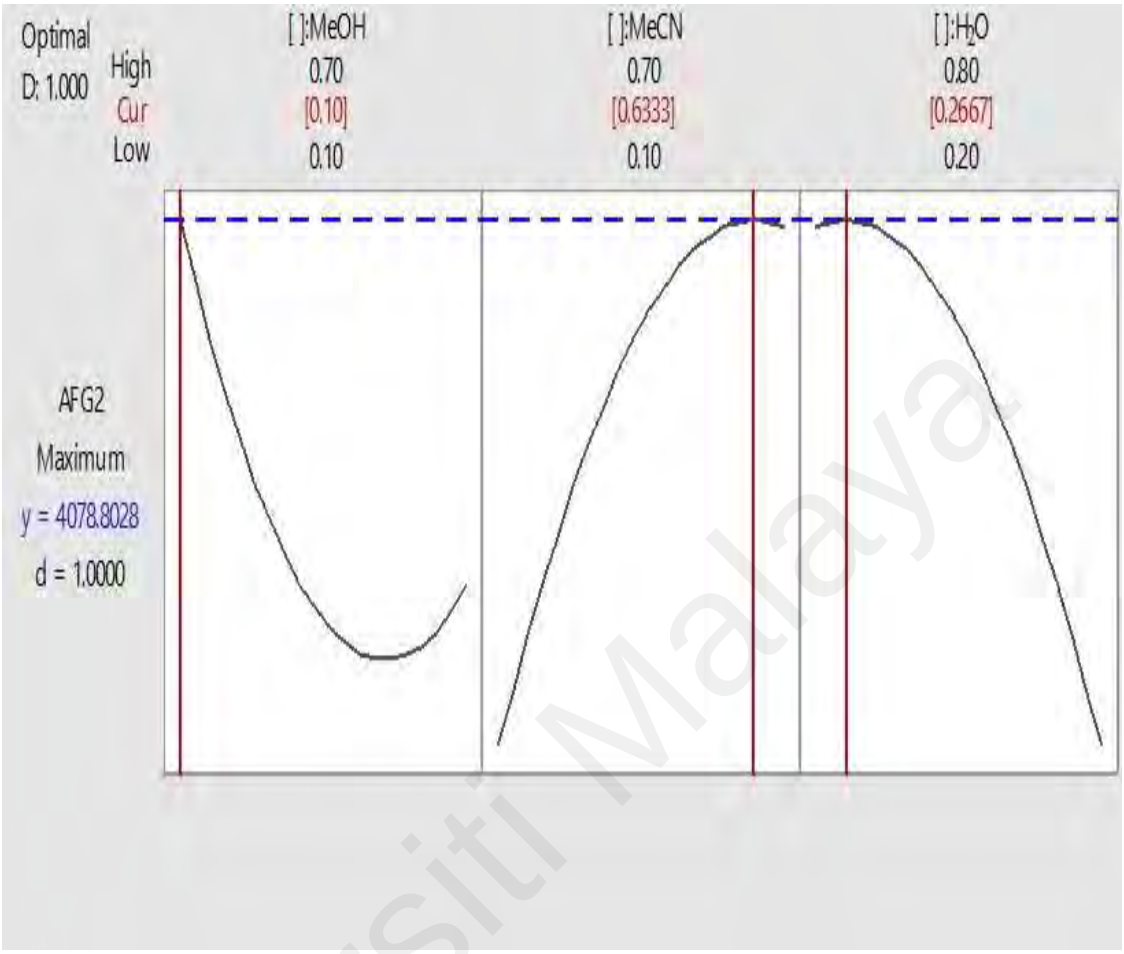

Figure 4.51: Response optimizer for maximum component desirability.

(b) Extraction Solvents Volume

The peak area of target analytes increased as the volume increased from 3 to 8 mL (Fig. 4.52). When the volume of the mixture was above 8 mL, most of the peak area started decreasing. Thus, it was suggested that 8 mL of extraction solvent mixture gave the maximum extraction efficiency (maximum peak area).

