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DETERMINATION OF MYCOTOXINS IN BROWN RICE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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อดิศร เจตนะจิตร : การตรวจวัดสารพิษจากเชื้อราในข้าวกล้องโดยไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี-แทนเดมแมสสเปกโทรเมตรี (DETERMINATION OF MYCOTOXINS IN BROWN RICE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ธรรมนูญ หนูจักร, 105 หน้า.

ได้หาสภาวะที่เหมาะสมและตรวจสอบความใช้ได้ของวิธีการเตรียมตัวอย่างด้วยเทคนิค QuEChERS สำหรับการวิเคราะห์ปริมาณสารพิษจากเชื้อรา 9 ชนิด (อะฟลาทอกซินบี 1, อะฟลาทอกซินบี 2, อะฟลาทอกซินจี 1, อะฟลาทอกซินจี 2, ฟูโมนิซินบี 1, ฟูโมนิซินบี 2, ดีออกซีไนวาลีนอล, โอคราทอกซินเอ และ ซีราลีโนน) ด้วยอัลตราไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทรกราฟี-แทนเดมแมสสเปกโทรเมตรี สภาวะที่เหมาะสมสำหรับการเตรียมตัวอย่างด้วยเทคนิค QuEChERS ได้แก่ โดยการใช้การสกัดตัวอย่างข้าวกล้อง (1.0 กรัม) ด้วยอะซิโตไนไทรล์ที่มีกรดแอซิติก 10% (5.0 มิลลิลิตร) และที่มีเกลือ 4 ชนิด ได้แก่ แมกนีเซียมซัลเฟต (2.0 กรัม) โซเดียมคลอไรด์ (0.50 กรัม) โซเดียมซิเตรตไตรเบสิกไดไฮเดรต (0.50 กรัม) และโซเดียมซิเตรตไดเบสิกเซสควิไฮเดรต (0.25 กรัม) จากนั้นกำจัดสารรบกวนด้วยเทคนิค dispersive-solid phase extraction โดยใช้ตัวดูดซับผสม 3 ชนิด ได้แก่ octadecylsilane (50 มิลลิกรัม) primary and secondary amine (25 มิลลิกรัม) และ silica (25 มิลลิกรัม) พบว่าวิธีที่พัฒนาขึ้นสามารถใช้วิเคราะห์ปริมาณสารพิษจากเชื้อราที่มีปริมาณน้อยได้ ในระดับที่ต่ำกว่าค่าปริมาณสูงสุดที่สามารถพบได้ตามข้อกำหนดของสหภาพยุโรป ด้วยค่าขีดจำกัดของการตรวจวัดอยู่ในช่วง 1.4 ถึง 25 ไมโครกรัมต่อกิโลกรัม เมื่อนำข้าวกล้องที่ไม่พบสารพิษจากเชื้อรามาเติมสารพิษจากเชื้อราที่ความเข้มข้นที่ทราบค่า แล้วพบว่า ความแม่นและความเที่ยงของปริมาณวิเคราะห์เป็นที่ยอมรับได้ ด้วยร้อยละการกลับคืนของการสกัดอยู่ในช่วง 81 ถึง 101 และค่าส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในช่วง 5 ถึง 19 % สำหรับความเข้มข้นในช่วง 5.0 ถึง 1,000 ไมโครกรัมต่อกิโลกรัม จากการวิเคราะห์ตัวอย่างข้าวกล้องจำนวน 14 ตัวอย่าง พบว่า 6 ตัวอย่างมีการปนเปื้อนของสารพิษจากเชื้อราอย่างน้อย 1 ชนิด ได้แก่ ฟูโมนิซินบี 1 ในช่วง 2.49 ถึง 5.41 ไมโครกรัมต่อกิโลกรัม ฟูโมนิซินบี 2 ในช่วง 4.33±0.04 ไมโครกรัมต่อกิโลกรัม และซีราลีโนนในช่วง 6.10-14.88 ไมโครกรัมต่อกิโลกรัม

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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QuEChERS sample preparation was optimized and validated by ultra high performance liquid chromatography-tandem mass spectrometry method for determination of nine mycotoxins in brown rice: aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, fumonisin B1, fumonisin B2, deoxynivalenol, ochratoxin A and zearalenone. The following suitable QuEChERS conditions were obtained using the solvent extraction of 1.0 g of the brown rice with 5.0 mL of acetonitrile containing 10% acetic acid in presence of four salts (2.0 g of anh. $MgSO_4$, 0.50 g of NaCl, 0.50 g of sodium citrate tribasic dehydrate and 0.25 g sodium citrate dibasic sesquihydrate), and followed by the dispersive-solid phase extraction cleaning up step using mixed sorbents of octadecylsilane (50 mg), primary and secondary amine (25 mg) and silica (25 mg). This developed method allows to determine mycotoxins in trace levels below maximum limits of the EU regulation, with our method detection limits of 1.4-25 μg/kg. Using blank brown rice spiked with mycotoxins at known concentrations, acceptable accuracy and precision for quantitative analysis were obtained with the recoveries in a range of 81-101% and relative standard deviation of 5-19% for 5.0-1,000 μg/kg. Six out of fourteen real samples of brown rice were found to be contaminated with at least one of these mycotoxins, 2.49-5.41 μg/kg of fumonisin B1, 4.33±0.04 μg/kg of fumonisin B2 and 6.10-14.88 μg/kg of zearalenone.

Department: Chemistry Student's Signature .....

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LIST OF ABBREVIATIONS

A	slope of calibration curve
Abs	maximum absorption
ACN	acetonitrile
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
anh. $MgSO_4$	anhydrous magnesium sulfate
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
C	concentration in fraction unit
ºC	degree celsius
C18	octadecylsilane
CAV	cell accelerator voltage
CE	collision energy
CF	correction factor
CID	collision-induced dissociation
cm	centimeter
DC	direct current voltage
DON	deoxynivalenol

d-SPE	dispersive solid phase extraction
ESI	electrospray ionization
FA	formic acid
FB1	fumonisin B1
FB2	fumonisin B2
F_{exp}	experimental *F* value
FLD	fluorescence detector
g	gram
GC	gas chromatography
GCB	graphitized carbon black
h_0	leverage for the blank sample
HOAc	acetic acid
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography-tandem mass spectrometer
H_2SO_4	sulfuric acid
I	number of calibration samples
IAC	immunoffinity column
I.D.	internal diameter
$K_2Cr_2O_7$	potassium dichromate
kg	kilogram
L	liter

L	total number of levels of calibration
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantitation
M	molar
MDL	method detection limit
MeOH	methanol
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MQL	method quantitation limit
MW	molecular weight
MS	mass spectrometer
ms	millisecond
m/z	mass to charge ratio
NaCl	sodium chloride
NaOAc	sodium acetate
nm	nanometer
OTA	ochratoxin A
PSA	primary and secondary amine
psi	pound per square inch

PTFE	polytetrafluoroethylene
Q	total number of replicates at each level for calibration
Quad	quadrupole
QuEChERS	quick easy cheap effective rugged and safe
r^2	correlation coefficient
RF	radio frequency voltage
rpm	round per minute
RSD	relative standard deviation
SD	standard deviation
t_{exp}	experimental t value
SMX	sulfamethoxazole
S_p^2	pooled variance
SPE	solid phase extraction
$S_{y/x}$	residual standard deviation
S_y	pure error (a measure of the instrumental noise)
TOF	time-of-flight
Trap	ion trap
UHPLC	altra high performance liquid chromatography
UHPLC-MS/MS	altra high performance liquid chromatography-tandem mass spectrometer
UV	UV-visible detector
v/v	volume by volume

y_i	experimental response values for sample
$\hat{y}_i$	estimated response values for sample
y_{lq}	calibration response
$\bar{y}_l$	mean response
ZON	zearalenone
µg	microgram
µL	microliter
α	probability of rejecting the null hypothesis when it is true
ε	molar absorptivity
λ_{max}	maximum absorption wavelength
$\%\Delta$	difference in the slope value

CHAPTER I
INTRODUCTION

1.1 Problem definition

Rice (Oryza sativa L.) is categorized as cereals and gives high carbohydrate nutrient and other sources of energy for population more than a half of the world especially in Asian countries [1, 2]. It is also an important industrial plant of Thailand. There were reports that Thailand exported rice in 2014 as 10.97 million tons and at the beginning of 2015 as 11.00 million tons which was the highest in the world [3]. Therefore, rice is the agricultural product which earns much money for Thailand. In general, rice is well known as milled rice which is obtained by removing germ and bran from brown rice in order to improve the sensory and storage stability [2]. Thai people consume rice as the main course for a long period of time. Recently, they are more interesting in healthy food such as brown rice which has many benefits for their health. The brown rice is produced only by removing rice husk, but rice germ and rice bran are still remained and provide benefit for nutrients and sources of bioactive compounds such as strongly antioxidant γ-oryzanols or tocopherols in bran and germ, respectively [4]. However, the brown rice may be contaminated with some species of microorganism, such as fungi, during unsuitable process of harvesting and storage. The growth fungi in brown rice may cause toxins, called “mycotoxins”, which danger to consumers. For example, 8.3 μg/kg aflatoxin B1 (AFB1), one of the mycotoxins, in brown rice has been recently reported in previous work [5].

Typically, mycotoxins are the products from secondary metabolism process of the fungi and can contaminate in many kinds of food. The contamination may be occurred both pre- and post-harvest of the plants such as cereals, nuts, dried fruits, spices, etc. Moreover, mycotoxins contamination can be found in livestock products such as meat and milk when livestock consumed the mycotoxins contaminated feed [6]. The major mycotoxins include AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), zearalenone (ZON), deoxynivalenol (DON),

fumonisin B1 (FB1) and fumonisin B2 (FB2). More details are given in Section 2.1. If people consume the mycotoxins-contaminated food, mycotoxins will cumulate in their body, and give hepatotoxic, nephrotoxic and/or neurotoxic effects.

According to Commission Regulation (EC) No. 1881/2006, which sets maximum levels for certain contaminants in foodstuffs in the European Union (EU), the maximum limits of mycotoxins in cereals and/or rice are regulated as follows: 5.0 μg/kg for AFB1; 10.0 μg/kg for the sum of AFB1, AFB2, AFG1 and AFG2; 5.0 μg/kg for OTA; 100 μg/kg for ZON; 1,250 μg/kg for DON; and 2,000 μg/kg for the sum of FB1 and FB2 as detailed in Section 2.2. Therefore, an effective analytical method to determine trace levels of mycotoxins in brown rice is necessary for consumer food safety.

1.2 Literature review

The separation-based analytical techniques for determination of mycotoxins include gas chromatography (GC) and high performance liquid chromatography (HPLC).

In a few work on GC determination, the derivatization of mycotoxins is required in order to convert the non-volatile mycotoxins to volatile derivatives. For example, trimethylsilylimidazole and trimethylchlorosilane were used as derivatizing agents in order to determine DON in beers [7], while *N,O*-bis(trimethylsilyl) acetamide, trimethylchlorosilane and *N*-trimethylsilyimidazole were used as derivatizing agents in order to determine DON, ZON and others in popcorn [8], and ZON, DON and others in wheat [9].

High performance liquid chromatography (HPLC) has been widely used for the analytical separation and determination of mycotoxins in a variety of samples such as snacks [10], spices [11], bread [12], eggs [13], wheat flour [14], apple juice [15], dried fruit [16] and maize or corn [17-19]. HPLC detectors for mycotoxin determination include UV-visible detectors or photodiode array detectors [15, 20,

21]; fluorescence detectors [7, 17, 20, 22, 23]; and mass spectrometers, either MS or MS/MS [7, 24-32].

Ultra high performance liquid chromatography (UHPLC) [33] is a powerful alternative separation technique to conventional HPLC because UHPLC provides higher resolution and faster separation owing to the smaller particle size of its stationary phase, typically less than 2 μm. In addition, UHPLC systems consume a smaller amount of the mobile phase, leading to less organic waste. A hyphenated technique of UHPLC with MS or MS/MS is widely used for the quantitative determination and identification of compounds in samples with complex matrices. UHPLC-MS/MS has previously been used to determine mycotoxins in various samples: milled rice [34]; nuts and seeds [29]; eggs [31]; rice, corn, wheat and peanut [24]; and rice, wheat, oats, barley and corn [26]. However, prior to LC-MS analysis, sample preparation is required to remove matrices that may interfere with the detection of mycotoxins, reduce separation efficiency, shorten the column life and contaminate the MS interface.

Previous studies of sample preparation techniques for the determination of mycotoxins have involved simple solvent extraction [19, 25, 26], ultrasonic extraction [18] or solvent extraction and solid phase extraction (SPE) [22, 24, 27]. The disadvantages of these procedures include the use of a large amount of organic solvent, matrix interferences for a single step of solvent extraction, long time requirements due to the multiple steps of SPE, and expensive SPE cartridges. Recently, in comparison between QuEChERS and other techniques such as matrix solid phase dispersion, solid-liquid extraction, and SPE, for sample preparation of barleys to determine some mycotoxins, QuEChERS was found to extract these mycotoxins from the sample with better recovery [35]., where QuEChERS stands for quick, easy, cheap, effective, rugged and safe.

The QuEChERS sample preparation method, a simple two-step technique based on solvent extraction in the presence of salts and dispersive-solid phase extraction (d-SPE) for clean-up, has been reported for the determination of mycotoxins in various samples using the following procedures: acetonitrile (ACN)

extraction and d-SPE using octadecylsilane (C18) sorbent [8, 9]; ACN extraction without d-SPE [10, 12, 35]; ACN (5 % formic acid, FA) extraction without d-SPE [29]; ACN (1 % FA) extraction without d-SPE [11]; ACN (1 % acetic acid, HOAc) extraction without d-SPE [36]; methanol (MeOH) with 1 % HOAc extraction without d-SPE [13, 31]; ACN:MeOH extraction and d-SPE using primary and secondary amine (PSA) sorbent [14]; and ACN (10 % FA) extraction and d-SPE using mixed C18, alumina-neutral and PSA sorbents [34]. Salts, that induce phase separation of the extraction solvent and water, include anhydrous magnesium sulfate (anh. $MgSO_4$) and sodium chloride (NaCl) [8-11, 14, 29, 30, 35, 36], sodium sulfate and sodium acetate (NaOAc) [13, 31]. The ratio of $MgSO_4$ and NaCl salts are typically used at 4:1 [8-11, 29, 30, 35, 36] and 2:1 [14]. Additionally, a sample size of 5 to 15 g is typically used for 5- to 10-mL ACN solvent extraction from QuEChERS standard method and other previous works [34, 37]. However, in complex matrices, a reduce sample size is required for reducing matrix interferences and getting satisfactory recovery as shown in following previous reports: spices such as red chilli and white and black pepper (1 g with 5-mL ACN extraction) [11], snacks (2 g with 10-mL ACN extraction) [10], nuts and seeds (2 g with 5-mL ACN with 5% FA extraction) [29].

Compared with conventional solvent extraction and SPE, each step of QuEChERS sample preparation is easily performed. A small amount (5-10 mL) of organic solvent and less than 1 g of cheap d-SPE sorbent are applied in a plastic centrifuge tube, then vortexed and centrifuged for 2-6 min; this method could be partially automated for high throughput analysis demands. Therefore, the QuEChERS sample preparation method provides the advantages suggested by its name.

The detection limits of the method using sample preparation technique as above to determine of mycotoxins with UHPLC-MS/MS were as follows: 0.05, 0.25, 0.05, 0.5, 0.01, 0.1, 5, 10 and 10 ng/mL for AFB1, AFB2, AFG1, AFG2, OTA, ZON, DON, FB1 and FB2, respectively [25, 26], 0.05, 0.05, 0.05, 0.05, 1, 0.5, 0.5, 0.1 and 0.5 ng/mL for AFB1, AFB2, AFG1, AFG2, FB1, FB2, DON, ZON and OTA, respectively [24], 10, 1, 5 and 10 ng/mL for FB1, AFB1, OTA and ZON, respectively [27], 0.5 and 0.3 ng/mL for DON and ZON, respectively [28].

1.3 Aim, scope and expected benefits of this work

As previously mentioned, UHPLC-MS/MS determination of mycotoxins in milled rice has recently reported using the QuEChERS sample preparation [34]. A similar QuEChERS procedure was also used for this preliminary study on the UHPLC-MS/MS determination of mycotoxins in three types of brown rice, black brown rice, red brown rice and brown brown rice. However, the mycotoxin extraction recovery was found to be less than 50 %, in order black brown rice < red brown rice < brown brown rice. In addition, the lower the recovery, the darker the extract. These different results between brown and milled rice may be due to the presence of more complex matrices in brown rice than milled rice. Therefore, this work aims to optimize and validate QuEChERS sample preparation particularly for the determination of nine mycotoxins in brown rice prior to UHPLC-MS/MS.

Firstly, UHPLC-MS/MS was optimized for simultaneous separation and determination of nine mycotoxins: AFB1, AFB2, AFG1, AFG2, FB1, FB2, DON, OTA and ZON: gradient profile and type of UHPLC mobile phase, UHPLC column temperature, and MS/MS detection such as mass to charge ratios (m/z) of each analyte including molecular ions, product ions and their suitable collision energy. Secondly, the QuEChERS sample preparation of brown rice was optimized for UHPLC-MS/MS determination of nine mycotoxins: various acidity extraction solvents with and without buffering agent, types of single and mixed sorbents, the amount of salts and volume ratios of water and extraction solvent. Thirdly, the optimized QuEChERS sample preparation for UHPLC-MS/MS determination of nine mycotoxins was validated In order to obtain a reliable method: analytical limits, linearity and calibration, matrix effect, accuracy and precision. Finally, the validated UHPLC-MS/MS method with QuEChERS sample preparation was used to determine mycotoxins in real samples of black brown rice, red brown rice and brown brown rice.

In this work, it is expected to obtain the validated and realized UHPLC-MS/MS method with the QuEChERS sample preparation for simultaneous separation and determination of mycotoxins in brown rice, along with the QuEChERS advantages suggested by its name (quick, easy, cheap, effective, rugged and safe)

CHAPTER II
THEORY

2.1 Mycotoxins

Mycotoxins, discovered in early 1960s, are the product from secondary metabolism process of fungi. The Food and Agriculture Organization (FAO) of the United Nations indicates that mycotoxins can infect in almost 25% of global food. The contamination occurs both pre- and post-harvest of the plants [38]. The structures of some of the most abundant mycotoxins are shown in Figure 2.1 including aflatoxin (AFB1, AFB2, AFG1 and AFG2), OTA, ZON, DON and fumonisin (FB1 and FB2).