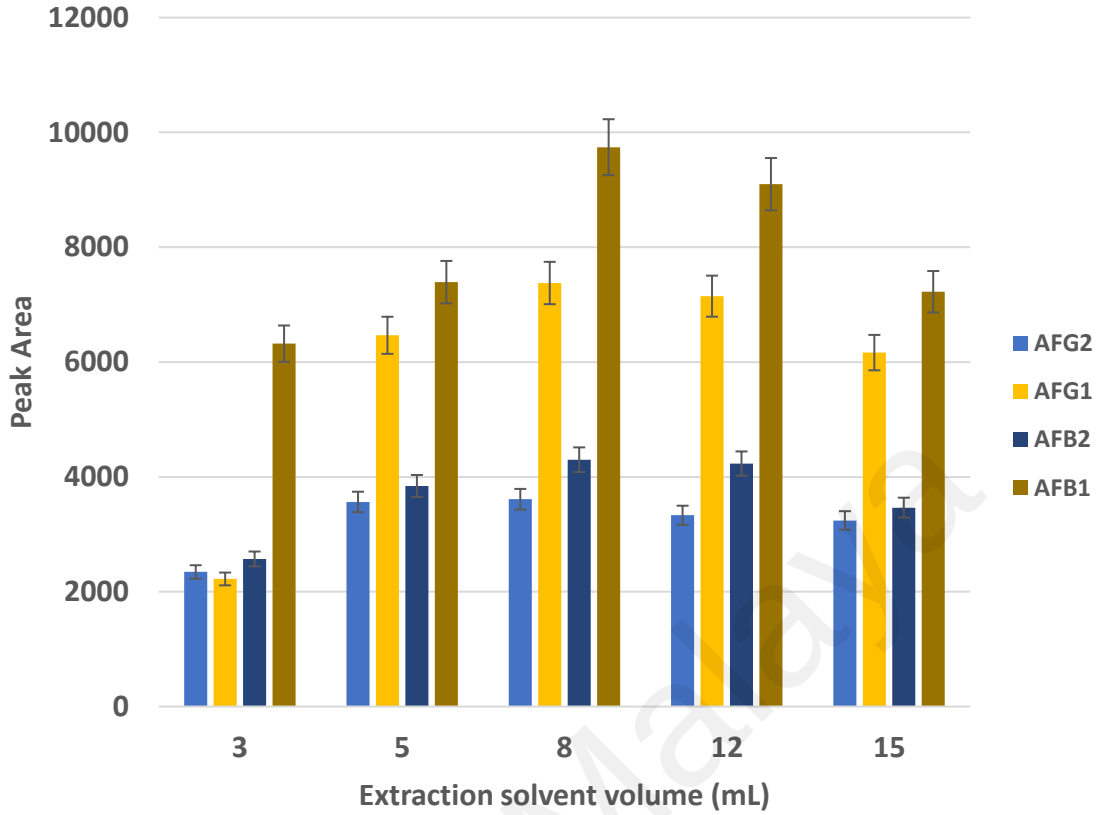

Figure 4.52: Effect of extraction solvents volume on aflatoxins extraction

(c) Extraction Time

The ultrasound extraction time ranging from 5 to 30 min was evaluated in the study. Figure 4.53 shows that the extraction efficiency of aflatoxins increased gradually with the increase of the extraction time, and achieved maximum efficiency at 15 min. Extraction efficiency gradually decrease when extraction time passed beyond 15 min which may be caused by aflatoxin degradation (Manoochehri et al., 2014). Therefore, 15 min was selected as the optimum extraction time.

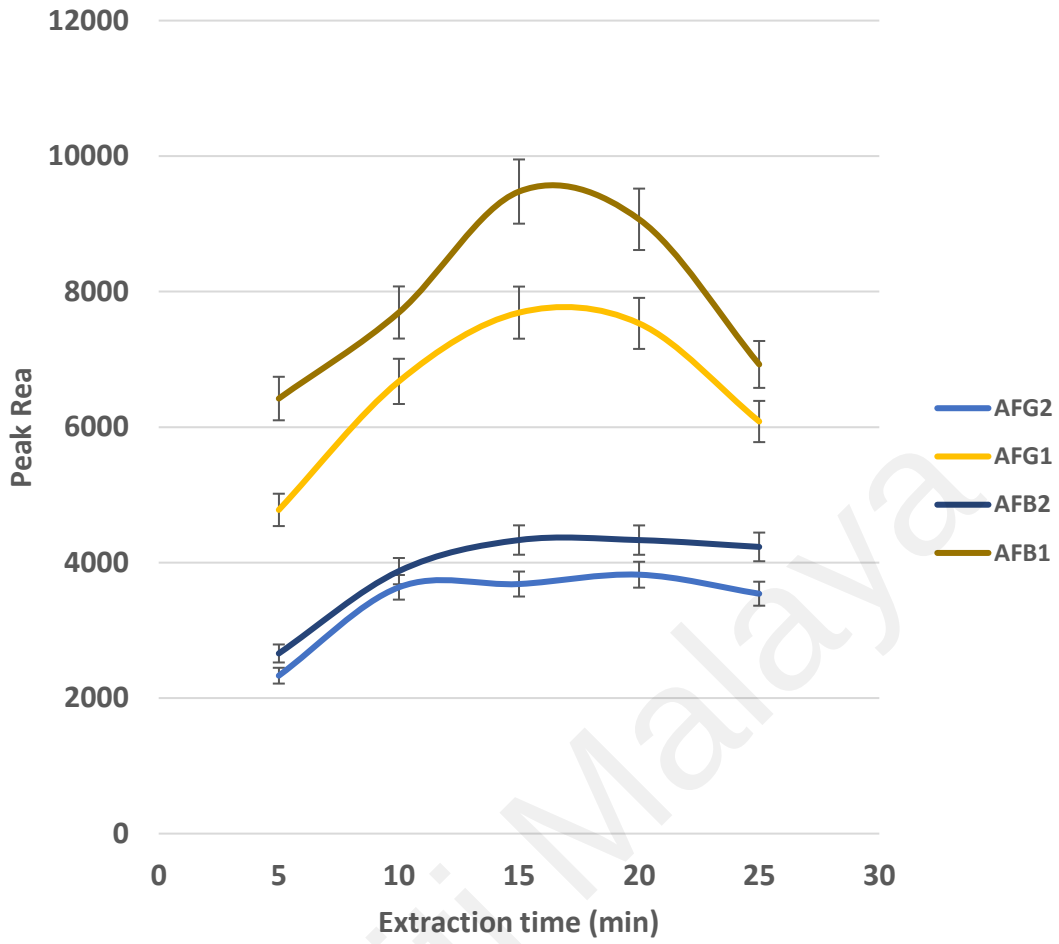

Figure 4.53: Effect of extraction time in aflatoxins extraction

4.2.5 Method Performance

4.2.5.1 Aflatoxins Analysis

Under the optimum conditions, the analytical performance of U-Shaped HF(2)LPME for fruit juice and UAE-U-Shaped HF(2)LPME (for solid food, i.e. dried fruits and nuts products) were evaluated. The matrix-matched calibration curves for aflatoxins (AFB1, AFB2, AFG1, and AFG2) were constructed using seven standard calibrators (i.e., 0.1, 0.5, 1, 5, 10, 15, and 30 µg/L or µg/Kg). The method accuracy, precision, and matrix effect were investigated using non-contaminated food sample spiked at three different levels (1, 10, and 30 µg/L or µg/Kg). As shown in Table 4.16, linear regression lines were obtained with coefficients of determination higher than 0.9986 in the range between

the limit of quantitation and 30 µg /L or µg/Kg for all of the analytes. The limits of detection (LOD) and limits of quantitation (LOQ) were calculated as 3 and 10 times the signal-to-noise ratios, respectively. Limits of detection ranging from 0.04 to 0.09 µg /L or µg/Kg were achieved for all aflatoxins studied. The limits of quantitation were in the range of 0.08 to 0.20 µg /L or µg/Kg. These methods revealed good enrichment factors of 708.15 - 765.52 (Table 4.16) for the extraction and purification of multi-aflatoxins from liquid and solid food samples.

Method accuracy was determined by evaluating the measured concentration of the target aflatoxins (AFB1, AFB2, AFG1, and AFG2) spiked in non-contaminated food samples with the known concentration. The results are illustrated in Table 4.17 and were within the recovery criteria for method development (80-120%) in the range of 90.13-107.39%, (SANTE-11813, 2017). The precision was in the range of 1.01-8.12% for intra-day precision and 1.10-9.39% for inter-day precision. In general, the relative standard deviation (RSD) of inter- and the intra-day precision were less than 10% which is within the precision criteria of method development (SANTE-11813, 2017). Figures 4.54-4.56 show the MRM chromatogram for aflatoxins extracted under optimum parameters from different food matrices.

Table 4.16: The analytical performance of the developed method

Matrices		High-water content		High-sugar content		High-fat content	
Sample		Apple	Grape	Raisin	Dried Fig	Non-roasted peanuts	Roasted pistachio
Linearity Range		0.1-30		0.1-30		0.1-30	
AFB1	R^2	0.9992	0.9993	0.9994	0.9994	0.9992	0.9991
	LOD	0.04	0.04	0.06	0.06	0.07	0.08
	LOQ	0.08	0.08	0.09	0.09	0.10	0.10
	EF	745.12	744	727.88	729.6	746.33	734.92
AFB2	R^2	0.9991	0.9991	0.9991	0.9990	0.9988	0.9990
	LOD	0.05	0.06	0.05	0.05	0.07	0.07
	LOQ	0.08	0.09	0.09	0.10	0.20	0.20
	EF	737.48	730.2	752.4	735.6	738	708.15
AFG1	R^2	0.9990	0.9993	0.9988	0.9989	0,9987	0.9989
	LOD	0.06	0.06	0.07	0.08	0.08	0.08
	LOQ	0.13	0.10	0.10	0.10	0.15	0.20
	EF	744.6	765.52	736.8	734.4	734.4	731.32
AFG2	R^2	0.9990	0.9991	0.9989	0.9991	0.9986	0.9989
	LOD	0.06	0.05	0.05	0.05	0.08	0.09
	LOQ	0.09	0.09	0.09	0.09	0.10	0.20
	EF	723.98	726.38	737.25	759.6	715.73	712.58

EF: Enrichment factor, LOD: Limit of detection, LOQ: limit of quantification, R^2: regression coefficient

Comparison of response from matrix-matched standards and solvent standards indicated that there was no sample matrix effect (Table 4.18), where no clear ion suppression or enhancement was observed. This agreed with the findings which support that HF-LPME techniques minimize the impact of matrix effects. (Kataoka, 2010; Tan & Abdulra'uf, 2012 ;Alsharif et al., 2017). The validation for U-Shaped HF-LPME and UASLE-U-Shaped HF-LPME as extraction and clean-up method prior to LC-ESI-QQQ-MS analysis has shown good accuracy in the determination of aflatoxins in various liquid and solid food samples. The methods satisfied the performance requirements established by the European Union (EU) legislation(European Commission 2006).

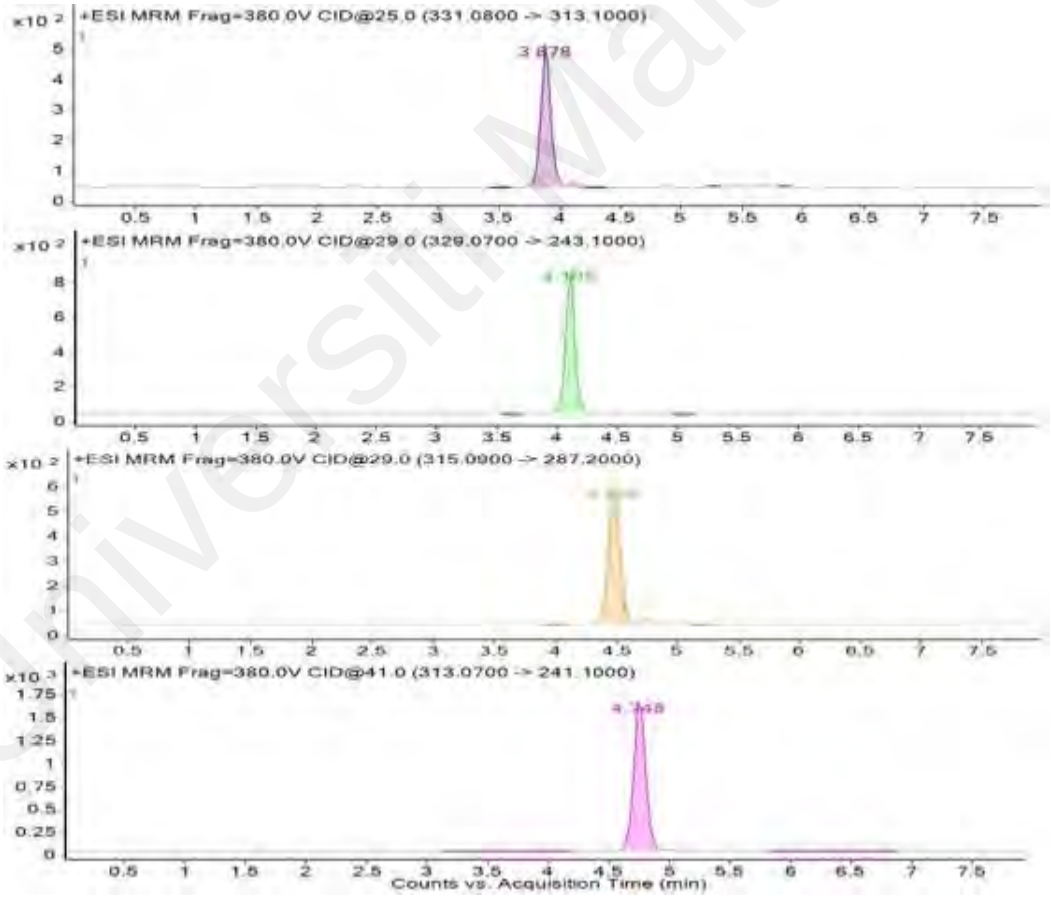

Figure 4.54: MRM chromatogram for aflatoxins under optimum parameters from spiked apple juice.