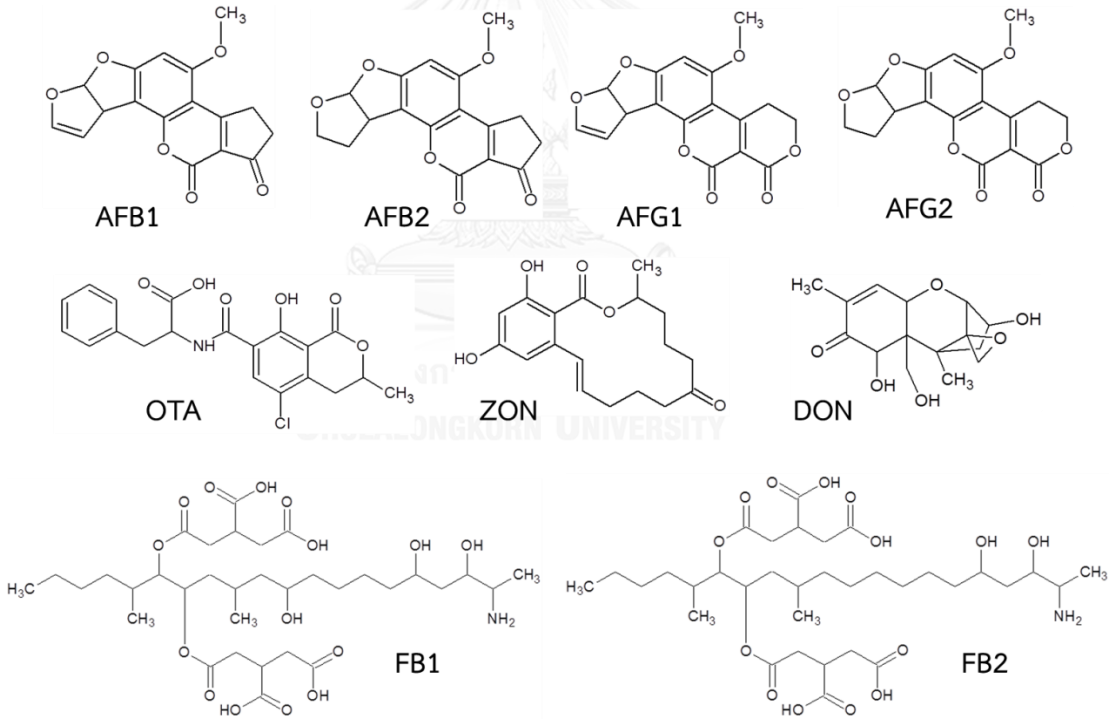


Figure 2.1 The structures of main mycotoxins. Adapted from [39, 40].

2.1.1 Aflatoxin

Aflatoxins are produced by *Aspergillus flavus* or *Aspergillus parasiticus*. Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) are found in many kinds of food [41]. They are categorized as carcinogenic,

mutagenic, immunosuppressive and teratogenic substances [41, 42]. AFB1 is the most toxic species of aflatoxin, with LD50 of 0.5-10 mg per one gram of animal body weight [24].

2.1.2 Ochratoxin

Among of four types of ochratoxin A, B C and D found in nature, ochratoxin A (OTA) has the most toxicity and is produced by *Aspergillus ochraceus* and *Penicilium verrucosum* [24, 41]. OTA is commodity found in barley, oat, wheat, coffee bean, etc [42]. In a human body, OTA is a kidney toxin, but can damage liver with its high concentration. Additionally, OTA was found as a carcinogen in mice [41].

2.1.3 Zearalenone

Zearalenone (ZON) is produced by *Fusarium graminearum* or *Fusarium culmorum* [41]. It is always found in corn and wheat, and sometimes in barley, oat, rice and dehydrated fruit [24]. ZON can cause estrogenic toxicity and precocious development of mammal [41].

2.1.4 Deoxynivalenol

Deoxynivalenol (DON), categorized in a group of trichothecenes type B, is also produced by *Fusarium graminearum* or *Fusarium culmorum*, similar fungi for ZON [24, 41]. DON can cause an immunosuppressive effect and may cause kidney problems in animals [41]. It is found in raw materials and processed products in agriculture [24].

2.1.5 Fumonisins

Three types of fumonisin B1, B2 and B3 (FB1, FB2 and FB3) are produced by *Fusarium verticillioides* and *Fusarium proliferatum* [41]. In general, FB1 was mostly found in corn and corn-based products in food and feed [24]. It can

cause esophageal tumors in certain human populations and liver toxicity in animals [41].

2.2 Mycotoxin regulations

The European Union issues the maximum limit of mycotoxin in foodstuffs [43-45], such as rice and related product as shown in Table 2.1. In Thailand, the notification of the Ministry of Public Health No.98 (1986) specifies that the total aflatoxins in foodstuffs must not exceed 20 μg/kg, while maximum level of 10 μg/kg for the EU regulation of rice and related products.

Table 2.1 Maximum limit of mycotoxins in rice and related products by Commission of the European Communities

Mycotoxins	Commodity from regulation	Maximum levels (μg/kg)
AFB1	Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5.0
Sum of AFB1, AFB2, AFG1 and AFG2	Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	10.0
OTA	Unprocessed cereals	5.0
DON	Unprocessed cereals other than durum wheat, oats and maize	1,250
ZON	Unprocessed cereals other than maize	100
Sum of FB1 and FB2	Unprocessed maize and applies to unprocessed cereals placed on the market for first-stage processing	2,000

2.3 High performance liquid chromatography and ultra high performance liquid chromatography

High performance liquid chromatography (HPLC) is an analytical technique used for separation of compounds in a liquid mobile phase mechanically pumped through a column containing a stationary phase. The HPLC separation mechanism is based on the difference in interactions of the compounds on the stationary phase and/or the mobile phase. Typically, HPLC instrument consists of mobile phase reservoirs, a pump, an injector, a column, a detector and data processor [46]. In comparison with conventional HPLC, ultra high performance liquid chromatography (UHPLC) or ultra performance liquid chromatography (UPLC) refers to HPLC with a smaller particle size of stationary phase (less than 2 μm) and higher driven pressure pump (up to 1,400 bar) [33].

In comparison with HPLC, the smaller particle size of UHPLC stationary phase reduces solvent consumption due to the smaller UHPLC column diameter, and also results in a decrease in band broadening due to Eddy diffusion and resistance to mass transfer. This leads to an increase in the plate number and better separation efficiency, and therefore, allows higher linear flow of mobile phase to provide the shorter analysis time in UHPLC with comparable resolution in UHPLC and HPLC. However, the high linear flow requires high backpressure to drive mobile phase through the smaller closed-packing of UHPLC stationary phase. Conventional HPLC instrument may have limitation for use of a UHPLC column at high backpressure. This high backpressure can be performed using a high pressure resistance pump with the proper fitting and may be also reduced by increasing the column temperature and using the monolithic column instead of packed column [47].

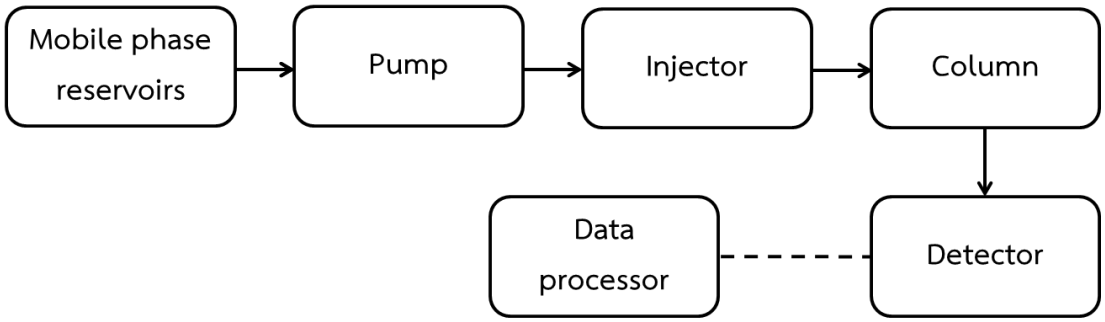


Figure 2.2 HPLC or UHPLC system diagram. Adapted from [48].

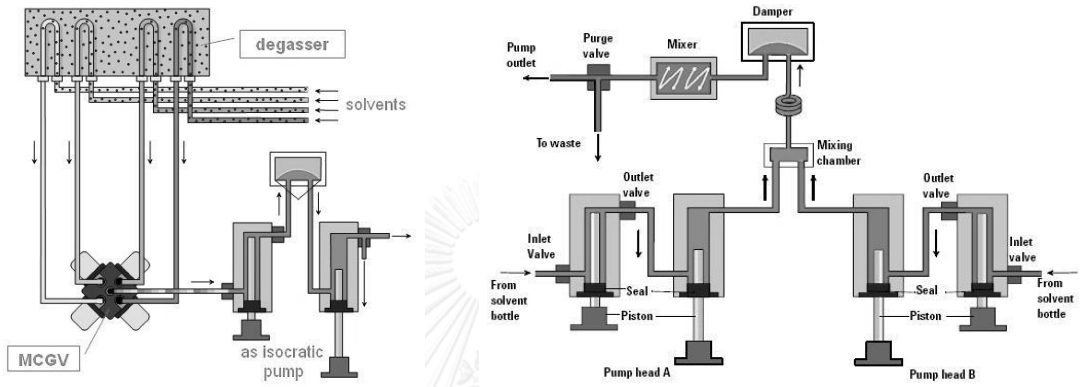
2.3.1 Mobile phase reservoirs

The mobile phase reservoirs in HPLC and UHPLC are the same, and used for storing the mobile phase before being pumped to the HPLC or UHPLC system. The reservoirs are typically made from glass. The characteristics of mobile phase include analyte-dissolving solvent, non-reaction with stationary phase material and analyte, high purity, detector compatibility and miscible solvents. In general, the mobile phase is driven by either isocratic elution or gradient elution. The former is performed by using a single solvent or mixed solvents with a desired constant ratio of mixing, while the latter by using mixed solvents with programing ratio of mixing [48].

2.3.2 Pump

Pump is used for controlling and driving the mobile phase from the reservoirs to a column and maintaining the precise backpressure for particular and precise flow rate of mobile phase. The stationary phase with a smaller particle size cause the higher resistance against the mobile phase flow, therefore the HPLC system requires a high pressure pump to drive the mobile phase [48]. Two types of the HPLC pump are separated by the number of solvent types used as the mobile phase including a quaternary pump and a binary pump. The quaternary pump, a similar structure as the isocratic pump, can provide a gradient of up to 4 different

solvents mixed at low pressure [49]. In contrast, the binary pump can be used with 2 different solvents mixed at high pressure. The binary pump consists of two independent pumps for both solvent channels connected with mixing chamber. The composition or gradient can be used in a range of 0% to 100% and has a higher precision compared to the quaternary pump [50].



(a) (b)

Figure 2.3 Schematic of (a) the quaternary pump [49] (b) the binary pump [50].

2.3.3 Injector

The HPLC or UHPLC injector is used to introduce a sample solution into a column. The good characteristics of the injector should be suitable for all types of samples and be performed under high backpressure with precise and accurate volume of a sample solution injected [48]. Various sample introduction devices can be manually and automatically performed by using a valve system providing excellent precision and easy use. When the valve is in the load position, the sample loop (generally 10 to 50 µL in volume) is filled with a two- to five-fold excess of sample in order to ensure that the previous sample has been flushed. The valve is turned from load to inject position in order to connect the sample loop to the high-pressure mobile phase stream, and then the sample is sent to the column [51].

2.3.4 Column

The HPLC or UHPLC column containing with stationary phase, is responsible for separating analytes in the presence of mobile phase driven by the pump. The HPLC column is typically used with 50 to 300 mm length, 2-4 mm column internal diameter and 1.7-10 μm particle size of the stationary phase [48].

Two types of partition chromatography: normal phase and reversed phase, are different based on the polarities of the mobile phase and stationary phases. In normal phase, polar stationary phase and non-polar solvent mobile phase are performed, while, in reversed phase, stationary phase is non-polar and mobile phase is polar solvent. In normal phase, the least polar component is eluted first, increasing the polarity of the mobile phase then decrease the elution time. In the other hand, with reversed phase, the most polar component elutes first, and increasing the mobile phase polarity increase the elution time. It has been estimated that more than three-quarters of all HPLC separation are currently performed with reversed phase, bonded silica with octyl (C8) or octyldecyl (C18) packing. The long-chain hydrocarbon groups in the surface of the particle, giving a brushlike, non-polar hydrocarbon surface. The mobile phase used with these stationary phase is often an aqueous solution such as methanol, acetonitrile or tetrahydrofuran [52].

2.3.5 Detector

The HPLC detector plays an important role for monitoring the compounds separated from the column. The chromatographic detector can response to the physical and/or chemical properties of analyte passing the detector, resulting in a change in electrical signal related to the amount of analyte. The good characteristics of the detector are high sensitivity and selectivity for analyte of interest, high stability with a change in temperature or pressure and responsible to all solutes. The most widely used of HPLC detectors are a UV-visible

spectrophotometer, a fluorescence detector and a mass spectrometer (MS) [48]. Advantages of MS over the first two types of detectors include higher sensitivity, better selectivity and more reliable confirmation of particular analyte using its structural information obtained from its molecular ion and fragmented ions. This work involves only with MS, and therefore its details are given in Section 2.4.

2.4 Mass spectrometry

Mass spectrometry (MS) is an analytical technique for measuring mass to charge ratio (m/z) of ionized atoms or molecules. MS can be used for either qualitative analysis of structural information by using a molecular ion and fragmented ions, or quantitative analysis of analyte by using ion abundant or intensity of detection response. Normally, a mass spectrometer consists of an ion source, a mass analyzer and an ion detector. The mass analyzer and the ion detector have to be operated at low pressure, therefore a pumping system is required [53]. In the case of a hyphenated technique of HPLC or UHPLC with MS, the common ion source used is electrospray ionization (ESI) as detailed in the following section.

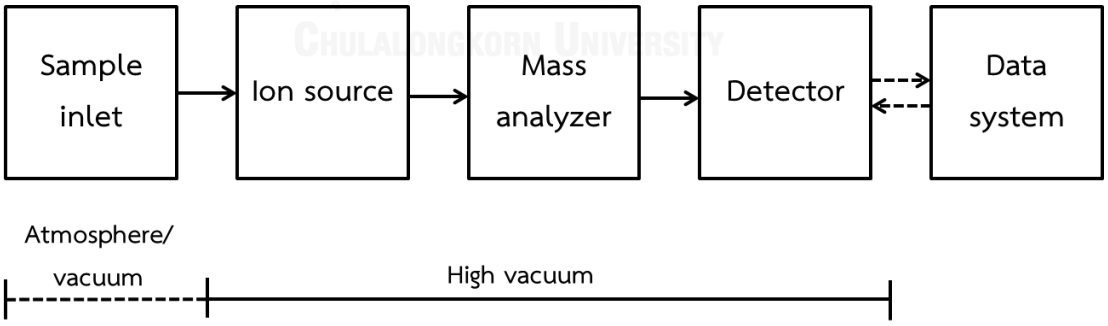


Figure 2.4 The components of mass spectrometer. Adapted from [54].

2.4.1 Ion source: electrospray ionization

ESI is the ion source suitable for a solution of polar and thermally-stable compounds eluted from HPLC through a metal capillary into the ESI ion source. When a sample solution is driven to the end of a capillary that has a high voltage potential across in the surface, it is sprayed out into a heated chamber. Then charged droplets are expelled into the ionization chamber and subjected to a counterflow of a sheath gas or drying gas (usually nitrogen) that evaporates the solvent from the droplets. Therefore, the charge density of each droplet increases until the electrostatic repulsive forces exceed the surface tension of the droplet (the Rayleigh limit), then the droplets break apart into smaller droplets. This process continues until solvent-free sample ions are left in the gas phase and pass into mass analyzer (Figure 2.4) [53-55].

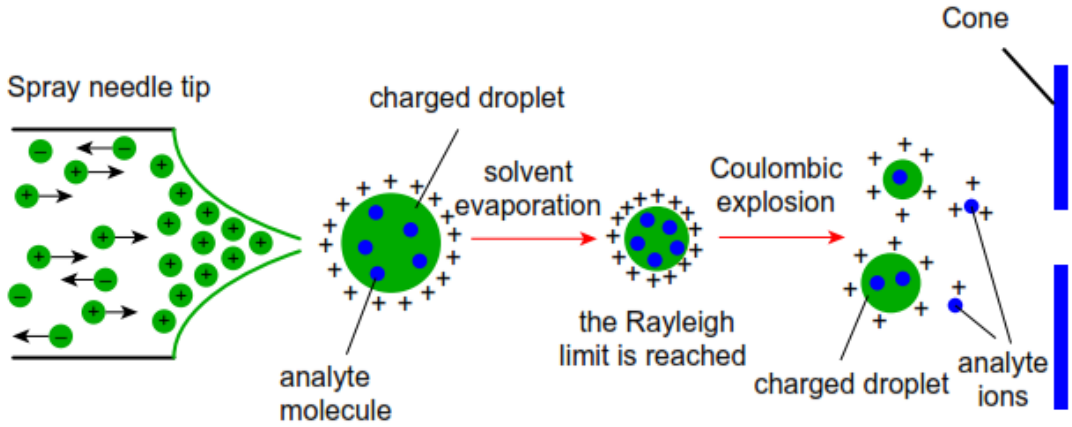


Figure 2.5 Schematic representation of electrospray ionization. Reproduced from [53].

2.4.2 Mass analyzer

A mass analyzer, a key part of a mass spectrometer, is used to separate ions according to the mass to charge ratio (m/z). Various types of mass analyzer used are for example, quadruple (Quad), time-of-flight (TOF), and ion trap (Trap) configurations. Additionally, two or more of single type of mass analyzers,

called tandem mass spectrometer (tandem-MS), are designed in order to enhance instrument performance, such as triple quadrupoles (Quad-Quad) and quadrupole time-of-flight (Quad-TOF) [53]. This work involves Quad-Quad, therefore Quad and Quad-Quad are given in the following sections.

2.4.2.1 Quadrupole

Quadruple (Quad) mass analyzer consists of four metal rods by opposite two-pairs of rods. The two opposite metal rods are connected by electricity: radio frequency (RF) voltage and also direct current (DC) voltage. These RF and DC voltages generate an oscillating electrostatic field in the area between the rods. Depending on the ratio of RF amplitude to the DC voltage, ions oscillate in this electrostatic field. Ions of the correct m/z undergo a stable oscillation of constant amplitude and travel down the quadrupole axis. These ions pass through the analyzer to reach the ion detector. Ions of an incorrect m/z (too small or too large) undergo an unstable oscillation and are removed from a mass analyzer system by continuously increasing of the oscillation amplitude and striking one of the metal rods. Therefore, quadrupole is responsible for a mass filter. A desired mass range and mass resolution depend on length, diameter and shape of metal rods [53, 54].

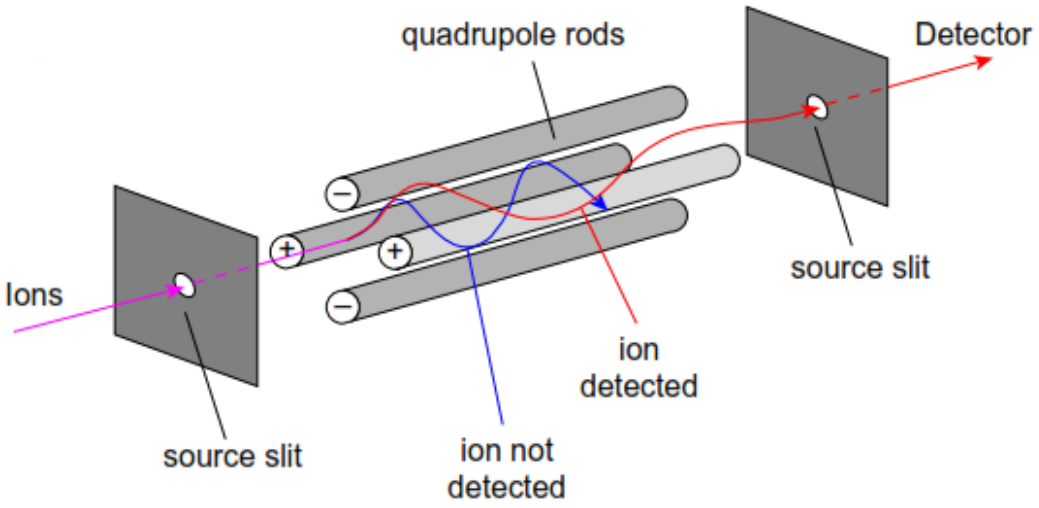


Figure 2.6 Schematic of a quadrupole mass analyzer. Reproduced from [53].