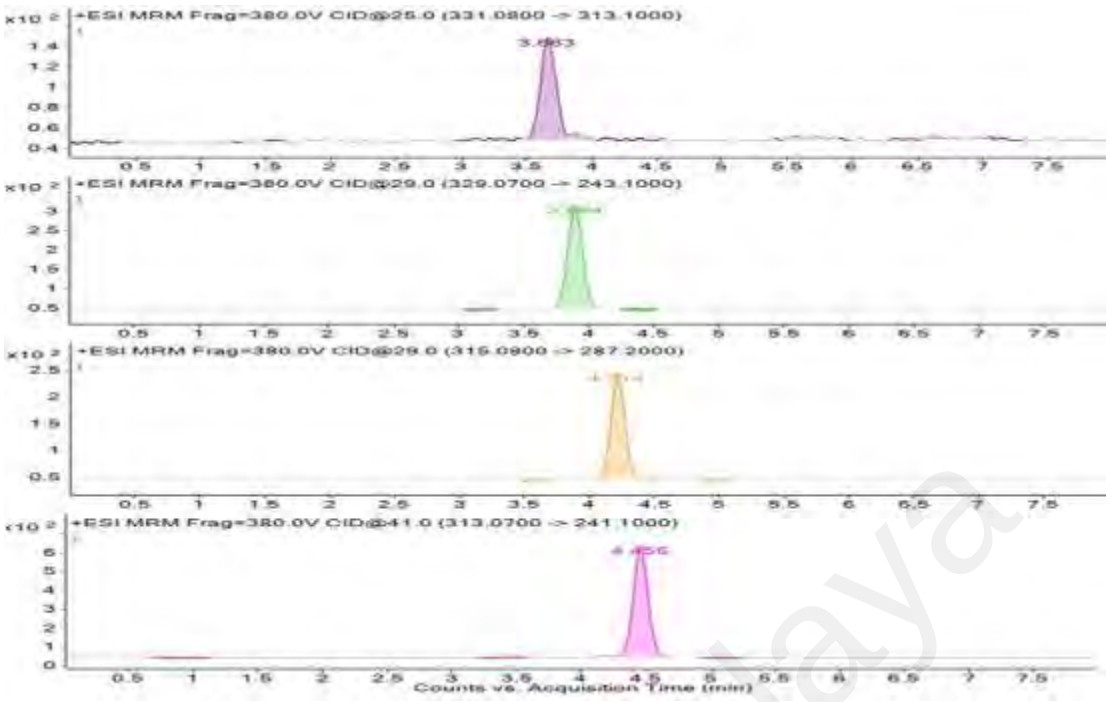

Figure 4.55: MRM chromatogram for aflatoxins under optimum parameters from spiked roasted peanut sample.

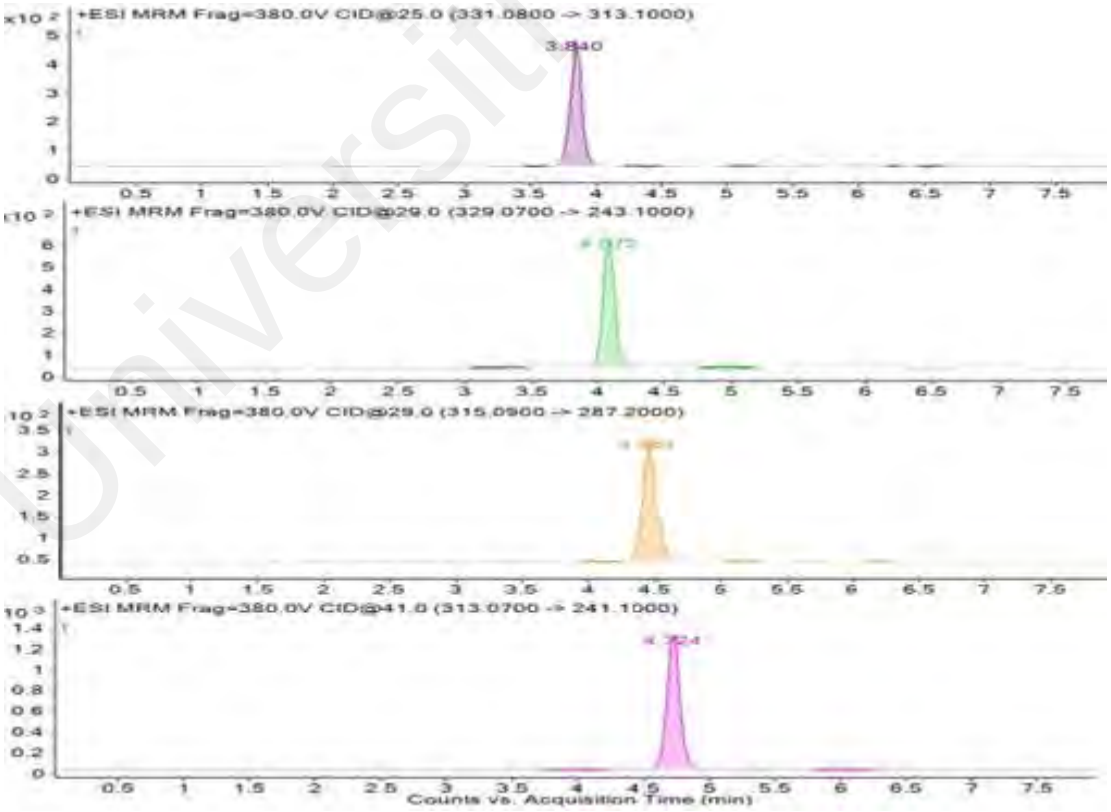

Figure 4.56: MRM chromatogram for aflatoxins under optimum parameters from spiked raisin sample.