2.4.2.2 Triple quadrupole

Triple quadrupole (Quad-Quad) is configured three quadrupoles. The first quadrupole (Q1) is used to select the specific ions passing into the second quadrupole (Q2). The specific ions from Q1 are collided with the collision nitrogen or argon gas at high ionization voltage and high pressure. This process, called collision-induced dissociation (CID), causes the fragmentation of specific ion to produce the produce ions or daughter ions. Therefore, Q2 in fact is a collision cell, not a mass analyzer. Finally, the product ions are selected and separated by the third quadrupole (Q3) prior to detection of desired m/z ions [53].

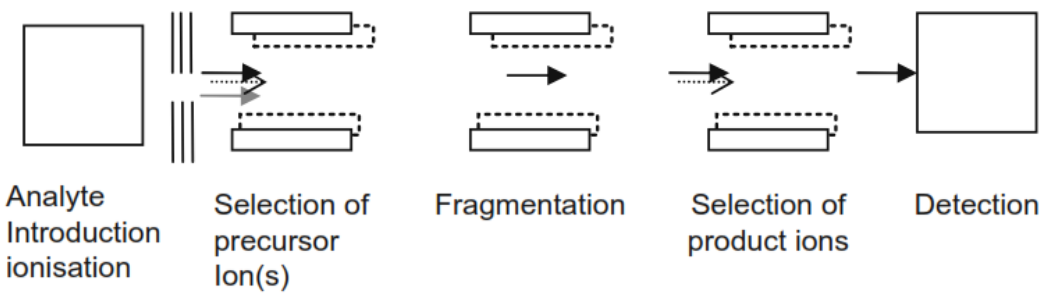


Figure 2.7 Schematic diagram of a triple quadrupole mass analyzer. Reproduced from [53].

2.4.2.3 Mode of operation in tandem MS

Multiple-reaction monitoring (MRM) is one of the modes of operation in tandem MS which was widely used for determination of known compounds. In the MRM mode, two stages of mass filtering are employed on a triple quadrupole mass spectrometer. In the first stage, an ion of interest (molecular ion) is selected in Q1 and induced to fragment by collisional excitation with a neutral gas in a pressurized collision cell (Q2). In the second stage, the product ions derived from the molecular ion are analyzed for their mass in Q3. Only a small number of specific fragment ions (product ions) are analyzed for their mass in Q3. This targeted MS analysis using MRM enhances the lower detection limit as compared to full scan

MS/MS analysis by allowing rapid and continuous monitoring of the specific ions of interest [56, 57].

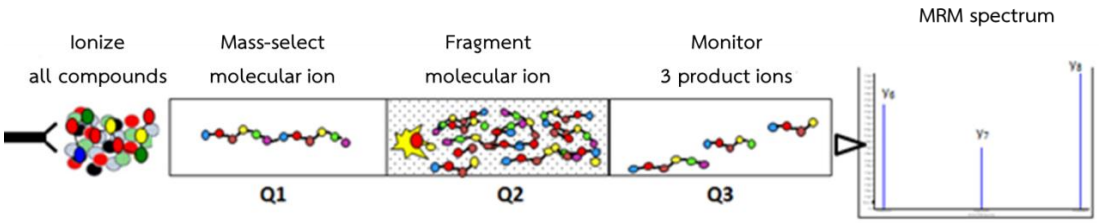


Figure 2.8 Schematic of multiple-reaction monitoring. Reproduced from [56].

2.4.3 Ion detector

The ion detector is responsible for measuring electrical signal of each ion passing from the mass analyzer and through the detector slit. Mostly, the common design is an electron multiplier being horned-shaped, as shown in Figure 2.9. The inside surface of this shape contains coated dynodes. A dynode is an electrode that is designed by electron crashing on the surface of dynode causes two or more electrons emitted from this surface. As the stream of ions enters from the mass analyzer through the slit and contacts the inside surface of the detector, corresponding stream of electrons is ejected. The electron signal amplifies each time this stream of electrons bounces off the wall, therefore, at the base of the horn, there is a significant signal, as illustrated in Figure 2.9. This amplification electronic signal results in a peak in the mass spectrum where the intensity of this signal is plotted on the y-axis. The different ions enter one at a time through the detector slit as a result of their separation in the mass analyzer creating their own unique signal. In this case, the mass spectrum of each ion is found at a particular mass to charge ratio, which is plotted on the x-axis [58].

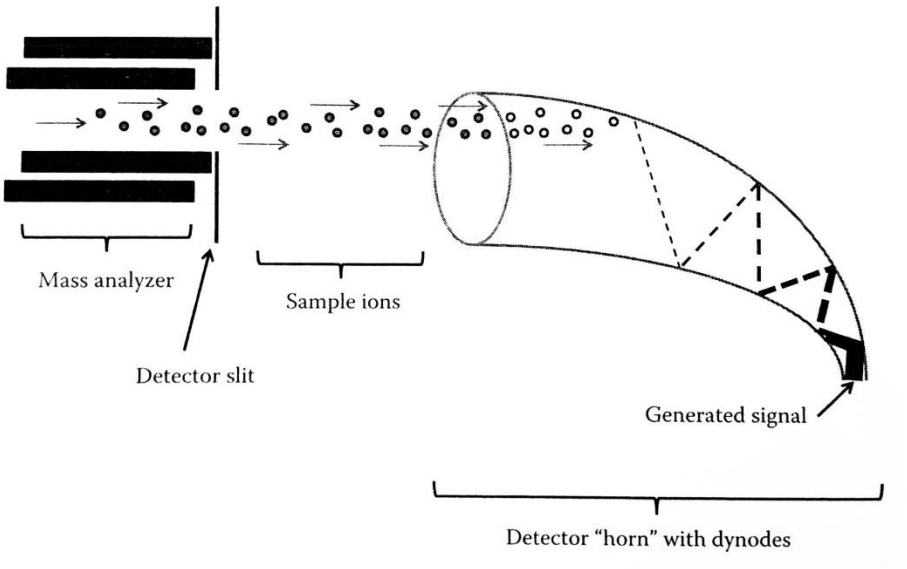


Figure 2.9 Schematic of the horned-shaped electron multiplier mass. Reproduced from [58].

2.5 Sample preparation techniques

Real samples contain both analytes and complex matrices. Although many advanced technologies have been established to enhance high performance in HPLC separation and tandem-MS detection, the sample preparation for real samples is also required prior to HPLC, HPLC-MS, HPLC-MS/MS or UHPLC-MS/MS analysis. The purpose of sample preparation is to extract the analytes from the samples and also to remove matrices that may interfere with the detection of analytes, reduce separation efficiency, shorten the column life and contaminate the MS interface. The ideal sample preparation should be fast, precise, accurate, low cost and high throughput [59]. Therefore, QuEChERS (quick, easy, cheap, effective, rugged and safe) sample preparation was chosen in this work with results as previously in Section 1.2 and its details are given in the following section.

2.6 QuEChERS sample preparation

QuEChERS sample preparation was developed by Anastassiades and coworker in 2003, intendedly for determination of pesticides in vegetables and fruits [60].

However, this technique has been widely used for determination other compounds in other matrices covering soil, water sediments, packaged foods, animal tissues, milk, fish, shrimp, fruit and vegetable as given in the recent review articles [59, 61]. QuEChERS [61] is an easy technique based on two extraction steps; i) the liquid partition with an organic solvent and ii) cleanup with dispersive-solid phase extraction (d-SPE).

QuEChERS technique provides a good recovery and reproducibility and less expensive than other techniques. Therefore, this technique is widely used in many laboratories. Especially the standard methods from AOAC International (AOAC Official Method 2007.01) and the Committee of European Normalization (European EN 15662 Method) are used for analysis of pesticides in many matrices as well as adopted these methods for analyzing other compounds in other matrices.

2.6.1 Solvent extraction

In the first QuEChERS step, the homogenous sample is placed in a centrifuge tube, and water-miscible organic solvent is added, in the presence of salts with or without buffering agents, to extract the sample by using a manual vortex or automatic shaker and then centrifuge the mixture in the tube prior to the next cleaning-up step. Key factors affecting QuEChERS solvent extraction have been studied: extraction solvent, salts, buffer agents and pH, and water content in a sample.

In dried sample, addition of water is required in order to hydrate and swell the matrices in a sample and to reduce the interaction between analytes and matrices leading to a good extraction efficiency [11]. The most widely used extraction solvents for QuEChERS include acetonitrile (ACN), ethyl acetate (EtOAc) and acetone. However, ACN is mostly preferred because, in the presence of salts added, ACN results in better phase separation between extraction solvent and water, and less water residue and lipophilic matrices in extraction solvent.

Salts, particularly sodium chloride (NaCl) and anhydrous magnesium sulfate (anh. $MgSO_4$), added in the QuEChERS solvent extraction induce phase separation between extraction solvent and water and enhance the extraction efficiency owing to salting out effect. The anh. $MgSO_4$ salt also removes water in the extraction solvent resulting in better partitioning of organic analytes in extraction organic solvent. In addition, an endothermic reaction of magnesium sulfate dissolving in water, with 40-45ºC of a heated solution, enhances partitioning of non-polar analytes in the extraction solvent

pH is another factor that is considered especially for the weak acidic or basic compounds to exist their neutral form at particular pH range. Addition of buffering agent should be done in pH control. For example, a citrate buffer containing sodium citrate tribasic dihydrate and sodium citrate dibasic sesquihydrate provides the pH in a range of 4.0-7.5, while an acetate buffer containing acetic acid and sodium acetate provides the pH in the range of 3.5-6.0 [48].

2.6.2 Dispersive-solid phase extraction

Dispersive-solid phase extraction (d-SPE) is used for a cleanup step of a QuEChERS technique. After centrifuging the mixture in the solvent extraction step, the solvent extract is transferred to another tube containing a small amount of sorbent to remove matrices and dehydrated salt, such as anh. $MgSO_4$, to remove the water residue in the organic solvent phase. The mixture is shaken by vortex, and then centrifuged to separate the solid from solution and then the solution is used to analyze by instruments. Ideally, a good sorbent should strongly adsorb or interact with matrix interferences but not or weakly with analytes of our interest. Types of sorbent widely used are for example, octadecylsilane (C18), primary and secondary amine (PSA), graphitized carbon black (GCB), silica, etc.

Octadecylsilane (C18) is an octadecyl moiety-bonded silica sorbent that is non-polar, and therefore, it can removes non-polar compounds such as lipid and small polar compounds [59, 62].

Primary and secondary amine (PSA) categorized as ion-exchange silica sorbent containing primary and secondary amine moiety that is polar, and therefore, it can remove polar compounds such as the sugars, fatty acid and others organic acids as well as pigments such as anthocyanidines. The interaction between matrix and sorbent are hydrogen bonding and electrostatic attraction [59, 60, 62].

Graphitized carbon black (GCB) sorbent, non-porous material [63], can retain the planar molecules. It can remove visible plant pigment from organic extracted such as chlorophyll, carotenoids and sterols that found in food. GCB cannot remove fatty acids from matrix interferences which affect the efficiency of ionization. GCB can attract with neutral, acidic and basic compounds. The mechanism of these attractions involves non-specific (such as van der Waals interaction) and anion exchange [59, 60, 62, 64].

Silica (SiO_2) is polar sorbents containing silanol groups (-Si-OH). It can remove polar matrix interferences in samples. The hydrophilic matrix components are retained in the polar sorbent, and therefore this sorbent is suitable for cleaning up fatty food samples because it can retain some lipids. The interaction between matrix and sorbent include hydrogen bonding, dipole-dipole interaction, π-π interaction and induced dipole-dipole interaction [62, 65].

2.6.3 Extraction efficiency of QuEChERS sample preparation

To improve the method performance and reduce error that occur in several steps of QuEChERS, the internal standard is always used in the process. A good internal standard has not to react with the sorbent in d-SPE step and should provide high recovery of compounds in organic phase. In most cases, the internal standard is added at the first step of extraction procedure. In some cases, the sample has complex matrices such as high fat contain, the internal standard is added in the last step of the method.

CHAPTER III

EXPERIMENTAL

3.1 Instruments and apparatus

3.1.1 Ultra high performance liquid chromatograph (UHPLC), Agilent Technologies Model 1290 (CA, USA), consists of vacuum degasser, binary pump, agilent jet weaver, autosampler and column oven

3.1.2 Mass spectrometer (MS), Agilent Technologies Model 6490 (CA, USA), consists of a triple quadrupoles mass analyzer, electrospray ionization (ESI) interface and MassHunter software processing

3.1.3 C18 UHPLC column: ACQUITY UPLC® HSS T3, 100 mm × 2.1 mm I.D., 1.8 µm, Waters (USA)

3.1.4 UV-visible Spectrophotometer, Shimadsu Model UV-1601 (Tokyo, Japan)

3.1.5 Milli-Q ultra pure water system, Merck (Germany)

3.1.6 Balance (4 digits), Sartorius Model AC211S-00MS (Germany)

3.1.7 Centrifuge, Kubota Corporation Model 8800 (Tokyo, Japan)

3.1.8 Micropipettes 10-100, 20-200, 100-1000, 500-2500 and 1000-5000 µL, Eppendorf (Germany)

3.1.9 Centrifuge tubes with screw cap 15 and 50 mL

3.1.10 Evaporator with stream nitrogen, Metalblock thermostats, VCM (Bielefeld, Germany)

3.1.11 Volumetric flask 25, 50, 100, 250, 500, 1000 mL

3.1.12 Nylon membrane filter 47 mm. 0.2 µm, Alltech Associates Inc (IL, USA)

3.1.13 PTFE Syringe filters 13 mm, 0.22 µm, Membrane solution (TX, USA)

3.1.14 Glass syringes 10 mL

3.1.15 HPLC amber vial 2 mL with PTFE caps, Agilent technologies (USA)

3.2 Chemicals

3.2.1 Mycotoxin standards and internal standard

Eight solid mycotoxins standards were purchased from Sigma-Aldrich (MO, USA): AFB1, AFB2, AFG1, AFG2, FB1, OTA, ZON and DON. A standard solution FB2 (50 µg/mL in ACN:water (50:50, v/v)) was obtained from Fluka (Buchs, Switzerland). The purity of FB1 and DON are 97.00% and 98.00%, respectively. Sulfamethoxazole (SMX), used as an internal standard, was also purchased from Sigma-Aldrich (MO, USA).

3.2.2 Organic Solvents

HPLC grade acetonitrile and methanol used for QuEChERS and LC-MS grade methanol used for UHPLC-MS/MS analysis were purchased from JT Baker (Center Valley, PA, USA). Benzene (analytical grade) used for dissolving standards was purchased from Carlo Erba (Ronado, MI, USA), chloroform (analytical grade) from VWR (Fontenay-sur-Bios, France), ethyl acetate (ultra-residue analyzed grade) from JT Baker (Phillipsburg, NJ, USA), acetic acid (analytical grade) from Merck (Darmstadt, Germany), formic acid (analytical grade) from Merck (Darmstadt, Germany) and sulfuric acid (analytical grade) from Fisher (UK).

3.2.3 Salts and sorbents

Anhydrous magnesium sulfate (anh. $MgSO_4$) was obtained from Panreac (Barcelona, Spain), sodium chloride (NaCl) and potassium dichromate ($K_2Cr_2O_7$) from Univar (Ingleburn, NSW, Australia), sodium citrate tribasic dihydrate, sodium citrate dibasic sesquihydrate, sodium acetate (NaOAc) and silica from Merck (Darmstadt, Germany), ammonium formate, 99% from Acros Organics (NJ, USA) and

primary and secondary amine (PSA), octadecylsilane (C18) and graphitized carbon black (GCB) from Supelco® (Bellefonte, PA, USA).

3.2.4 Brown rice sample

Three types of brown rice samples, such as black brown rice, red brown rice and brown brown rice, were obtained from the supermarkets in Bangkok. A blank sample was obtained from these brown rice without mycotoxins preliminarily determined by our UHPLC-MS/MS. A blank sample of black brown rice was used for optimization and validation for determination of mycotoxins. Then, this validated method was also applied for red brown rice and brown brown rice.

3.3 Mycotoxin standard preparation and measurement concentration

3.3.1 Preparation of stock mycotoxin standard and internal standard solutions

3.3.1.1 A separately standard solution of AFB1, AFB2, AFG1 and AFG2 each at 40 mg/L, were separately prepared by dissolving 1 mg of each standard with benzene:ACN (98:2, v/v) in a 25-mL volumetric flask.

3.3.1.2 A standard solution of OTA at 40 mg/L was prepared by dissolving 1 mg of OTA with benzene:HOAc (99:1, v/v) in a 25-mL volumetric flask.

3.3.1.3 A standard solution of ZON at 400 mg/L was prepared by dissolving 10 mg of ZON with benzene in a 25-mL volumetric flask.

3.3.1.4 A standard solution of DON at 200 mg/L was prepared by dissolving 5 mg of DON with ethyl acetate:MeOH (95:5, v/v) in a 5-mL volumetric flask (concentration is 196.0 mg/L at 98.00% purity).

3.3.1.5 A standard solution of FB1 at 40 mg/L was prepared by dissolving 1 mg of FB1 with ACN:water (50:50, v/v) in a 25-mL volumetric flask (concentration is 38.80 mg/L at 97.00% purity).

3.3.1.6 An internal standard solution of SMX at 1,000 mg/L was prepared by dissolving 25 mg of SMX with MeOH in a 25-mL volumetric flask.

3.3.2 Measurement and calculation of exact concentration of mycotoxin standards

Since mycotoxins are not stable in a solution form, the exact concentration should be measured prior to analytical analysis. In this work, the exact concentrations (c) of the prepared AFB1, AFB2, AFG1, AFG2, OTA and ZON standard solutions in Section 3.3.1.1-3.3.1.3 were determined, according to an Official Methods of Analysis of AOAC International [66] and a Beers Lambert law's equation Abs=εbc, by measuring the UV absorbance of a solution (Abs) and comparing with its known molar absorptivity (ε), where b is the light pathlength of 1.00 cm. Due to unknown absorptivity for the rest three mycotoxins, the exact concentration of FB2 was obtained from the certified value from Sigma-Aldrich (Seelze, Germany), while the exact concentrations of DON and FB1 were obtained from calculation of their purity of 98.00 and 97.00%, respectively.

According to the Official Methods of Analysis of AOAC International [66], the method accuracy must be measured before determination of exact concentration of mycotoxins, by comparison of measured and known absorptivity of $K_2Cr_2O_7$. Using a UV-visible spectrophotometer, $K_2Cr_2O_7$ solutions at 3 concentrations (0.0625, 0.125 and 0.25 mM in 0.009 M sulfuric acid) were prepared, and their UV absorbance were scanned in a wavelength range of 300-400 nm. The maximum absorption near 350 nm was observed and used for calculation of the molar absorptivity of each concentration. In comparison with known molar absorptivity of 3160 $L.mol^{-1}.cm^{-1}$ for a $K_2Cr_2O_7$ solution. the correction factor (CF) was obtained by

taking the ratio of molar absorptivity from known to measured value. In this work, the acceptable accuracy for determination of molar absorptivity was obtained with the average CF of 1.01 ± 0.01 lying in the acceptable CF of 0.95-1.05, indicating that this method can be used to determine the exact concentration of mycotoxins in the following paragraph.

Before measuring the absorbance, mycotoxin standard solutions were 4 times and 10 times diluted for each aflatoxin (in benzene:ACN (98:2, v/v)) and ZON (in benzene), respectively. OTA and other diluted standard solution were then measured for their UV absorbance by scanning the wavelength in 250-400 nm. The maximum absorption near 350 nm (for aflatoxin), 333 nm (for OTA) and 317 nm (for ZON) were used to calculate the concentration of their standard solutions by the equation: concentration (mg/L) = Abs × M × 1000/ε (when M is the molecular mass) and other values as shown in Table 3.1. The results are shown in Table 3.2.

Table 3.1 Molecular mass (M), molar absorptivity (ε), and maximum absorption wavelength of mycotoxins (λ_{max}) [66]

Mycotoxin	M	Solvent	ε ($L.mol^{-1}.cm^{-1}$)	λmax (nm)
AFB1	312	Benzene:ACN (98:2, v/v)	19800	350
AFB2	314	Benzene:ACN (98:2, v/v)	20900	350
AFG1	328	Benzene:ACN (98:2, v/v)	17100	350
AFG2	330	Benzene:ACN (98:2, v/v)	18200	350
OTA	403	Benzene:HOAc (99:1, v/v)	5550	333
ZON	318	Benzene	6060	317

Table 3.2 Summary of the mycotoxin concentration

Analyte	λ_{max} (nm)	maximum absorption (Abs)	Concentration of measured standard (mg/L)	Dilution factor	Concentration of stock standard (mg/L)
AFB1	348.8	0.678	10.68	4	42.73
AFB2	349.8	0.682	10.25	4	40.99
AFG1	355.0	0.758	14.54	4	58.16
AFG2	356.2	0.494	8.96	4	35.83
OTA	333.5	0.673	48.87	1	48.87
ZON	317.0	0.765	40.14	10	401.4

3.3.3 Working mixed mycotoxin standard preparation

A working mixed mycotoxin standard was prepared by diluting individual stock solution in MeOH to give the final solution containing desired amounts of each mycotoxin and 25 µg/L SMX.

3.4 Optimization of mass spectrometric detection

Mycotoxin standards were individually prepared in ACN, except for FB1 and FB2 in CAN:water (50:50, v/v) at concentration of 1 mg/L for each aflatoxin and each fumonisin, and 20 mg/L for OTA, ZON, and DON, while SMX internal standard at 20 mg/L in MeOH was prepared. MS/MS detection for mycotoxins was optimized using a UHPLC-MS/MS system without a UHPLC column, and 0.1% FA-containing water:methanol (50:50, v/v) mobile phase with a flow rate of 0.3 mL/min.

3.4.1 Major parameters of MS detection

3.4.1.1 Molecular ion

The molecular ion of each mycotoxin and internal standard were observed by using an MS2Scan mode. The mass to charge ratios (m/z) were set

to cover the molecular weight of each mycotoxin and internal standard. Both positive and negative polarity modes were used; however, only the mode yielding higher sensitivity was chosen for each particular analyte.

3.4.1.2 Product ion and collision energy optimization

Agilent MassHunter Optimizer software was used to find the product ions and suitable collision energy of each mycotoxin. The product ion yielding the highest abundance was selected as a quantitative m/z ion, while the lower-abundance ion was selected as a qualitative m/z ion. Then, MRM mode was used to find the suitable collision energy. The MS conditions used are shown in Section 4.1.

3.4.2 Minor parameters of MS detection

In order to obtain highest sensitivity for mycotoxins detection, the following other MS parameters were optimized after optimization of UHPLC separation: capillary voltage, nozzle voltage, gas flow, nebulizer pressure, sheath gas flow, gas temperature, sheath gas temperature and dwell time.

3.5 Optimization of UHPLC separation

Mycotoxin standards used for optimization of UHPLC separation were prepared in ACN:water (50:50, v/v) at 10, 100, 200, 1000 and 2000 µg/L for each aflatoxin, ZON, OTA, each fumonisin and DON, respectively. UHPLC-MS/MS was performed by using a UHPLC column: ACQUITY UPLC® HSS T3, C18 column 100 mm × 2.1 mm I.D., 1.8 µm, and 10-µL injection of a sample solution. The gradient mobile phase programming, at flow rate of 0.3 mL/min, was assigned to be A for water containing 0.1% FA and 5 mM ammonium formate, and B for MeOH. The gradient profile for elution of mycotoxins was optimized and results are given in Section 4.2. The optimum gradient profile was chosen for studying in the following sections.

3.5.1 Type of aqueous mobile phase

To obtain high sensitivity and a symmetric peak shape, the amounts of FA and ammonium formate added in aqueous mobile phase (mobile phase A) were optimized as shown in Table 3.3. The results are shown in Section 4.2.1.

Table 3.3 Type of aqueous mobile phase

Code	Concentration of formic acid	Concentration of ammonium formate
A1	0.1%	-
A2	0.1%	5 mM
A3	0.1%	10 mM
A4	0.5%	-
A5	0.5%	5 mM
A6	0.5%	10 mM
A7	1%	-
A8	1%	5 mM
A9	1%	10 mM

3.5.2 Solvent for dissolving standard solution or samples

To provide a high peak area and a symmetric peak shape of all analytes, the solvent used for dissolving standard solutions or samples was optimized using 6 types (S1-S6) as shown in Table 3.4. The results are shown in Section 4.2.2.

Table 3.4 Types of solvent for dissolving standard solutions or samples

Code	Type of solvent
S1	ACN
S2	MeOH
S3	ACN:water (50:50)
S4	MeOH:water (50:50)
S5	ACN:0.1% FA in water (50:50)
S6	MeOH:0.1% FA in water (50:50)

3.5.3 Column temperature

In general, temperature affects the separation of mycotoxins in a UHPLC column. If column temperature is set at room temperature or a little higher, the separation system will not be stable. The column temperature was set at 30, 35, and 40 °C by using 5 runs of mycotoxin standard solutions injected (n = 5) for each column temperature in the same day.

3.6 Optimization of sample preparation

To obtain high recovery and low limit of detection for trace levels of mycotoxins in brown rice, the following parameters of the QuEChERS sample preparation method were studied: acidity of extraction solvent, buffer types, the volume ratio of added water and extraction solvent, the amount of salt and sorbent type. A sample blank of black brown rice (three batches, n = 3) spiked with nine standard mycotoxins at known levels, was used as sample in this section.

3.6.1 QuEChERS procedure

The QuEChERS procedure for determination mycotoxins in brown rice was basically performed by the followings, unless otherwise stated. Firstly, a blank sample was blended, homogenized and weighed (1.0 g) into a 50 mL-centrifuge tube. After mycotoxins standards were spiked in blank sample at known levels (50 µg/kg

for AFB1, AFB2, AFG1, AFG2, FB1 and FB2, 200 µg/kg for OTA and ZON and 400 µg/kg for DON), water (5.0 mL) was added and vortexed for 30 sec. In order to extract the mycotoxins into organic solvent, 5.0-mL ACN with 10% HOAc was added in the mixture and vortexed for 1 min at high speed. After that, the following four salts were added to the mixture in the tube: 2.0-g anh. $MgSO_4$ and 0.50-g NaCl to induce separation between ACN and aqueous phases, and 0.50-g sodium citrate tribasic dihydrate and 0.25-g sodium citrate dibasic sesquihydrate for buffering reagents. After being vigorously shaken by hand for 1 min, the mixture was centrifuged at 3,000 rpm for 5 min. A 2.0- mL supernatant was then transferred into another 15 mL-centrifuge tube containing 300 mg of anh. Finally, $MgSO_4$, and mixed sorbents 50 mg of C18, 25 mg of PSA and 25 mg of silica, and followed by vortexing for 1 min and centrifuging at 3,000 rpm for 5 min. It should be noted that the added $MgSO_4$ salt is for the removal of water residue, while the added sorbents are for the removal of matrix interferences.

Prior to UHPLC-MS/MS analysis, a 1.0-mL aliquot from an upper part of the extract was evaporated to dryness under a stream nitrogen, and then 1 mL of the final dissolving solvent was reconstituted, and the mixture was vortexed for 30 sec, where the final dissolving solvent contain 50:50 MeOH:0.1% FA-containing water, and also 0.5 µg/L of an SMX internal standard. Prior to a UHPLC-MS/MS injection, the final solution was filtered through a 0.22 µm-PTFE syringe filter. It should be noted that the 50:50 MeOH:0.1% FA-containing water solvent used for dissolving the extracted mycotoxins, instead of the original 10% HOAc-containing ACN, is due to obtain a symmetric peak and high peak area for quantitative analysis.

3.6.2 Acidity of extraction solvent

The experiment was performed as mentioned in Section 3.6.1, except that the nine types of extraction solvent (M01-M09) were varied as shown in Table 3.5. The results are shown in Section 4.3.1. It should be noted that the percentage recovery is calculated using Equation 3.1.

$$\%\text{Recovery} = 100 \times C_1/C_2 \quad \textbf{(3.1)}$$

where C_1 refers to the determined amount of the analyte and C_2 refers to the known amount of spiked analyte.

Table 3.5 Types of extraction solvent for QuEChERS extraction procedure

Code	Extraction solvent
M01	ACN
M02	ACN with 0.1% FA
M03	ACN with 1% FA
M04	ACN with 5% FA
M05	ACN with 10% FA
M06	ACN with 0.1% HOAc
M07	ACN with 1% HOAc
M08	ACN with 5% HOAc
M09	ACN with 10% HOAc

3.6.3 $MgSO_4$, NaCl and buffering salts

3.6.3.1 Buffer types

In order to maintain an aqueous buffer solution, additional salts may be included with anh. $MgSO_4$ and NaCl, such as sodium citrate tribasic dihydrate and sodium citrate dibasic sesquihydrate for a citrate buffer according to the Committee of European Normalization (European EN 15662 Method) and other previous works [29, 30, 34, 36], or sodium acetate for an acetate buffer according to the standard methods from AOAC International (AOAC Official Method 2007.01) and other previous works [13, 31]. The experiment was performed as mentioned in Section 3.6.1, except that the three types of mixed salts (M10-M12) for inducing phase separation in QuEChERS solvent extraction step were varied as shown in Table 3.6. The results are shown in Section 4.3.2.

Table 3.6 Types of buffer and mixed salts for inducing phase separation in QuEChERS solvent extraction

Code	Type of buffer	Mixed salts
M10	No buffer	2.0 g of anh. $MgSO_4$ + 0.50 g of NaCl
M11	Citrate buffer	2.0 g of anh. $MgSO_4$ + 0.50 g of NaCl + 0.50 g of sodium citrate tribasic dihydrate + 0.25 g of sodium citrate dibasic sesquihydrate
M12	Acetate buffer	2.0 g of anh. $MgSO_4$ + 0.50 g of NaCl + 0.50 g of NaOAC

3.6.3.2 The amount of salt

The experiment was performed as mentioned in Section 3.6.1, except that the four types of anh. $MgSO_4$ and NaCl weight ratio (M13-M16) were varied as shown in Table 3.7. The results are shown in Section 4.3.2.

Table 3.7 Weight ratio of anh. $MgSO_4$ and NaCl salts for inducing phase separation in QuEChERS solvent extraction

Code	Weight of anh.$MgSO_4$ (g)	Weight of NaCl (g)
M13	0.50	0.125
M14	1.0	0.25
M15	2.0	0.50
M16	4.0	1.0

3.6.4 The volume ratio of water and extraction solvent

The experiment was performed as mentioned in Section 3.6.1, except that the four types of water and extraction solvent volume ratio (M17-M20) were varied as shown in Table 3.8. The results are shown in Section 4.3.3.

Table 3.8 Volume ratio of water and extraction solvent for QuEChERS extraction procedure

Code	Volume of water (mL)	Volume of extraction solvent (mL)
M17	5	5
M18	5	10
M19	10	5
M20	10	10

3.6.5 Sorbent types

In the d-SPE step, various sorbents were used in order to remove matrix interferences from the extracted solution compromisingly with acceptable recovery of mycotoxins as followed.

3.6.5.1 Single sorbent

Four types of single sorbent, C18, PSA, GCB and silica, were individually used to compare with no any sorbent. The experiment was performed as mentioned in Section 3.6.1, except that types of sorbent were varied as shown in Table 3.9. The results are shown in Section 4.3.4.1.

Table 3.9 Types of single sorbent for d-SPE clean-up step

Code	Type of single sorbent
M21	None
M22	C18 50 mg
M23	PSA 50 mg
M24	GCB 50 mg
M25	Silica 50 mg

3.6.5.2 Mixed sorbents

The experiment was performed as mentioned in Section 3.6.1, except that six types of mixed sorbents were varied as shown in Table 3.10, in comparison with the C18 sorbent. The results are shown in Section 4.3.4.2.

Table 3.10 Types of mixed sorbent for d-SPE clean-up step

Code	Type of mixed sorbent
M22	C18 50 mg
M26	C18 50 mg + PSA 50 mg
M27	C18 50 mg + GCB 50 mg
M28	C18 50 mg + Silica 50 mg
M29	C18 50 mg + PSA 25 mg + GCB 25 mg
M30	C18 50 mg + PSA 25 mg + Silica 25 mg
M31	C18 50 mg + GCB 25 mg + Silica 25 mg

3.7 Method validation

3.7.1 Analytical limits

According to the modern IUPAC recommendation [67], the limit of detection (LOD) is defined as the minimum analyte concentration that can be discriminated from the blank, controlling the risks of false positives and false negative. In order to obtain LOD and limit of quantitation (LOQ) values from a matrix-matched calibration plot, the blank sample was extracted using QuEChERS sample preparation as described in Section 3.6.1. The known amounts of mycotoxins in the QuEChERS extract were determined by UHPLC-MS/MS as described in Section 4.1 and 4.2. The calibration plots were performed with four triplicate concentration level of each analyte spiked in the final solvent. It should be noted that the method dectection limit (MDL) and the method quantitation limit (MQL) are estimated from LOD and LOQ, respectively with the QuEChERS sample preparation for 1 g of sample and 5 mL of the final extraction solvent. The results are shown in Section 4.4.1.

3.7.2 Standard calibration curve

Standard calibration curves were established using mixture standard solutions in 50:50 MeOH:0.1% FA-containing water, with five concentration levels and three replicates for each level as shown in Table 3.11. The linear regression plots were performed using the relative response, the ratio of peak area for the analyte to internal standard, against the analyte concentration. The results are shown in Section 4.4.2.

Table 3.11 Concentration range of standard calibration curve

Analyte	Concentration range (µg/L)				
	Level 1	Level 2	Level 3	Level 4	Level 5
AFB1	1.0	2.5	5.0	7.5	10
AFB2	1.0	2.5	5.0	7.5	10
AFG1	1.0	2.5	5.0	7.5	10
AFG2	1.2	2.5	5.0	7.5	10
FB1	2.0	5.0	10	15	20
FB2	2.0	5.0	10	15	20
OTA	2.0	5.0	10	15	20
ZON	2.6	5.0	10	15	20
DON	15	50	100	150	200

3.7.3 Matrix-matched calibration curve

Matrix-matched calibration curves were established using five concentration levels of mycotoxin standards spiked in the QuEChERS extract of a blank sample, with three replicates for each concentration level as shown in Table 3.12. The linear regression plots were performed using the relative response, the ratio of peak area for the analyte to internal standard, against the analyte concentration. The results are shown in Section 4.4.2.

Table 3.12 Concentration range of matrix-matched calibration curve

Analyte	Concentration range (µg/L)				
	Level 1	Level 2	Level 3	Level 4	Level 5
AFB1	1.0	2.5	5.0	7.5	10
AFB2	1.0	2.5	5.0	7.5	10
AFG1	1.0	2.5	5.0	7.5	10
AFG2	1.2	2.5	5.0	7.5	10
FB1	2.0	5.0	10	15	20
FB2	2.0	5.0	10	15	20
OTA	2.0	5.0	10	15	20
ZON	2.6	5.0	10	15	20
DON	15	50	100	150	200

3.7.4 Matrix effect

In order to investigate matrix effect, which may interfere with increasing or decreasing the detection signal of analytes, two curves for the standard calibration curve and the matrix-matched calibration curve were compared. The results are shown in Section 4.4.2.

3.7.5 Accuracy and precision

Accuracy and precision in the quantitative analysis method were evaluated by spiking mycotoxins standards in a blank sample of black brown rice at three concentration levels for ten batches each on three different days, as shown in Table 3.13. The amount of mycotoxins after QuEChERS extraction was determined by UHPLC-MS/MS analysis using matrix-matched calibration curves. The accuracy is expressed by the percentage recovery as Equation 3.1, while the precision is expressed by the percentage relative standard deviation in the recovery. The results are shown in Section 4.4.3.

Table 3.13 Spiking concentration of nine mycotoxins in blank sample for accuracy study

Analyte	Spiking concentration in blank sample (µg/kg)		
	Low	Medium	High
AFB1	5.0	25	50
AFB2	5.0	25	50
AFG1	5.0	25	50
AFG2	6.0	25	50
FB1	10	50	100
FB2	10	50	100
OTA	10	50	100
ZON	13	50	100
DON	75	500	1,000

3.8 Application to real samples

The developed and validated QuEChERS method used for the UHPLC-MS/MS determination of mycotoxins in the black brown rice was also applied to that in red brown rice and brown brown rice. The recovery of mycotoxins extracted from a spiked blank sample of the red brown rice and the brown brown rice at known levels (25 µg/kg for AFB1, AFB2, AFG1 and AFG2, 50 µg/kg for FB1, FB2, OTA and ZON and 500 µg/kg for DON) was observed by using matrix-matched calibration curves. The fourteen brown rice samples including three black brown rice samples, six red brown rice samples and five brown brown rice sample, were obtained from various supermarkets in Bangkok, and were analyzed under the developed and validated method. The results are shown in Section 4.5.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Optimization of mass spectrometric detection

From Section 3.4.1, the molecular ion, product ion, collision energy (CE) and cell accelerator voltage (CAV) of each mycotoxin and internal standard (SMX) were observed, and the results are shown in Table 4.1. Both positive and negative polarity modes were chosen at the same time. Most of mycotoxins are operated in the positive polarity mode, except OTA and ZON for in the negative mode. The molecular ion forms of all mycotoxins are $[M+H]^+$ and $[M+H]^-$ for positive and negative polarity modes, respectively.

From Section 3.4.2, the following minor parameters of MS detection were optimized: capillary voltage (both positive and negative), nozzle voltage, gas flow, nebulizer pressure, sheath gas flow, gas temperature, sheath gas temperature and dwell time. The optimization data and optimum conditions are shown in Table 4.2.