Table 4.17: The accuracy of the developed method

Matrices		High water content						High sugar content						High fat content					
Sample		Apple juice			Grape juice			Raisin			Dried fig			Non-roasted peanuts			Roasted pistachio		
Myco	C	RR	Intra	Inter	RR	Intra	Inter	RR	Intra	Inter	RR	Intra	Inter	RR	Intra	Inter	RR	Intra	Inter
	1	98.73	2.38	2.42	97.11	2.79	2.92	95.23	7.44	7.11	97.48	4.67	4.99	93.92	5.89	7.02	96.90	4.90	5.62
AFB1	10	100.23	1.17	3.55	99.39	3.55	3.47	95.80	5.84	6.90	96.02	3.72	4.10	98.46	3.11	3.98	98.01	6.22	6.03
	30	99.11	1.57	1.92	101.10	1.10	2.22	100.12	2.44	5.83	98.30	2.51	2.38	100.16	2.63	3.87	99.98	3.76	4.11
	1	96.53	3.11	3.78	95.12	3.47	4.77	97.77	1.34	1.10	96.39	7.21	7.96	97.23	5.90	6.55	93.12	5.82	4.55
AFB2	10	98.19	1.01	2.48	97.92	1.39	3.91	103.23	3.32	3.88	99.73	4.90	5.29	98.21	4.85	7.90	95.28	2.30	3.71
	30	100.27	1.39	4.67	99.05	4.71	4.83	99.98	3.55	4.43	98.12	4.55	5.18	98.98	2.01	4.01	94.88	1.82	4.10
	1	97.33	3.44	4.33	98.12	6.89	6.38	97.12	3.74	3.39	98.80	2.11	2.38	97.34	5.77	5.23	97.10	2.44	2.39
AFG1	10	100.87	2.10	3.91	107.39	3.78	4.20	99.11	2.45	4.09	99.78	2.73	4.23	99.09	3.16	6.68	99.98	3.97	4.07
	30	99.66	3.45	2.47	100.72	4.16	5.33	98.51	4.73	4.88	96.08	4.89	5.23	97.34	3.74	5.14	95.47	5.85	6.19
	1	91.33	4.71	6.74	94.63	7.37	8.65	96.90	4.97	5.33	98.45	4.67	6.47	93.89	7.34	7.49	90.13	8.12	9.39
AFG2	10	98.08	2.69	2.76	98.30	5.21	6.97	98.64	3.01	3.85	105.20	2.13	3.98	95.22	5.11	6.36	95.07	7.40	7.95
	30	100.19	5.81	7.13	97.64	5.02	7.11	99.38	2.95	4.20	100.19	3.91	4.01	97.18	3.46	5.99	99.85	4.99	5.03

C: Aflatoxins spiked concentration(µg /L or µg/Kg) ,RR: Relative recovery.

Table 4.18: Values of matrix effect determined for aflatoxins in food samples

Sample	Apple juice		Grape juice		Raisin		Dried Fig		Non-roasted peanuts		Roasted pistachio	
(10 µg/L or µg/Kg)*	ME	RSD	ME	RSD	ME	RSD%	ME	RSD%	ME	RSD%	ME	RSD%
AFB1	0.12	4.74	0.16	1.54	-0.028	1.34	-0.024	1.03	-1.40	4.15	1.83	3.51
AFB2	0.03	1.05	-0.13	4.75	-0.01	2.81	-0.14	4.05	0.61	3.35	0.01	3.16
AFG1	0.34	2.90	0.10	3.89	-0.02	2.01	0.05	2.06	2.81	1.30	1.74	1.28
AFG2	-1.23	4.77	0.04	1.33	0.47	3.66	-2.43	5.65	3.83	1.83	3.45	2.02

ME: Matrix effect, RSD: Relative standard deviation. * µg/L for liquid samples, µg/Kg for non-liquid samples

4.2.5.2 Ochratoxin A Analysis

A linear dynamic range of 0.08-20 µg/L or µg/Kg, presented R^2 higher than 0.9906 for all samples (Tables 4.19 and 4.20). Under optimum conditions, the method LOD values ranged between 0.02-0.04 µg/L and 0.07-0.1 µg/Kg for liquid and solid food samples, respectively. LOQ values ranged from 0.08-0.1 µg/L and 0.4-0.8 µg/Kg for liquid and solid food samples, respectively (Tables 4.19 and 4.20).

The intra-day precision was in the range of 0.96-7.24%, and the inter-day precision range was from 1.22-11.87% in the liquid samples. While in the solid food samples, the intra-day precision was in the range of 1.55 to 12.41%, and the inter-day precision range was from 1.55 to 11.87%. In general, the relative standard deviation (RSD) of inter- and the intra-day precision were less than 20% within the accepted precision criteria of method development (SANTE-11813, 2017).

The enrichment factor is defined as the ratio of target analyte concentration in the acceptor phase to the initial concentration in the donor phase. The method revealed good enrichment factors of 612- 748.5 (Tables 4.19 & 4.20) for the extraction and purification of OTA from liquid and solid food samples.

The validation for QuEChERS U-Shaped HF-LPME as extraction and clean-up method prior to LC-ESI-QQQ-MS analysis has shown good precision and accuracy in the determination of OTA in various liquid and solid food samples. Moreover, the method performance satisfied the requirements established by the European Union (EU) legislation(European Commission 2006) . Figure 4.57 shows the multiple-reaction monitoring (MRM) chromatogram for OTA by the developed method from the spiked food sample (10 µg/kg).

Table 4.19: Range, R^2, precision, LOD, LOQ, and enrichment factor of OTA for liquid food samples

Sample	Range (µg/L)	R^2	LOD (µg/L)	LOQ (µg/L)	Intra			Inter			EF
					1(µg/L)	10(µg/L)	20(µg/L)	1(µg/L)	10(µg/L)	20(µg/L)	
Pure water	0.08-20	0.9993	0.02	0.08	1.09%	0.96%	1.08%	2.84%	1.22%	2.41%	748.50
Apple juice	0.1-20	0.9991	0.04	0.09	9.01%	4.30%	2.80%	8.52%	10.93%	3.01%	738.60
Grape juice	0.1-20	0.9990	0.04	0.09	12.78%	4.40%	7.15%	5.44%	9.52%	8.34%	732.97
Orange juice	0.2-20	0.9986	0.04	0.09	6.94%	1.30%	14.36%	4.45%	5.64%	4.15%	711.60
Pomegranate juice	0.2-20	0.9981	0.04	0.1	3.72%	6.45%	3.54%	11.87%	8.95%	10.78%	699.07

EF:Enrichment factor, Intra: intraday precision; Inter: interday precision, LOD: Limit of detection, LOQ:limit of quantification, R^2: regression coefficient

Table 4.20: Range, R, precision. LOD, LOQ, and enrichment factor of OTA for non-liquid food samples