Table 4.1 Molecular ion, product ion, collision energy (CE) and cell accelerator voltage (CAV) of each mycotoxin and SMX

Analyte	Polarity	Molecular ion		Q_1/Q_2	Product ion	CE	CAV	Height	Area
		form	m/z						
AFB1	Positive	$[M+H]^+$	313.1	Q_1	241.0	40	3	29590	152441
				Q_2	284.9	21	7	23365	121712
AFB2	Positive	$[M+H]^+$	315.1	Q_1	287.0	25	3	15046	74800
				Q_2	259.0	29	3	14818	73461
AFG1	Positive	$[M+H]^+$	329.1	Q_1	243.1	26	3	23716	105229
				Q_2	310.9	19	7	15038	66373
AFG2	Positive	$[M+H]^+$	331.1	Q_1	312.9	24	7	10142	47450
				Q_2	189.0	46	7	7743	35527
FB1	Positive	$[M+H]^+$	722.4	Q_1	334.3	45	5	22867	160398
				Q_2	352.1	40	5	21596	150232
FB2	Positive	$[M+H]^+$	706.4	Q_1	336.3	40	5	17938	124187
				Q_2	318.3	41	7	9265	63234
DON	Positive	$[M+H]^+$	297.1	Q_1	249.1	7	3	85522	575320
				Q_2	203.1	9	5	54508	365960

Q_1 = quantitative m/z, Q_2 = qualitative m/z

Table 4.1 (continued)

Analyte	Polarity	Molecular ion		Q_1/Q_2	Product ion	CE	CAV	Height	Area
		form	m/z						
OTA	Negative	$[M-H]^-$	402.1	Q_1	358.1	16	5	424427	2502526
				Q_2	166.8	39	3	139112	826839
ZON	Negative	$[M-H]^-$	317.1	Q_1	131.0	30	3	155602	970649
				Q_2	174.9	24	7	151025	935495
SMX	Positive	$[M+H]^+$	254.0	-	91.9	25	3	622361	4619205

Q_1 = quantitative m/z, Q_2 = qualitative m/z

Table 4.2 MS parameter, optimization data and optimum condition

MS parameter	Optimization data	Optimum condition
Capillary voltage (Positive and Negative)	2,500 V / 3,000 V / 3,500 V	3,000 V
Nozzle voltage	500 V / 1,000 V / 1,500 V	1,000 V
Gas flow	12 L/min / 14 L/min / 16 L/min	16 L/min
Nebulizer	10 psi / 20 psi / 30 psi / 40 psi	20 psi
Sheath gas flow	9 L/min / 10 L/min / 11 L/min / 12 L/min	11 L/min
Gas temperature	150 °C / 200 °C / 250 °C	150 °C
Sheath gas temperature	300 °C / 350 °C / 400 °C	400 °C
Dwell time	10 ms/cycle / 30 ms/cycle / 50 ms/cycle / 100 ms/cycle / 200 ms/cycle	50 ms/cycle

4.2 Optimization of UHPLC separation of mycotoxins

From Section 3.5, the mobile phase reservoir A refers to water containing 0.1% FA and 5 mM ammonium formate, while B refers to MeOH. The optimum gradient profile for elution of mycotoxins was as follows: 0 min: 25% B; 7 min: 85% B; 8 min: 85% B; 9 min: 25% B; 14 min: 25% B. The optimized times of 9, 5 and 14 min were obtained for the data collection, the re-equilibration time and the total run time, respectively. Typically, for several peaks in a chromatogram showing with one displayed window, the peak resolution value of 1.5 refers to the achieved baseline resolution in chromatographic separation including UHPLC, but that of 2.0 is recommended for quantitative analysis. However, UHPLC with the MS/MS detection provides high selectivity for analytes with deferent transitions of a particular molecular ion to its product ion. The chromatogram for each analyte peak can be displayed for the particular window, and therefore, the overlapped peaks can be monitored using the different displayed windows. For example as shown in Figure

4.1, OTA and ZON with the retention times of 5.36 and 5.41, respectively, seemed to have the overlapped peak if their peaks were displayed in the same window or chromatogram. In this case, the transitions of their molecular to product ion are 402.1 to 358.1 m/z for OTA and 317.1 to 131.0 m/z for ZON. By using different displayed windows to monitor each peak, the peak area of each peak can be individually obtained for quantitative analysis. It can be seen from Figure 4.1 that the individual UHPLC-MS/MS chromatogram provides a satisfactory MS/MS detection of all mycotoxins. The elution order of mycotoxins are as follows: DON, AFG2, AFG1, AFB2, AFB1, FB1, FB2, OTA and ZON. Therefore, the optimum gradient profile was chosen for studying in the following parameters.

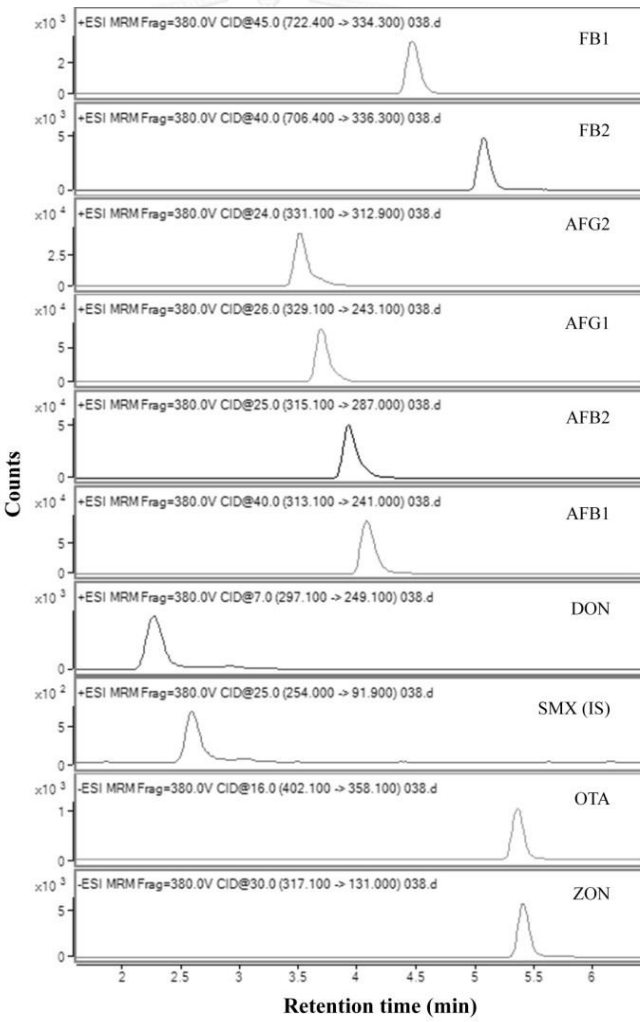


Figure 4.1 UHPLC-MS/MS chromatogram of mycotoxins standard solution.

4.2.1 Type of aqueous mobile phase

From Section 3.5.1 with keeping the mobile phase B as MeOH, nine sets of the aqueous mobile phase A with different amounts of FA and ammonium formate were varied. Results showed that the water containing 0.1% FA and 0-10 mM ammonium formate (A1-A3 in Table 3.3) gave poor tailing peaks of FB1 and FB2, and the water containing 0.1-1% FA without ammonium formate (A1, A4 and A7 in Table 3.3) gave lower sensitivity for detection of AFB1, AFB2, AFG1 and AFG2. In order to achieve symmetric peak shape and high detection sensitivity, the water containing 0.5% FA and 5 mM ammonium formate was chosen for the aqueous mobile phase A.

4.2.2 Solvent for dissolving standard solution or samples

Among six types of solvents used for dissolving standard solutions or samples from Section 3.5.2, results showed that the ACN and MeOH (S1-S2 in Table 3.4) gave the lowest sensitivity for detection of FB1 and FB2, and the solvent containing ACN (S1, S3 and S5 in Table 3.4) gave asymmetric peak shape of DON. In order to achieve symmetric peak shape and high detection sensitivity, the MeOH-0.1% FA in water (50/50) (S6 in Table 3.4) solvent was found to compromisingly provide a good peak shape and highest peak area of mycotoxins.

4.2.3 Column temperature

From Section 3.5.3, the column temperatures were varied at 30, 35, and 40 °C. A slightly increase in the retention time of last analyte, ZON, was found to be 5.4, 5.5 and 5.7 min for 30, 35 and 40 °C, respectively. By evaluating the percentage relative standard deviation (%RSD) in the retention time of each mycotoxin as shown in Table 4.3, the results showed that %RSD of retention time of all mycotoxins at 40 °C were the lowest from other temperature in the study. This indicates that the retention time at 40 °C are more stable than others. Therefore, 40 °C was used for column temperature in the future study.

Table 4.3 %RSD of retention time in different UHPLC column temperature

Analyte	%RSD of retention time		
	30 ºC	35 ºC	40 ºC
AFB1	0.60	0.61	0.24
AFB2	0.49	0.56	0.23
AFG1	0.49	0.59	0.24
AFG2	0.45	0.51	0.24
FB1	0.84	0.86	0.28
FB2	1.05	1.06	0.26
OTA	0.95	0.90	0.23
ZON	0.89	0.89	0.24
DON	0.53	0.47	0.22

4.3 Optimization of sample preparation

4.3.1 Acidity of extraction solvent

ACN without and with acid (FA or HOAc at 0.1, 1, 5 and 10%) was used for extraction solvent as details in Section 3.6.2. The results of recovery (n = 3) of neutral mycotoxins and acidic mycotoxins are shown in Figures 4.2 and 4.3, respectively.

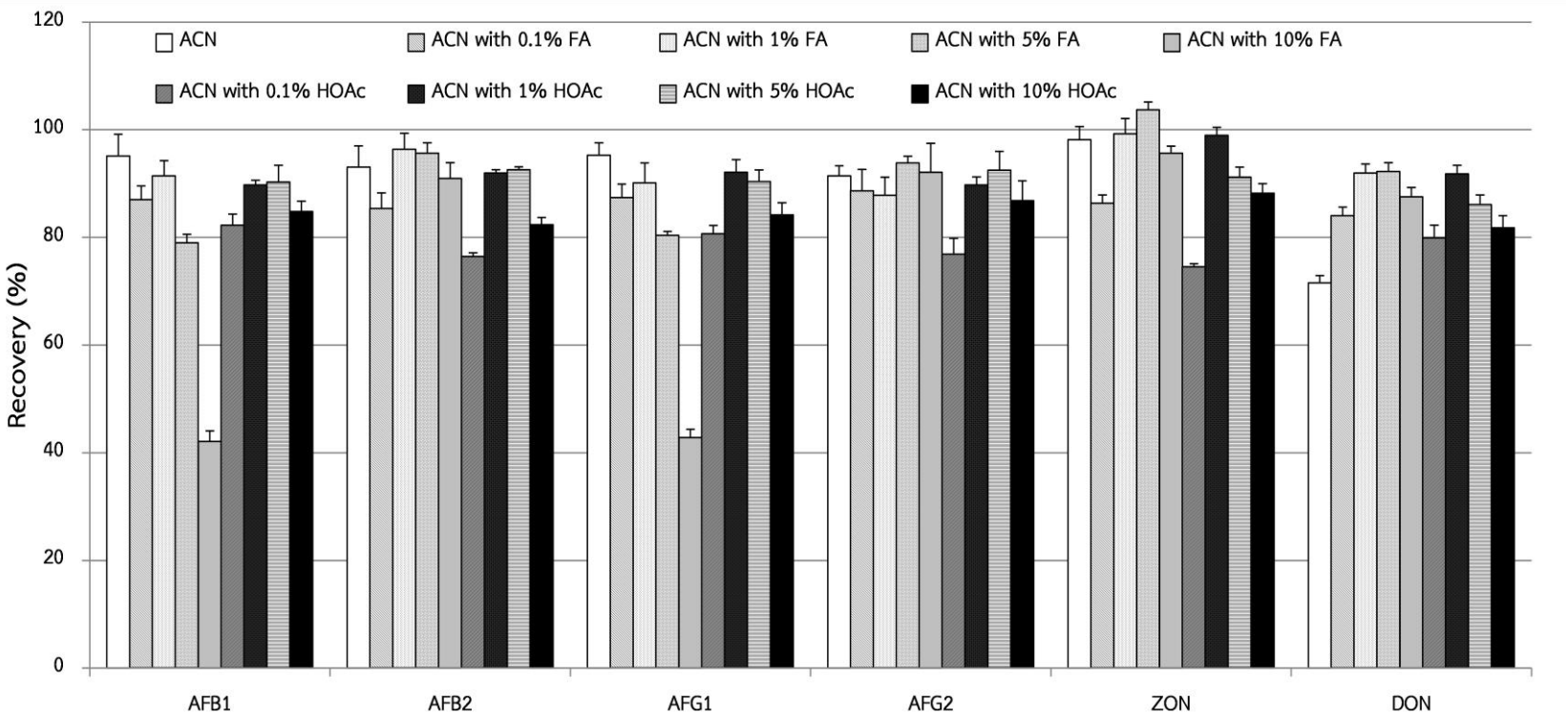


Figure 4.2 Average recovery (*n* = 3) of neutral mycotoxins obtained from the QuEChERS sample preparation using ACN with and without acid for solvent extraction of a blank sample spiked with nine mycotoxins.

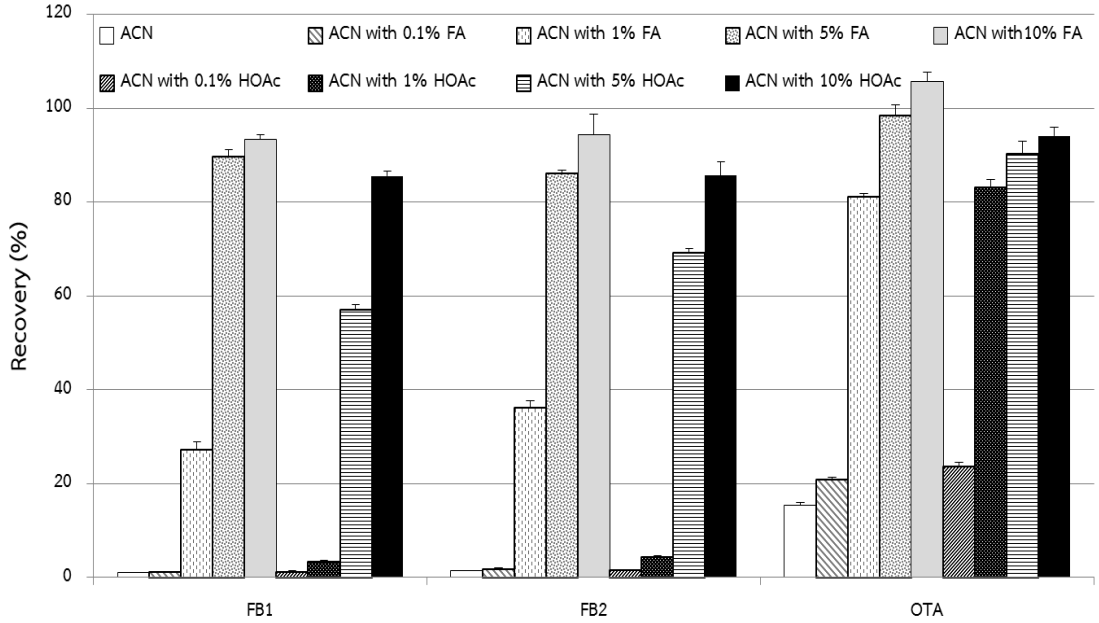


Figure 4.3 Average recovery (*n* = 3) of acidic mycotoxins obtained from the QuEChERS sample preparation using ACN with and without acid for solvent extraction of a blank sample spiked with nine mycotoxins.

As seen in Figure 4.3, an increase of 0 to 10% FA or HOAc in ACN provided better extraction efficiency for all acidic mycotoxins because FA or HOAc promotes a neutral form of acidic mycotoxins into the ACN phase. The average recoveries of all acidic mycotoxins from ACN with 5% FA, 10% FA and 10% HOAc are in acceptable region of 80-110% [68] for analyte concentration of 50-400 µg/kg. While, similar extraction efficiency, with recovery of 77-104 %, for neutral mycotoxins as seen in Figure 4.2 being within the acceptable recovery, except for the recovery of 72 ± 1 % for DON using ACN without acid, 42 ± 2 % for AFB1 and 43 ± 2 for AFG1 using ACN with 10 % FA and 76 ± 1 % for AFB2 and 75 ± 1 % for ZON using ACN with 0.1% HOAc. However, ACN with 10 % FA gave poorer recoveries for AFB1 and AFG1 (42-43 %) than ACN with 5% FA (79-80 %) and 10% HOAc (84-85 %). The individual experiments for AFB1 and AFG1 using ACN with 5% FA, 10% FA and 10% HOAc were repeated three times on different days, and the same trends were obtained. It was observed that the use of ACN with 10% FA resulted in darker brown ACN extract

from brown rice, implying that increased pigment matrices reduce the extraction efficiency or may interfere with the detection of AFB1 and AFG1. Therefore, ACN with 10% HOAc, giving recoveries within 82-94% for all mycotoxins, was chosen as extraction solvent in this work.

4.3.2 $MgSO_4$, NaCl and buffering salts

$MgSO_4$ and NaCl salts, commonly with a ratio of 4:1, are widely used to induce phase separation in the QuEChERS solvent during the extraction step [8-11, 29, 30, 35, 36]. To maintain an aqueous buffer solution, additional salts may be included with these two salts, such as sodium citrate tribasic dehydrate and sodium citrate dibasic sesquihydrate for a citrate buffer, according to the Committee of European Normalization (European EN 15662 Method) and other previous works [29, 30, 34, 36]; or sodium acetate for an acetate buffer, according to standard methods from the AOAC International (AOAC Official Method 2007.01) and other previous works [13, 31]. In this work as detailed in Section 3.6.3, anh. $MgSO_4$ and NaCl (4:1 by weight) with and without buffer (citrate or acetate) were compared for QuEChERS extraction. The results of recovery (n = 3) of nine mycotoxins are shown in Figure 4.4, in most case, the highest recovery is obtained in the order acetate buffer > citrate buffer > no buffer, except for FB1, FB2 and OTA where the order of highest recovery was citrate buffer > acetate buffer; in particular, acetate buffer yielded only 49 ± 1% for FB1 and 75 ± 1% for FB2. Therefore, citrate buffer, giving recoveries within 82-94% for all mycotoxins, was chosen in this work.

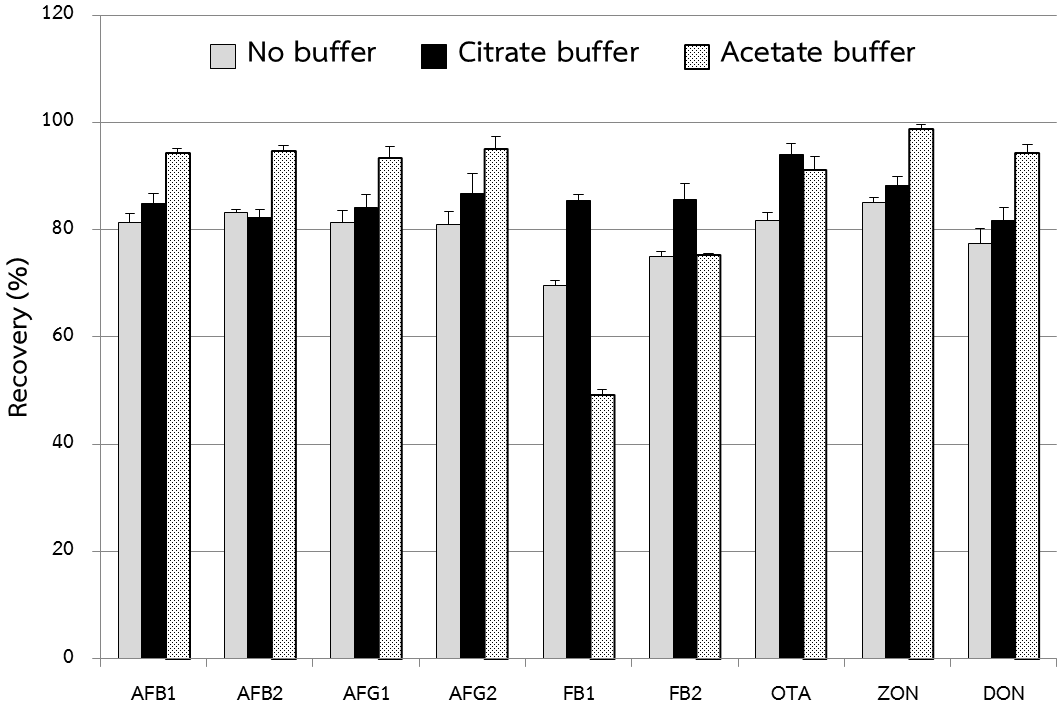


Figure 4.4 Average recovery (*n* = 3) of nine mycotoxins obtained from the QuEChERS sample preparation using with and without buffer for QuEChERS extraction of a blank sample spiked with nine mycotoxins.

In addition, the weight ratio (g) of anh. $MgSO_4$:NaCl in citrate buffer was varied as 0.50:0.125, 1.0:0.25 and 4.0:1.0, as well as 2.0:0.50. The results of recovery (*n* = 3) of nine mycotoxins are shown in Figure 4.5. By setting acceptable recoveries in a range of 80-110%, only one (DON), three (DON, FB1 and ZON), nine (all mycotoxins) and seven (all except FB1 and FB2) out of nine mycotoxins were found to be within an acceptable range for anh. $MgSO_4$:NaCl ratios of 0.50:0.125, 1.0:0.25, 2.0:0.5 and 4.0:1.0, respectively. Therefore, the salts mixture containing anh. $MgSO_4$:NaCl (2.0:0.5) were chosen in this work, providing recoveries of 82-94% for all mycotoxins.

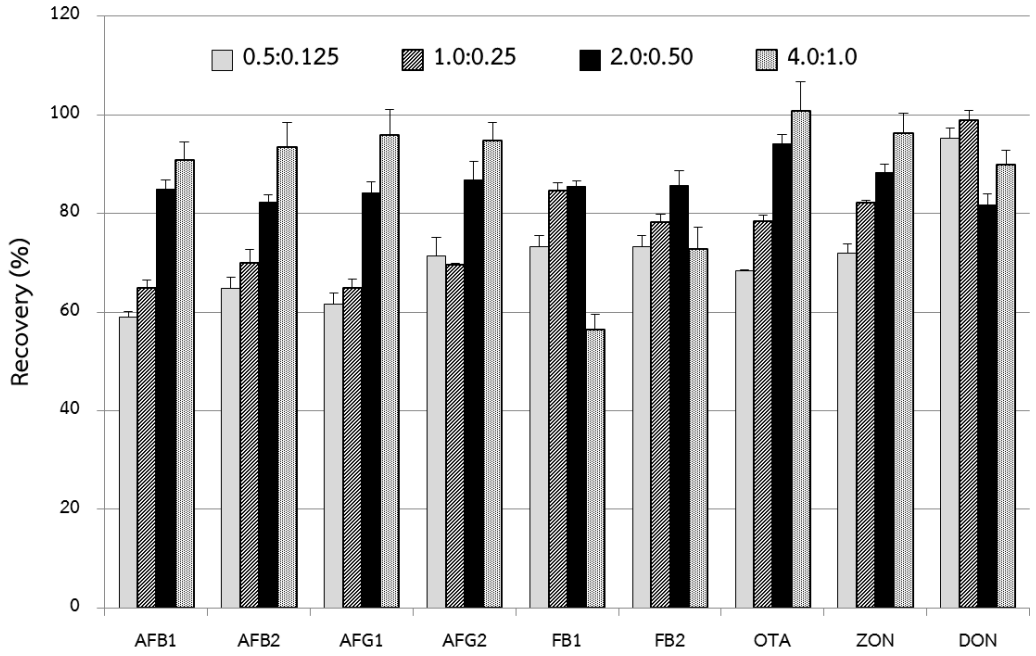


Figure 4.5 Average recovery (*n* = 3) of nine mycotoxins obtained from the QuEChERS sample preparation using different weight ratio (g) of anh. $MgSO_4$ and NaCl for QuEChERS extraction of a blank sample spiked with nine mycotoxins.

4.3.3 The volume ratio of water and extraction solvent

A volume ratio of water and extraction solvent were varied as QuEChERS extraction from Section 3.6.4. The results of recovery (*n* = 3) of nine mycotoxins are shown in Figure 4.6. The average recoveries of AFB1, AFB2, AFG1, AFG2 and DON, using a volume ratio (mL) of added water and extraction solvent, 5:10, 10:5 and 10:10, are not in acceptable region 80-110 %, except for the recovery of 89 ± 5 % for DON using water and extraction solvent ratio of 10:5. While, the average recoveries of rest mycotoxins are within acceptable region. Therefore, water and extraction solvent ratio of 5:5, giving recoveries within 82-94% for all mycotoxins, was chosen in this experiment.

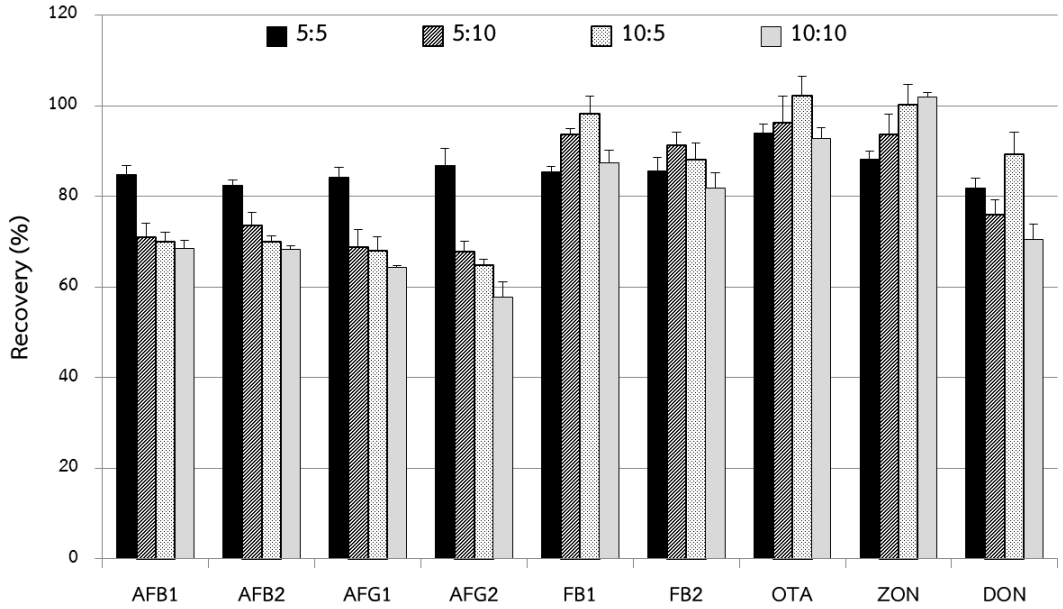


Figure 4.6 Average recovery (*n* = 3) of nine mycotoxins obtained from the QuEChERS sample preparation using different volume ratio (mL) of water and extraction solvent for QuEChERS extraction of a blank sample spiked with nine mycotoxins.

4.3.4 Sorbent types

4.3.4.1 Single sorbent

In order to remove the matrices such as pigments and other component, d-SPE was performed varying different types of sorbent as detailed in Section 3.6.5.1. The results of recovery (*n* = 3) of nine mycotoxins are shown in Figure 4.7, d-SPE clean-up procedures with C18 sorbent and without any sorbent were found to provide recoveries in a range of 78-99% for all mycotoxins, but three (FB1, FB2 and OTA), three (AFB1, AFB2 and OTA) and two (AFB1 and AFG1) out of nine mycotoxins were recovered at less than 80% for PSA, GCB and silica, respectively; in particular, values of 27 ± 3% for FB1, 30 ± 2% for FB2 and 69 ± 2% for OTA were found for PSA sorbent, and 59 ± 2% was found for OTA with GCB sorbent. However, it was noticed that, after the d-SPE clean-up, the C18 sorbent and

no sorbent gave a darker brown extract than did the other three sorbents, implying the higher amount of pigment remaining in the extract, while PSA and GCB produced clearer extract than did other sorbents. It should be noted that the amino group from PSA can bind with the carboxylic acid group from FB1, FB2 and OTA leading to reduce the recovery of their analytes in an extract solution. In order to compromise between acceptable recovery for all nine mycotoxins and high removal of matrix interferences, therefore, mixed sorbents were performed in the following study.

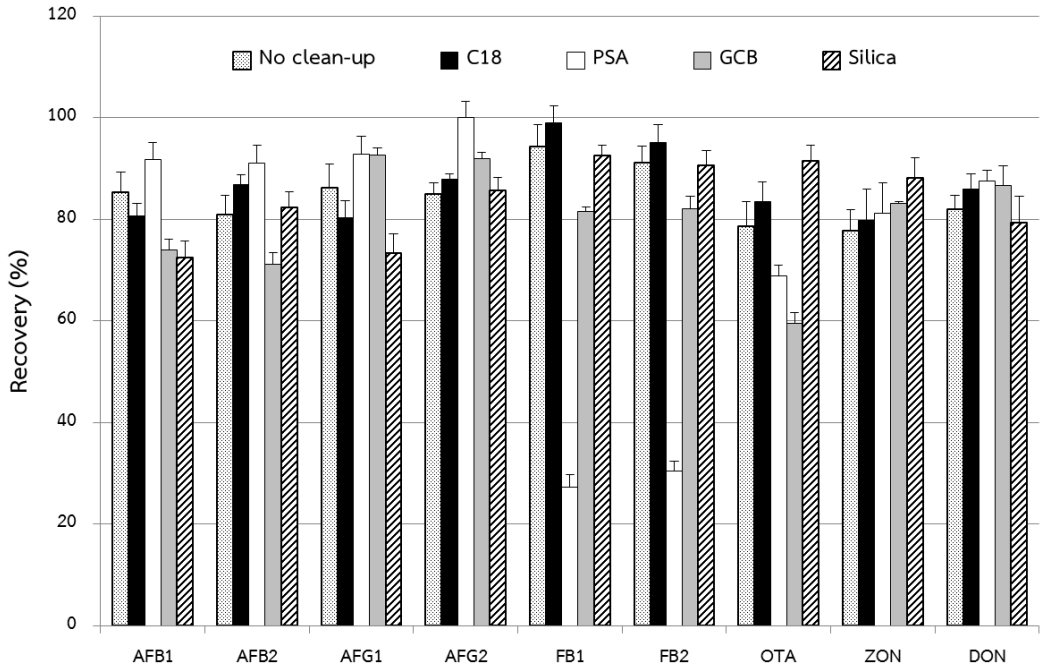


Figure 4.7 Average recovery (*n* = 3) of nine mycotoxins obtained from the QuEChERS sample preparation using 50 mg four different single sorbents along with 300 mg of anh. $MgSO_4$ for d-SPE clean-up for QuEChERS extraction of a blank sample spiked with nine mycotoxins.

4.3.4.2 Mixed sorbent

In order to additionally remove pigment matrices, total 100 mg of mixed sorbents, C18:other sorbents, were varied for the d-SPE clean-up step from Section 3.6.5.2. The results of recovery (n = 3) of nine mycotoxins are shown in Figure 4.8. Mixed sorbent containing C18:PSA:silica were found to provide recoveries within an acceptable range, while, three (FB1, FB2 and OTA), three (AFB1, AFB2 and OTA), three (AFB1, AFG1 and ZON), four (FB1, FB2, OTA and ZON) and six (AFB1, AFB2, AFG1, FB1, OTA and ZON) out of nine mycotoxins were found to be less than 80% for mixed sorbents containing C18:PSA, C18:GCB, C18:silica, C18:PSA:GCB and C18:GCB:silica, respectively. Therefore, total 100 mg of mixed sorbent consisting of C18:PSA:silica was used for the d-SPE step.

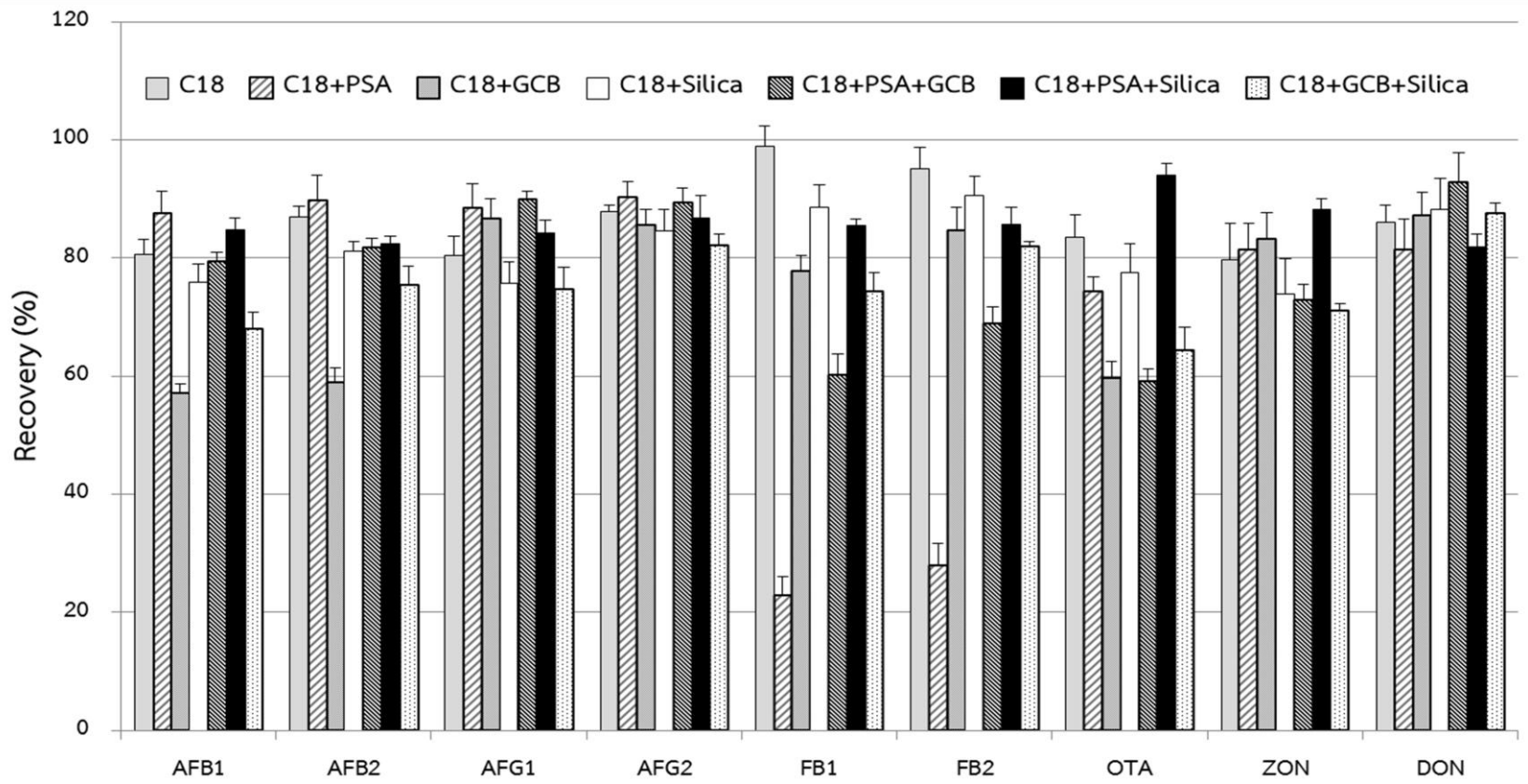


Figure 4.8 Average recovery (n = 3) of nine mycotoxins obtained from the QuEChERS sample preparation using 50 mg four different mixed sorbents along with 300 mg of anh. $MgSO_4$ for d-SPE clean-up for QuEChERS extraction of a blank sample spiked with nine mycotoxins.

4.4 Method validation

4.4.1 Analytical limits

The old definition of LOD is based on the analyte concentration giving a signal-to-noise ratio of 3.0 or may be estimated from 3 times the standard deviation in the known concentration of analyte spiked in blank samples. This LOD definition is now abandoned by IUPAC due to an underestimation [69, 70]. According to the modern IUPAC recommendation [67], LOD is defined as the minimum analyte concentration that can be discriminated from the blank, controlling the risks of false positives and false negative, and therefore, may be expressed by Equation 4.1.

$$\text{LOD} = \frac{3.3\, S_{x/y}}{A} \sqrt{1 + h_0 + \frac{1}{I}} \qquad \textbf{(4.1)}$$

where A is the slope of a linear plot between the signal against the analyte concentration, $S_{y/x}$ is the residual standard deviation, I is the number of calibration samples, and h_0 is the leverage for the blank sample as described in Equation 4.2.

$$h_0 = \frac{\bar{c}_{cal}^{2}}{\sum_{i=1}^{I} (c_i - \bar{c}_{cal})^2} \qquad \textbf{(4.2)}$$

where $\bar{c}_{cal}$ is the mean calibration concentration, and c_i is each of the calibration concentration

Similar concepts also apply to LOQ with the factor of 10 instead of 3.3 in Equation 4.1, to ensure a maximum relative prediction uncertainty of 10 as given in Equation 4.3.

$$\text{LOQ} = \frac{10\, S_{x/y}}{A} \sqrt{1 + h_0 + \frac{1}{I}} \qquad \textbf{(4.3)}$$

Table 4.4 shows results of calibration plot data obtained from four triplicate concentration levels of each analyte spiked in the final solvent (*I* of 12 in Equation 4.1). It should be noted that MDL and MQL are estimated from LOD and LOQ, respectively with the QuEChERS sample preparation for 1 g of sample and 5 mL of the final extraction solvent. It can be concluded that this method allows for the determination of trace levels of mycotoxins with the MDL and MQL value in ranges of 1.4-25 and 4.1-75 µg/kg, respectively, below the maximum limit of EU regulation except for OTA with MQL of 7.5 µg/kg slightly higher than ML of 5.0 µg/kg. In the case that the LOD values were estimated from 3 times the standard deviation in the known concentration of analyte spiked in blank samples, MDL values were obtained to be 0.10 µg/kg for AFB1, AFB2, AFG1 and AFG2, 0.40 µg/kg for FB1, 0.3 µg/kg for FB2, 0.60 µg/kg for OTA, 0.25 µg/kg for ZON, and 9.0 µg/kg for DON. The old MDL definition significantly underestimates 4-20 times the modern MDL definition.