Sample	Range (µg/kg)	R^2	LOD (µg/kg)	LOQ (µg/kg)	Intra-day			Inter-day			EF
					1(µg/kg)	10(µg/kg)	20(µg/kg)	1(µg/kg)	10(µg/kg)	20(µg/kg)	
Raisin	0.4-20	0.9991	0.07	0.4	8.55%	4.3%	9.73%	3.41%	4.85%	3.94%	714.07
Dried Fig	0.5-20	0.9990	0.07	0.5	2.33%	2.37%	2.43%	3.97%	3.64%	4.88%	709.05
Wheat flour	0.4-20	0.9989	0.08	0.4	2.11%	7.07%	2.66%	2.66%	12.40%	6.63%	708.52
Barley flour	0.4-20	0.9986	0.08	0.4	6.06%	4.08%	9.88%	1.80%	4.66%	5.08%	699.00
Non-roasted peanuts	0.8-20	0.9967	0.1	0.8	9.41%	3.37%	12.44%	3.96%	3.33%	8.77%	708.07
Roasted pistachio	0.8-20	0.9968	0.1	0.8	6.74%	8.62%	6.82%	4.28%	12.36%	8.03%	686.92
Chili	0.8-20	0.9906	0.1	0.8	4.62%	7.31%	5.92%	5.35%	8.74%	7.18%	565.35
Spice	0.8-20	0.9907	0.1	0.8	1.55%	9.59	2.77%	9.72%	12.14%	13.05%	612.00

EF: Enrichment factor, Intra: intraday precision; Inter: interday precision, LOD: Limit of detection, LOQ: limit of quantification, R^2: regression coefficient

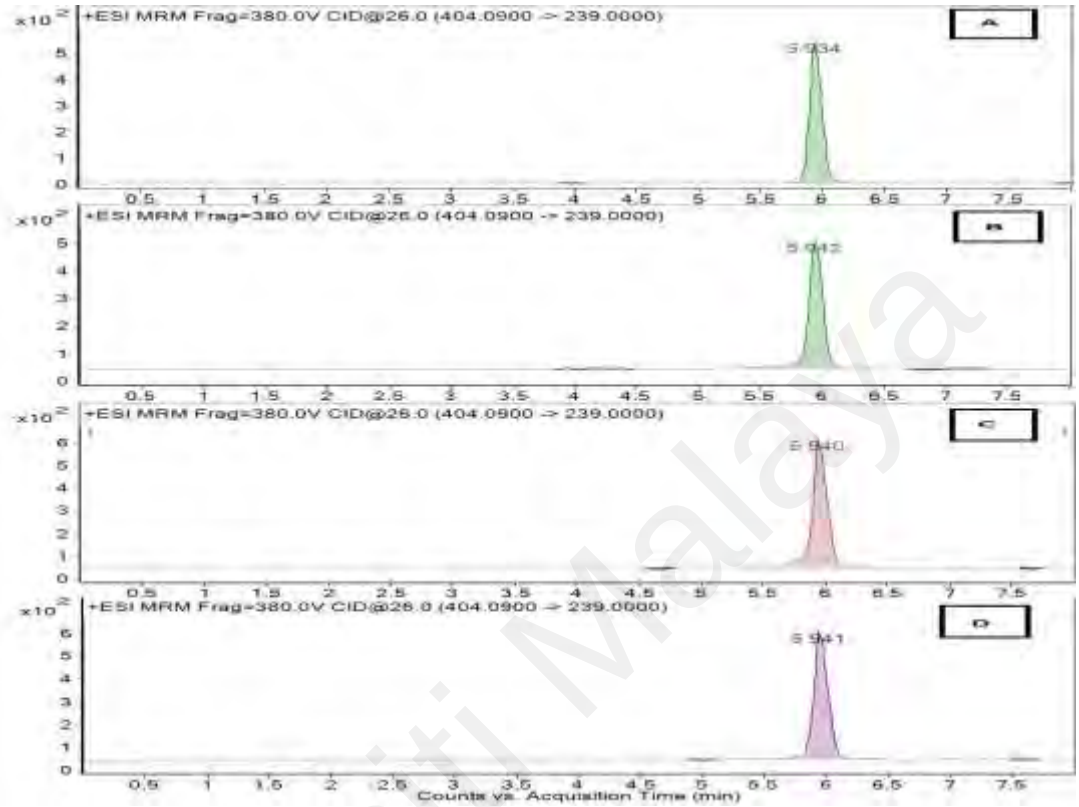

Figure 4.57: MRM chromatogram for OTA (10 µg/Kg); A-Pure standard; B-Spiked in pure water; C- Spiked in apple juice; D- Spiked in peanut, extracted by HF-LPME under optimum parameters.

The results are summarized in tables (Tables 4.21 and 4.22) in the satisfactory range of 75.38-99.85%, within the criteria recovery values of 70-120% (SANTE-11813, 2017). The results are shown in tables (Tables 4.23 and 4.24) revealed the comparison of response from solvent standards and matrix-matched standards after treated with the developed method. No ion enhancement was observed in this assessment, and slight ion suppression was observed for the chili and spice samples. These results are consistent with other reviews supporting the potential of the present method for preventing contaminants from entering the acceptor phase (Kataoka, 2010; Tan & Abdulra'uf, 2012 ; Alsharif et al., 2017).

Table 4.21: Recovery and relative standard deviation(RSD) of OTA spiked in blank fruit juice

Sample	Pure water		Apple Juice		Grape Juice		Orange Juice		Pomegranate juice	
Con(µg/L)	RR	RSD	RR	RSD	RE	RSD	RR	RSD	RR	RSD
1	99.85	1.9	83.18	9.01	89.35	12.78	91.60	6.94	85.58	3.72
10	99.80	0.96	98.48	4.30	97.73	4.40	94.88	1.30	93.21	6.45
20	99.60	1.08	98.44	2.80	96.89	2.80	97.53	14.36	95.54	3.54

RR: Relative recovery, RSD: Relative standard deviation.

Table 4.22: Recovery and relative standard deviation(RSD) of OTA spiked in blank solid food samples

Sample	Raisin		Dried fig		Wheat flour		Barley Flour		Non-roasted peanuts		Roasted pistachio		Chili		Spice	
(µg/Kg)	RR	RSD	RR	RSD	RR	RSD	RR	RSD	RR	RSD	RR	RSD	RR	RSD	RR	RSD
1	93.28	9.28	88.45	6.83	89.89	3.62	90.34	6.55	89.62	13.53	91.76	8.08	75.99	8.34	85.33	2.33
10	95.21	2.70	94.54	3.75	94.47	3.80	93.20	4.08	94.41	6.40	91.59	7.41	75.38	4.33	81.60	9.59
20	97.54	4.69	95.07	5.54	94.16	4.10	95.32	3.00	95.45	6.54	93.22	10.22	85.15	7.75	88.65	14.62

RR: Relative recovery, RSD: Relative standard deviation.

Table 4.23: Values of matrix effect determined for OTA in juice samples

Sample	Pure water		Apple juice		Grape juice		Orange juice		Pomegranate juice	
(µg/L)	ME	RSD	ME	RSD	ME	RSD	ME	RSD	ME	RSD
1	0.02	0.89	-0.12	4.74	-0.04	1.54	-0.06	7.57	-2.84	14.15
10	0.17	2.13	-0.03	1.05	-0.07	4.75	-0.03	5.25	-0.12	10.35
20	0.02	6.28	-0.05	2.90	-0.10	3.89	0.02	4.12	-1.77	9.30

ME: Matrix effect, RSD: Relative standard deviation.

Table 4.24: Values of matrix effect determined for OTA in non-liquid food samples

Sample	Raisin		Dried fig		Wheat flour		Barley Flour		Non-roasted peanuts		Roasted pistachio		Chili		Spice	
µg/L	ME	RSD	ME	RSD	ME	RSD	ME	RSD	ME	RSD	ME	RSD	ME	RSD	ME	RSD
1	-0.028	0.031	-0.024	0.03	0.02	0.39	0.01	0.5	-3.40	4.15	-2.83	3.51	-4.72	3.51	-2.78	0.87
10	-0.01	0.046	-0.14	0.05	-0.10	0.17	-0.02	0.25	-4.61	3.35	-5.01	3.16	-4.62	3.75	-2.13	0.84
20	-0.02	0.050	0.05	0.06	-0.20	0.44	0.05	0.12	-2.81	1.30	-3.74	1.28	-3.05	1.28	-1.89	1.30

ME: Matrix effect, RSD: Relative standard deviation.

4.2.6 Method Efficiency

A comparison was made between the results of this study with the method used for the analysis of aflatoxins in soya juice (Simão et al., 2016) (Table 4.25). The present study had a shorter extraction time (50 min) when compared to the reported method (60 min). This improvement can be attributed to the increase of surface area in the membrane. Also, the present method precision was better than those reported with RSD <10%. The improvement in method efficiency could be explained in better extraction kinetics displayed by the U-shaped design as compared to the rod-like design (Alsharif et al., 2017). In the case of ochratoxin A analysis, the present method also gave a better extraction time, as compared to the published method (Table 4.25). The use of U-Shape design with added extraction/dispersion solvent improved the efficiency of the developed method. The overall results of the present method demonstrated an improvement to the previously reported methods (González-Peñas et al., 2004; Romero-González et al., 2010).

4.2.1 Analysis of Mycotoxins in Food Samples

4.2.1.1 Occurrence of Aflatoxins in Food Samples

The juice samples were free of aflatoxins (AFB1, AFB2, AFG1, and AFG2). For solid samples, five samples, two peanuts, and three pistachio samples were tested positive for aflatoxins. The mean concentration in the peanuts sample was in the range from 1.74-7.78 µg/Kg within the permitted European Legal Maximum Limit for AFTs in non-roasted peanuts (8.00 and 15.00 for AFB1 and sum total aflatoxins, respectively) (European Commission, 2006). For pistachio samples, the aflatoxins content was in the range from 1.37-8.84 µg/Kg as illustrated in Table 4.26. One pistachio sample contained 8.84 µg/Kg of AFB1, exceeded the European limits (8 µg/Kg). Three pistachio samples were tested positive for aflatoxins and also exceeded the European legal maximum limit for AFTs in pistachios

products (15 µg/Kg). The total aflatoxins concentrations for three pistachio samples were (a)15.84 µg/Kg, (b)16.80 µg/Kg, and (c) 23.02 µg/Kg (European Commission, 2006).

4.2.1.1 Occurrence of Ochratoxin A in Food Samples

The results(4.27) showed no detectable amount of OTA in most samples, except in four samples. One wheat flour, two peanut, and one pistachio sample showed the presence of a trace amount of OTA. The mean concentration of OTA in wheat flour sample (high starch and protein) is 0.8 µg/Kg, below the European Legal Maximum Limit for OTA (European Commission, 2006) and FAO (AC04318739, 2004). In the high fat and low water content samples, OTA was detected in two peanuts (0.9 and 3.2 µg/Kg), and pistachio (1.4 µg/Kg) samples. The amounts of OTA in the nut samples were lower than the maximum level allowed in the European Legal Maximum Limit (European Commission, 2006).

Table 4.25: Comparison of the present with previously reported methods

Method	Mycotoxin	pH	Salt percentage	Extraction time (min)	Limit of quantification	Relative standard deviation (%)	Relative recovery (%)	Reference
U-Shaped-HF-DLLME	Aflatoxins	8	2	50	0.08-0.20 µg/L	<10	90.13-107.39	This study
U-Shaped-HF-DLLME	Ochratoxin A	2.6	7	60	0.09-0.80 µg/L	<13	83.18-98.48	This study
Rode-like-HF-DLLME	Aflatoxins	n.a.	2	60	0.03-0.10 µg/L	12-18	83-117	Simão et al., 2016
Rode-like-HF-LPME	Ochratoxin A	2	10	240	0.02–0.09 µg/L	12-21	>70	Romero-González et al., 2010
Rode-like-HF-LPME	Ochratoxin A	1.05	n.a.	120	0.25 µg/L	<10	77	González-Peñas et al., 2004

n.a. – not available

Table 4.26: Detection of aflatoxins in various real food samples

Sample	T.S	Positive (%)	Concentration* (RSD)			
			AFB1	FB2	AFG1	AFG2
Apple juice	10	0	<LOQ	<LOQ	<LOQ	<LOQ
Grape juice	10	0	<LOQ	<LOQ	<LOQ	<LOQ
Raisin	10	0	<LOQ	<LOQ	<LOQ	<LOQ
Dried Fig	10	0	<LOQ	<LOQ	<LOQ	<LOQ
Non-roasted peanuts	10	20	3.15(3.4), 7.78(2.11)	1.25(3.45), 2.82(7.31)	2.93(3.78), 4.35(2.35)	1.47(1.38), <LOQ
Roasted pistachio	10	30	4.82(4.17), 6.75(2.48), 8.48(8.55)	1.77(6.42), 1.37(9.02) ,5.19(3.50)	3.62(7.55), 2.74(4.90), 3.41(2.11)	2.63(8.23), 1.83(7.19), 5.94(4.42)