Table 4.4 Analytical limits obtained from internal standard calibration results: LOD, LOQ, MDL and MQL

Analyte	Conc. range (µg/L)	Calibration plot					MDL (µg/kg)	MQL (µg/kg)	ML (µg/kg)
		Slope	$S_{y/x}$	h_0	LOD (µg/L)	LOQ (µg/L)			
AFB1	0.06 – 2.5	5.82 ± 0.14	0.43	0.102	0.27	0.82	1.4	4.1	5.0 for AFB1 and totally 10.0
AFB2	0.05 – 2.5	3.18 ± 0.09	0.27	0.100	0.31	0.94	1.6	4.7	
AFG1	0.04 – 2.5	4.78 ± 0.14	0.44	0.099	0.33	1.0	1.7	5.0	
AFG2	0.06 – 2.5	2.72 ± 0.09	0.29	0.102	0.39	1.2	2.0	6.0	
FB1	0.25 – 5.0	0.858 ± 0.018	0.11	0.109	0.48	1.5	2.4	7.5	Totally 2,000
FB2	0.20 – 5.0	0.844 ± 0.020	0.12	0.106	0.54	1.7	2.7	8.5	
OTA	0.35 – 5.0	0.293 ± 0.006	0.038	0.114	0.47	1.5	2.4	7.5	5.0
ZON	0.15 – 5.0	0.335 ± 0.012	0.079	0.103	0.85	2.6	4.3	13	100
DON	6.0 – 50	0.0372 ± 0.0009	0.051	0.112	5.0	15	25	75	1,250

MDL LOD and LOQ were calculated using Eq. 4.1 and 4.3, respectively, with four triplicate concentration levels for each analyte (*I* of 12).

MDL and MQL were estimated from LOD and LOQ by using 1 g of sample with 5 mL of the final extraction solvent (dilution factor of 5).

4.4.2 Standard calibration curve: linearity and matrix effect

A standard calibration curve is typically obtained from the relationship between the response and the analyte concentration with multi-replicates. The established calibration plot should be included with all replicate data, not the average response for each concentration. As stated in Ref. [71] the r^2 value is a measure of the degree of linear association between concentration and noise, and therefore, the acceptable linearity is suggested to be evaluated using the statistical *F*-test as an indicator, a comparison of F_{exp} less than the critical $F_{(\alpha,I-2,I-L)}$ at particular confidence level where F_{exp} is the ratio of the residual variance ($S^2_{y/x}$) to the squared pure error (S^2_y) as Equations 4.4-4.6.

$$F_{exp} = \frac{S^2_{y/x}}{S^2_y} \quad \textbf{(4.4)}$$

$$S_{y/x} = \sqrt{\frac{\sum_{i=1}^{I} (y_i - \hat{y}_i)^2}{I-2}} \quad \textbf{(4.5)}$$

$$S_y = \sqrt{\frac{\sum_{l=1}^{L} \sum_{q=1}^{Q} (y_{lq} - \bar{y}_l)^2}{I-Q}} \quad \textbf{(4.6)}$$

where α is the probability of rejecting the null hypothesis when it is true, $S_{y/x}$ is the residual standard deviation, S_y is the pure error (a measure of the instrumental noise), y_i and $\hat{y}_i$ are experimental and estimated response values for sample *i*, y_{lq} is the calibration response for replicate *q* at level *l*, $\bar{y}_l$ is the mean response at level *l*, and *I*, *L* and *Q* are the total number of calibration samples, levels and replicates at each level, respectively.

In comparison of two calibration methods, the difference in slope A_1 and A_2 should be indicated using a difference in the slope value (%Δ) or the *t*-test as the following the equation.

$$\%\Delta = 100 \times (A_1\text{-}A_2)/A_2 \quad \textbf{(4.7)}$$

$$t_{exp} = \frac{\left|A_1\text{-}A_2\right|}{\sqrt{S_p^2\left[\frac{1}{\sum_{n_1=1}^{N_1}(c_{n1}-\bar{c}_1)^2}+\frac{1}{\sum_{n_2=1}^{N_2}(c_{n2}-\bar{c}_2)^2}\right]}} \quad \textbf{(4.8)}$$

$$s_p^2 = \frac{S_{y/x1}^2(N_1\text{-}2)+S_{y/x2}^2(N_2\text{-}2)}{N_1+N_2\text{-}4} \quad \textbf{(4.9)}$$

where A_1 is a slope of standard calibration curve, A_2 is a slope of matrix-matched calibration curve, t_{exp} is an experimental *t* value, S_p^2 is the pooled variance, N_1 and N_2 are the number of concentration values used to estimate each slope, c_{n1} and c_{n2} are each concentration value, $\bar{C}_1$ and $\bar{C}_2$ are average concentrations.

In order to investigate matrix effects in this work, which may interfere by increasing or decreasing the detection signal of analytes, two curves for the internal standard calibration and matrix-matched internal standard calibration were compared using five triplicate concentration levels of the mycotoxin standard (N_1 of 5 and N_2 of 5). The linear regression plots of relative response against concentration of each analyte, with the correlation coefficient $r^2 > 0.996$ for all analytes, are shown in Appendix B (Figure B.1-B.18), and estimated calibration data are summarized in Tables 4.5-4.7. It should be noted from Equations 4.5-4.6 that *I*, *L* and *Q* are equal to 15, 5 and 10 for five triplicate concentration levels in this work.

From Tables 4.5 and 4.6, using the *F*-test at 95% confidence level, the F_{exp} value less than the critical $F_{(0.05,13,10)}$ value of 2.9 indicates acceptable linearity of both an internal standard calibration curve and matrix-matched internal standard

calibration curve of each analyte. However, a difference in slope is observed by visual inspection.

It can be seen from Table 4.7 that a difference in the slope value (%Δ) are found to be out of the acceptable criteria within ±20% [11, 31] except for ZON with %Δ of 1.6. In addition, using the *t*-test with one-tail *t*-coefficient at 95% confidence level with 6 degree of freedom (N_1+N_2-4) for statistical comparison of two analytical methods, the higher t_{exp} for almost the analyte than critical *t* value of 2.4, except for ZON, indicate significant difference in the slope of two calibration methods. Results of both %Δ and *t*-test in this work imply that quantitative analysis should be performed by using the matrix-matched calibration method.

Table 4.5 Calibration parameters of standard calibration curve

Analyte	Conc. range (µg/L)	Calibration plot					
		Slope	Intercept	r^2	$S^2_{y/x}$	S^2_y	F_{exp}
AFB1	1.0 – 10	12.10 ± 0.18	-2.4 ± 1.1	0.9971	5.2	3.7	1.4
AFB2	1.0 – 10	7.19 ± 0.09	-0.8 ± 0.6	0.9978	1.4	1.9	0.74
AFG1	1.0 – 10	10.56 ± 0.16	-2.0 ± 1.0	0.9969	4.3	1.7	2.5
AFG2	1.2 – 10	6.06 ± 0.07	-1.4 ± 0.4	0.9982	0.80	0.51	1.6
FB1	2.0 – 20	0.224 ± 0.003	-0.12 ± 0.04	0.9974	0.0065	0.011	0.59
FB2	2.0 – 20	0.300 ± 0.004	-0.16 ± 0.05	0.9979	0.0091	0.011	0.83
OTA	2.0 – 20	0.0593 ± 0.0008	-0.010 ± 0.009	0.9979	0.00037	0.00069	0.54
ZON	2.6 – 20	0.323 ± 0.005	-0.06 ± 0.06	0.9972	0.014	0.021	0.67
DON	15 – 200	0.0744 ± 0.0008	0.46 ± 0.09	0.9986	0.040	0.088	0.45

Critical $F_{(0.05,13,10)}$ value of 2.9 with five triplicate concentration levels

Table 4.6 Slope, intercept and correlation coefficient (r^2) of matrix-matched calibration curve

Analyte	Conc. range (µg/L)	Calibration plot					
		Slope	Intercept	r^2	$S^2_{y/x}$	S^2_y	F_{exp}
AFB1	1.0 – 10	5.51 ± 0.09	0.2 ± 0.5	0.9968	1.2	2.9	0.41
AFB2	1.0 – 10	2.95 ± 0.04	1.0 ± 0.3	0.9971	0.31	0.63	0.49
AFG1	1.0 – 10	4.63 ± 0.08	0.4 ± 0.5	0.9962	1.0	2.3	0.43
AFG2	1.2 – 10	2.90 ± 0.04	0.1 ± 0.3	0.9975	0.25	0.58	0.43
FB1	2.0 – 20	0.900 ± 0.013	-0.10 ± 0.16	0.9972	0.11	0.25	0.44
FB2	2.0 – 20	0.885 ± 0.009	-0.27 ± 0.11	0.9987	0.050	0.11	0.45
OTA	2.0 – 20	0.296 ± 0.004	-0.09 ± 0.05	0.9974	0.011	0.010	1.1
ZON	2.6 – 20	0.318 ± 0.005	-0.02 ± 0.07	0.9962	0.018	0.021	0.86
DON	15 – 200	0.0367 ± 0.0006	-0.15 ± 0.07	0.9970	0.021	0.028	0.75

Critical $F_{(0.05,13,10)}$ value of 2.9 with five triplicate concentration levels

Table 4.7 Summary of matrix effect evaluation

Analyte	A_1	A_2	S^2_p	t_{exp}	%Δ	Statistic difference in slope
AFB1	12.1	5.51	3.2	36	119	Significant
AFB2	7.19	2.95	0.84	45	144	Significant
AFG1	10.6	4.63	2.6	35	128	Significant
AFG2	6.06	2.90	0.53	42	109	Significant
FB1	0.224	0.900	0.060	54	-75	Significant
FB2	0.300	0.885	0.030	66	-66	Significant
OTA	0.0593	0.296	0.0058	60	-80	Significant
ZON	0.323	0.318	0.016	0.77	1.6	Non-significant
DON	0.0744	0.0367	0.030	42	58	Significant

4.4.3 Accuracy and precision

As detailed in Section 3.7.5, a blank brown rice sample was spiked with mycotoxin standards at three concentration levels and ten batches each. After the QuEChERS extraction, the amount of mycotoxins in the spiked samples was determined by UHPLC-MS/MS using the matrix-matched calibration curves, in order to evaluate accuracy and precision in the determined amount, where the accuracy is expressed by the recovery and precision is expressed by %RSD of the recovery.

For each day analysis of each analyte, average recoveries were obtained with satisfactory accuracy, that is, with values in the range of 81-101% for 5.0-1,000 µg/kg; furthermore, all of the recovery data fell within the criteria for acceptable recovery [68] for the respective analyte concentration as shown in Table 4.8.

The recovery RSD value for intraday precision was obtained using ten batches from the QuEChERS sample preparation method, while that for interday precision was evaluated on three different days. For intraday precision, the acceptance limitations for the relative standard deviation (RSD_r) were calculated from modified Horwitz equation: $\%RSD_r < 0.66 \times 2^{(1-0.5 \log C)}$, where C is the analyte concentration in fraction units (analyte/sample, g/g). For interday precision, the acceptance values were decided by an analysis of variance (ANOVA): single factor analysis at 95% confidence level as shown in Table 4.8. Satisfactory RSD values for intraday precision were obtained, falling in the range of 5-19 for the analyte concentration of 5.0-1,000 µg/kg; all of the RSD data fell within an acceptable range ($\%RSD_r$), with values of 11-23. For interday precision, non-significant difference is obtained for all analyte by the *P*-value more than 0.05 at 95% confidence level.

Table 4.8 Accuracy and precision in the QuEChERS extraction recovery of mycotoxins spiked in blank sample at three level (*n* = 10)

Analyte	Conc. (µg/kg)	% Recovery (% RSD)				Overall	
		Acceptable criteria	Day 1	Day2	Day3	*P*-value for RSD	% Recovery (% RSD)
AFB1	5.0	60-115 (23)	92 (18)	83 (20)	89 (19)	0.45	88 (19)
	25	80-110 (18)	91 (10)	91 (12)	94 (9)	0.63	92 (10)
	50	80-110 (17)	87 (9)	83 (6)	91 (8)	0.086	87 (9)
AFB2	5.0	60-115 (23)	90 (17)	83 (17)	95 (11)	0.15	89 (16)
	25	80-110 (18)	91 (10)	92 (9)	97 (8)	0.25	93 (9)
	50	80-110 (17)	84 (10)	83 (7)	87 (8)	0.34	85 (9)
AFG1	5.0	60-115 (23)	81 (16)	95 (18)	89 (18)	0.15	88 (18)
	25	80-110 (18)	89 (9)	90 (11)	91 (9)	0.87	90 (9)
	50	80-110 (17)	82 (10)	83 (9)	88 (8)	0.16	84 (9)
AFG2	6.0	60-115 (23)	86 (19)	89 (19)	91 (14)	0.74	89 (17)
	25	80-110 (18)	88 (7)	94 (9)	87 (10)	0.15	90 (9)
	50	80-110 (17)	84 (8)	83 (8)	85 (9)	0.86	84 (8)
FB1	10	60-115 (21)	95 (13)	98 (16)	97 (14)	0.88	97 (13)
	50	80-110 (17)	91 (7)	91 (12)	93 (7)	0.68	92 (9)
	100	80-110 (15)	90 (6)	90 (5)	93 (7)	0.35	91 (6)
FB2	10	60-115 (21)	86 (18)	88 (18)	86 (18)	0.93	87 (17)
	50	80-110 (17)	89 (9)	90 (11)	90 (8)	0.93	89 (9)
	100	80-110 (15)	88 (7)	89 (6)	92 (7)	0.30	90 (7)
OTA	10	60-115 (21)	86 (17)	83 (13)	85 (16)	0.89	84 (15)
	50	80-110 (17)	93 (9)	92 (11)	91 (8)	0.95	92 (9)
	100	80-110 (15)	87 (7)	86 (7)	90 (9)	0.41	88 (8)
ZON	13	80-110 (20)	94 (12)	95 (12)	91 (4)	0.67	93 (13)
	50	80-110 (17)	97 (8)	98 (10)	96 (9)	0.86	97 (9)
	100	80-110 (15)	100 (4)	101 (4)	96 (9)	0.20	99 (6)
DON	75	80-110 (16)	85 (11)	85 (9)	86 (12)	0.90	85 (10)
	500	80-110 (12)	88 (8)	92 (8)	91 (7)	0.57	90 (8)
	1,000	80-110 (11)	86 (5)	88 (4)	89 (5)	0.22	88 (5)

P-value > 0.05 = non-significant difference

4.5 Application to real samples

The optimum QuEChERS method for black brown rice was used for UHPLC-MS/MS determination of mycotoxins in red brown rice and brown brown rice as detailed in Section 3.8. The recoveries of mycotoxins extracted from blank sample of red brown rice and brown brown rice are shown in Table 4.9.

Table 4.9 Average recovery of nine mycotoxins from red brown rice and brown brown rice (n = 2)

Analyte	Conc. (µg/kg)	Average recovery ± SD	
		Red brown rice	Brown brown rice
AFB1	25	90 ± 3	92 ± 2
AFB2	25	90 ± 2	95 ± 1
AFG1	25	91 ± 1	93 ± 1
AFG2	25	98 ± 1	94 ± 1
FB1	50	82 ± 1	91 ± 1
FB2	50	81 ± 1	97 ± 1
OTA	50	87 ± 2	84 ± 2
ZON	50	94 ± 2	85 ± 2
DON	500	83 ± 1	90 ± 2

From Table 4.9, all data of average recovery from red brown rice and brown brown rice lie in a range of 81-98% with being in acceptable region of 80-110% for analyte at the concentration of 25-500 µg/kg [68], indicating that this method can be applied to another two types of brown rice, red brown rice and brown brown rice. Then, this method was used for determination of mycotoxins in fourteen brown rice samples including three black brown rice (Black1-3), six red brown rice (Red1-6) and five brown brown rice (Brown1-5), obtained from various supermarkets in Bangkok. The results are shown in Table 4.10.

Table 4.10 Mycotoxins contamination in brown rice sample

Sample Code	Range of contamination (µg/kg)								
	AFB1	AFB2	AFG1	AFG2	FB1	FB2	DON	OTA	ZON
Black1	nd	nd	nd	nd	5.41±0.02[a]	4.33±0.04[a]	nd	nd	6.25±0.11[a]
Black2	nd	nd	nd	nd	nd	nd	nd	nd	nd
Black3	nd	nd	nd	nd	nd	nd	nd	nd	nd
Red1	nd	nd	nd	nd	nd	nd	nd	nd	nd
Red2	nd	nd	nd	nd	nd	nd	nd	nd	nd
Red3	nd	nd	nd	nd	nd	nd	nd	nd	14.88±0.20
Red4	nd	nd	nd	nd	3.51±0.04[a]	nd	nd	nd	nd
Red5	nd	nd	nd	nd	nd	nd	nd	nd	nd
Red6	nd	nd	nd	nd	nd	nd	nd	nd	nd
Brown1	nd	nd	nd	nd	2.64±0.04[a]	nd	nd	nd	nd
Brown2	nd	nd	nd	nd	nd	nd	nd	nd	6.10±0.14[a]
Brown3	nd	nd	nd	nd	nd	nd	nd	nd	nd
Brown4	nd	nd	nd	nd	2.49±0.05[a]	nd	nd	nd	nd
Brown5	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd refers to "not detected." [a]The determined amount is between MDL and MQL.

Six out of fourteen samples were found to be contaminated with at least one mycotoxin, as shown in Table 4.10. FB1, FB2 and ZON were detected at 2.49-5.41 µg/kg (four samples), 4.33±0.04 µg/kg (one sample) and 6.10-14.88 µg/kg (three samples) being lower than the maximum limit established by EU regulations (sum of FB1 and FB2 = 2,000 µg/kg and ZON = 100 µg/kg). Some UHPLC-MS/MS chromatograms of the determination of mycotoxins in brown rice are shown in Figures 4.9-4.12.