AFB1:Aflatoxin B1,AFB2: Aflatoxin B2 ,AFG1: Aflatoxin G1,AFG2: Aflatoxin G2,OTA:Ochratoxin A, Positive(%) : Percent of positive samples, n.d: Non-detected , T.S: Total samples.*µg/Kg, <LOQ: Lower than quantification limit

Table 4.27: Analytical results of real food samples for OTA

Sample type	samples analyzed	Positive samples	Concentration* (RSD)
Apple juice	5	0	<LOQ
Grape juice	5	0	<LOQ
Orange juice	5	0	<LOQ
Pomegranate juice	5	0	<LOQ
Raisin	5	0	<LOQ
Dried Fig	5	0	<LOQ
Wheat flour	5	1	0.8(14)
Barley flour	5	0	<LOQ
Non-roasted peanuts	5	2	0.9(17.3) - 3.2(8.4)
Roasted pistachio	5	1	1.4(17.25)
Chili	5	0	<LOQ
Spice	5	0	<LOQ

RSD: Relative standard deviation;. <LOQ: Lower than quantification limit ;* (µg/L or µg/Kg)),

CHAPTER 5: CONCLUSION

A U-Shaped hollow fiber liquid phase microextraction with added extraction/dispersion solvents combined with LC-MS/MS method for specific and accurate analysis of mycotoxins in food samples has been developed and validated. The use of the chemometric approach to the screening and subsequent optimization of instrument parameters has resulted in minimizing analysis time and maximizing the compounds peak area. Applying chemometrics in the LC-ESI-QQQ-MS optimization has revealed better performance by showing higher precision and improved detectability of the target mycotoxins.

In this study, the Plackett-Burman design (PBD) was used for the screening of critical factors affecting the response (TCPA & MRT) of mycotoxins in LC-ESI-QQQ-MS. It provided valuable information on each variable with a small number of experimental runs. The BBD has been utilized for optimization of the significant factors retained from PBD. The results showed that multivariate procedure leads to good analytical performance, i.e. repeatability (0.63-4.76%), IDL (1.41 -3.61 ng), and R^2 (0.9992-0.9999) based on the standard and spiked samples. The validated LC-MS/MS method combined with QuEChERS method was applied for the separation and determination of five mycotoxins in different food samples. Mycotoxins were detected in some food samples in the range of 0.41-16.93 µg/Kg. The present study has successfully demonstrated the multivariate method using PBD coupled with BBD for the optimization of LC-ESI-QQQ-MS/MS to simultaneous determination and separation of mycotoxins.

A U-Shaped HF-LPME, combined with LC-ESI-QQQ-MS/MS for determination of multi-aflatoxins and ochratoxin A in liquid and solid food samples was developed and validated using univariate and multivariate optimization methods. Combined optimization

methods (univariate and multivariate central composite design) were applied for U-Shaped HF-DLLME optimization for OTA in non-solid food samples, while only univariate was used for multi-aflatoxins in non-solid food samples. For solid food samples, the simple lattice design was used for optimization of Ultrasound-assisted solid-liquid extraction(UASLE) and QuEChERS.

The results of hollow fiber microextraction (combination of chemometric with hollow fiber microextraction technique and simplex lattice mixture designs) prior to LC-MS/MS optimization study have shown a successful method for the analysis of mycotoxins from various sample matrices (fruit juice as high water content and high sugar, wheat and barley flour as high starch and protein samples; dried raisin and dried fig as high sugar and low water content samples; peanut and pistachio as a high oil and low water content samples; chili and spices as samples containing complex component such as phenolic compounds and pigments). The precision and accuracy results were consistent with international standards. The results of the developed method were in accordance with the international guidelines. The linear dynamic range was in good fit with a correlation coefficient higher than 0.9906, LOD of 0.04-0.09 µg/L or µg/Kg and LOQ 0.80-0.20 µg/L or µg/Kg. Both intra- and inter-day precision were lower than 20% RSD, with intra-day and inter-day precision of 0.96-0.12% and 1.10-11.87%, respectively. The recoveries (75.38-107.39%) for the target analytes in the samples were in agreement with the European Commission Regulation (70-120%). The developed methods have demonstrated as excellent detection methods and clean-up procedure for Ultrasound-assisted solid-liquid extraction (UASLE) and QuEChERS for aflatoxins and OTA detection. The methods were successfully applied in the determination of AFTs and OTA in a variety of foods.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

a) List of Publications

1- **Alsharif, A. M. A**., Choo, Y.-M., & Tan, G.-H. (2019). Detection of five mycotoxins in different food matrices in the malaysian market by using validated liquid chromatography electrospray ionization triple quadrupole mass spectrometry. *Toxins*, 11(4), 196.

2- **Alsharif, A. M. A**., Choo, Y.-M., Tan, G. H., & Abdulra'uf, L. B. (2019). Determination of mycotoxins using hollow fiber dispersive liquid–liquid–microextraction (HF-DLLME) prior to high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). *Analytical Letters*, 1-15.

3- **Alsharif, A. M. A**., Tan, G.-H., Choo, Y.-M., & Lawal, A. (2017). Efficiency of hollow fiber liquid-phase microextraction chromatography methods in the separation of organic compounds: A Review. *Journal of chromatographic science, 55*(3), 378-391.

b) List of Paper Presented

1- **Alsharif, A. M. A**., Tan, G.-H., Choo, Y.-M., & Lawal, A. (2018). LC-MS/MS optimization using response surface methodology (RSM) for the separation and determination of aflatoxins and ochratoxin A. An oral paper presented at the First International Conference on Science and Technology (1st ICST2018) held in Sebha University. Sebha Libya. February 12–13, 2018