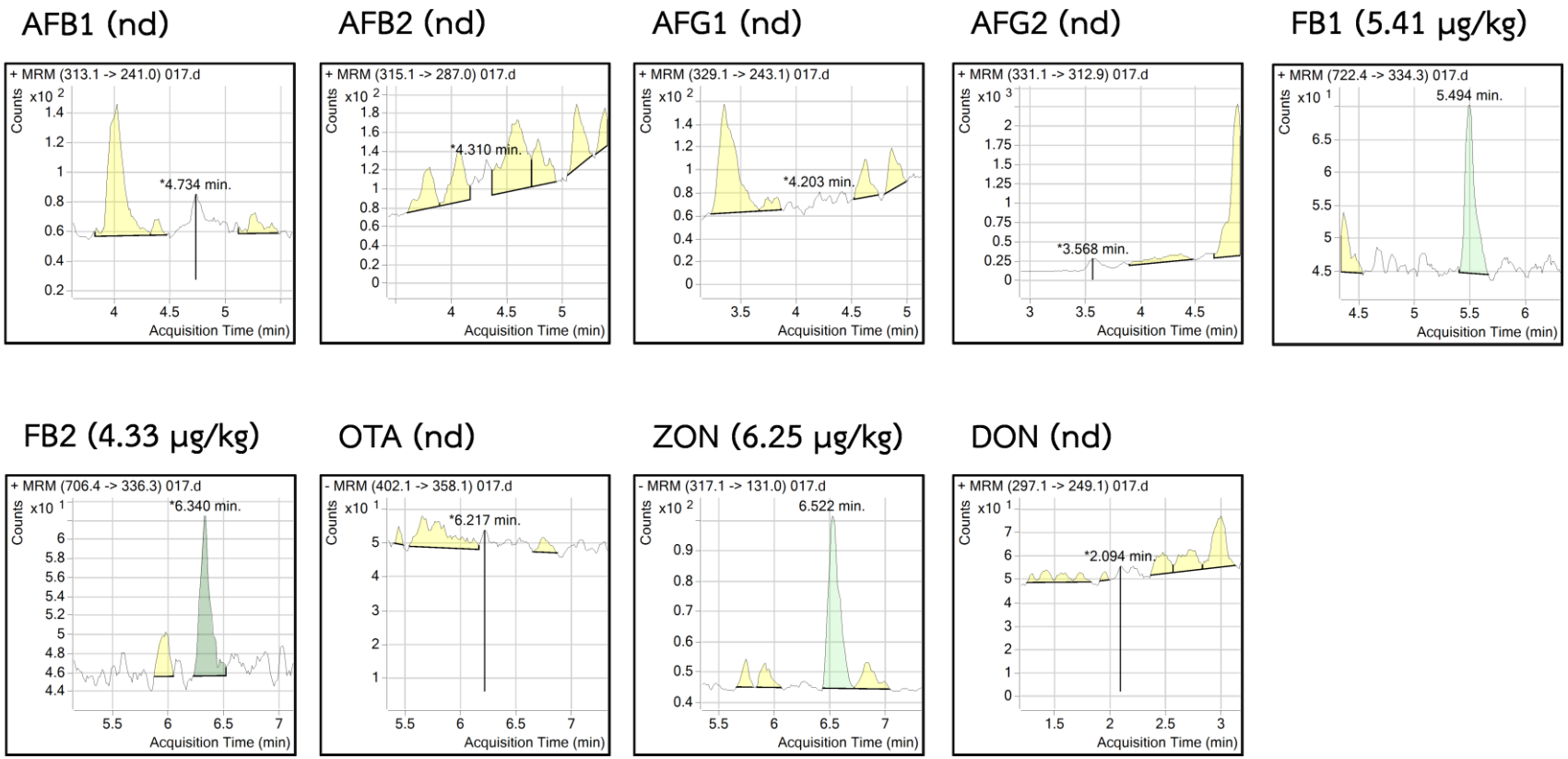


Figure 4.9 UHPLC-MS/MS chromatograms of mycotoxins obtained from black brown rice sample analysis (Code: Black 1).

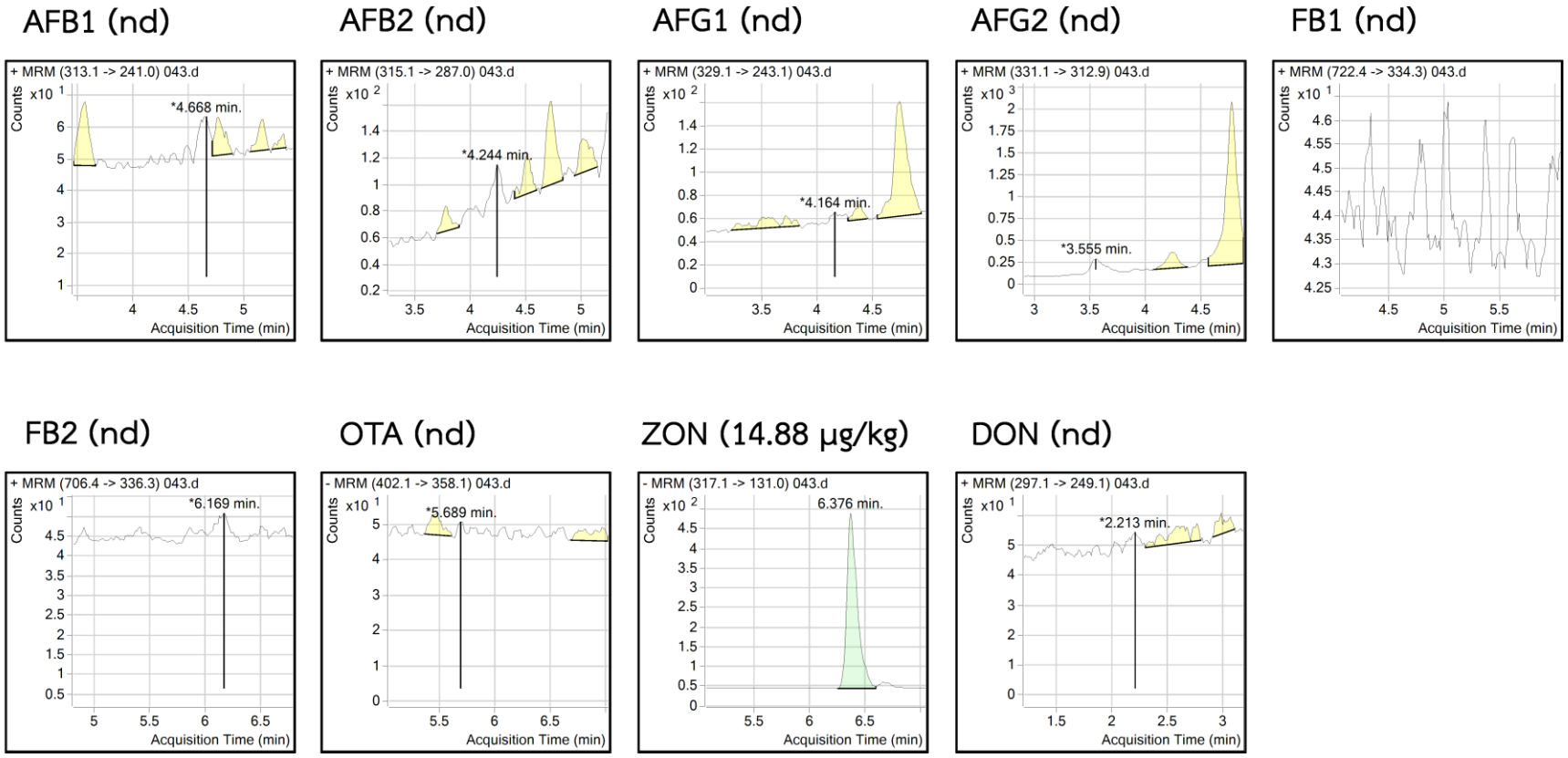


Figure 4.10 UHPLC-MS/MS chromatograms of mycotoxins obtained from red brown rice sample analysis (Code: Red 3).

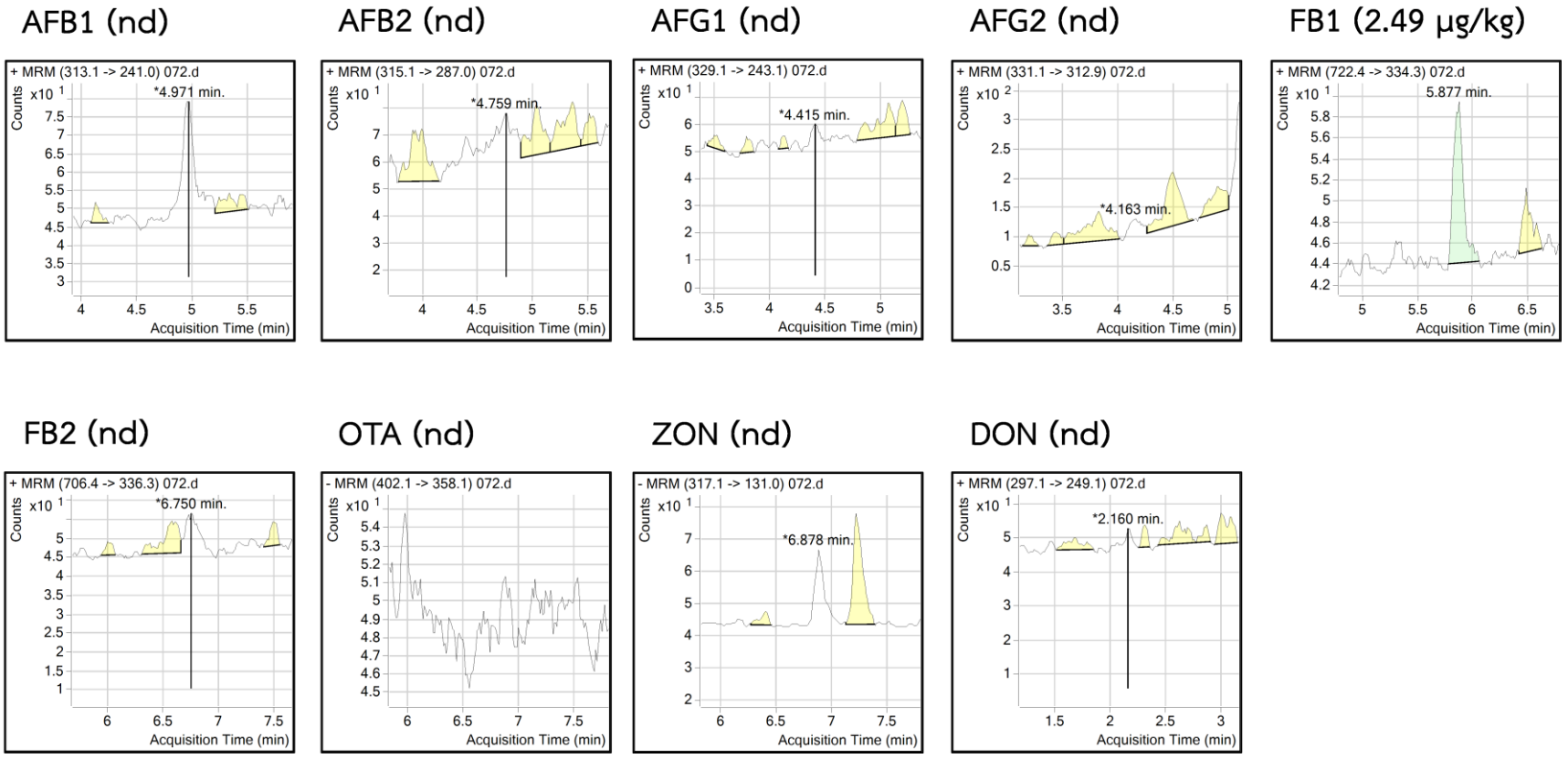


Figure 4.11 UHPLC-MS/MS chromatograms of mycotoxins obtained from brown brown rice sample analysis (Code: Brown 4).

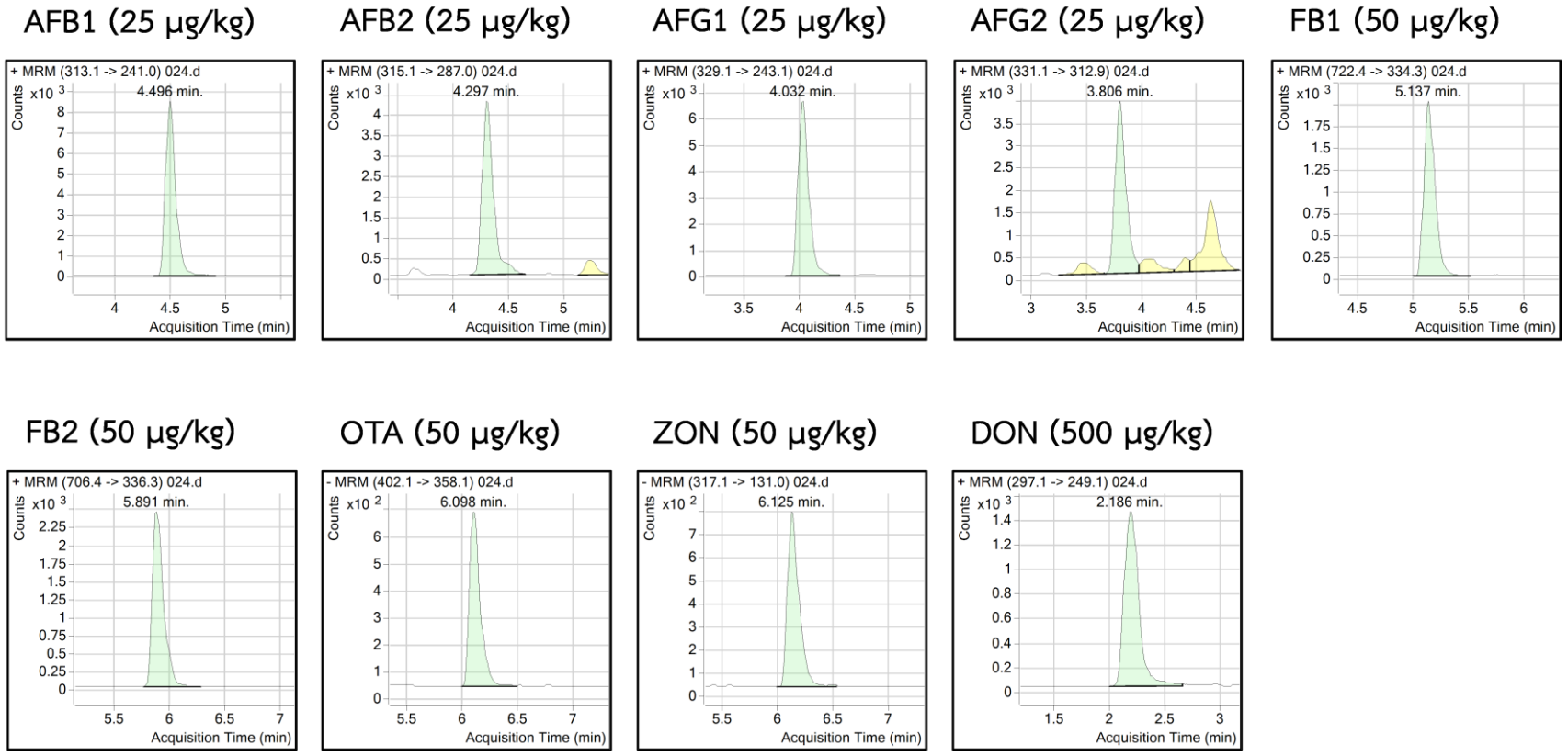


Figure 4.12 UHPLC-MS/MS chromatograms of mycotoxins spiked black brown rice sample analysis.

4.6 A comparison of this and previous works

Tables 4.11 and 4.12 show a comparison of suitable QuEChERS conditions and MDL for UHPLC-MS/MS determination of mycotoxins in brown rice from this work and milled rice from previous work [34]. Since higher matrix interferences in brown rice than milled rice, the brown rice sample size used in this work was reduced to 1 g, instead of 10 g used for milled rice, and also the ACN volume:sample size ratio used was 5 mL:1 g for brown rice, while 10 mL:10 g or 1 mL:1 g for milled rice. In our work, the ACN with 10% FA and ACN with 10% HOAc were also compared, but the former gave the poor extraction recovery of approximately 40% for AFB1 and AFG1, while the latter provided the acceptable recovery of 82-94% for all analytes. The results of this difference between our and previous work is not known. The same types of mixed salts were used with the same ratio, but the half total amounts in this work because the water volume and the extraction solvent volume was a half of that in previous work. The similar types of mixed sorbents, except for silica instead of alumina-N, were used but different amount due to the different volume of solvent extract for d-SPE and also different amount and types of interferences to be removed. Starting from the original rice sample to the final solvent before UHPLC-MS/MS analysis, dilution instead of preconcentration was performed for brown rice because the matrix interferences with the brown final solvent was still remained with brown rice. Although, mycotoxins MDL for brown rice analysis is poorer than that for milled rice due to the higher matrix interferences in brown rice than milled rice, our developed method allows to determine trace levels of mycotoxins below the ML values of EU regulation.

Table 4.11 Comparison of QuEChERS sample preparation for this and previous work

Parameters	This work	Previous work [34]
Sample		
Type	Brown rice	Milled rice
Matrix interferences	Much higher	Low
Sample weight	1 g	10 g
Water addition before extraction	5 mL	10 mL
Solvent extraction step		
Extraction solvent	5 mL of ACN with 10% HOAc	10 mL of ACN 10% FA
Salts anh. MgSO4 : NaCl : sodium citrate tribasic dihydrate : sodium citrate dibasic sesquihydrate	2.0 g : 0.50 g : 0.50 g : 0.25 g	4.0 g : 1.0 g : 1.0 g : 0.5 g
d-SPE clean-up step		
Starting extract volume	2 mL	8 mL
$MgSO_4$	300 mg	1,200 mg
C18	50 mg	250 mg
PSA	25 mg	400 mg
Other sorbent	Silica 25 mg	Alumina-N 250 mg
Final solution volume	1 mL of the extract was evaporated to dry and then 1 mL of reconstituted solvent was added	5 mL of the extract was evaporated to dry and then 1 mL of reconstituted solvent was added
Dilution/preconcentration factor from sample to the final solvent prior to UHPLC-MS/MS	5-folds diluted	2.5-folds concentrated

Table 4.12 Comparison of MDL from this and previous work

Analyte	MDL (µg/kg)	
	This work	Previous work [34]
AFB1	1.4	0.5
AFB2	1.6	0.5
AFG1	1.7	0.5
AFG2	2.0	1
FB1	2.4	1
FB2	2.7	0.5
OTA	2.4	0.5
ZON	4.3	0.5
DON	25	5

CHAPTER V
CONCLUSION

QuEChERS sample preparation was optimized and validated for a rapid and simultaneous UHPLC-MS/MS determination of nine mycotoxins in black brown rice: aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, fumonisin B1, fumonisin B2, ochratoxin A, zearalenone and deoxynivalenol.

The optimized UHPLC-MS/MS conditions for mycotoxins separation and detection were obtained using the ACQUITY UPLC® HSS T3 column controlled at temperature of 40 ºC and flow rate of 0.3 mL/min gradient mobile phase of MeOH:water containing 0.5% FA and 5 mM of ammonium formate. The triple quadrupoles mass analyzer with electrospray ionization (ESI) interface was performed. ESI was operated in both positive and negative modes under multiple-reaction monitoring (MRM). The optimized MS/MS conditions were as follows: capillary voltage of 3000 V, nozzle voltage of 1000 V, gas flow of 16 L/min, gas temperature of 150 ºC, nebulizer pressure of 20 psi, sheath gas flow of 11 L/min, sheath gas temperature of 400 ºC, fragmentor of 380 V and dwell time of 50 ms.

The QuEChERS sample preparation for 1.0 g of the blank black brown rice spiked with known concentrations of mycotoxin standards was optimized for the solvent extraction step and followed by the dispersive-solid phase extraction clean-up step. By setting acceptable recovery of 80-110% for mycotoxin concentration of 50-400 µg/kg, the following suitable conditions were obtained with i) the solvent extraction step using 5.0 mL of acetonitrile with 10% acetic acid in presence of four salts (2.0 g of anh. $MgSO_4$, 0.50 g of NaCl, 0.50 g of sodium citrate tribasic dehydrate and 0.25 g sodium citrate dibasic sesquihydrate), and ii) the dispersive-solid phase extraction clean-up step using mixed sorbents of octadecylsilane (50 mg), primary and secondary amine (25 mg) and silica (25 mg).

In method validation, the following parameters were validated for the QuEChERS method: method detection limits, method quantitation limits, standard calibration curve, matrix-matched calibration curves, matrix effect, accuracy and

precision. This developed method allows to determine mycotoxins in trace levels below maximum limits of the EU regulation, with our method detection limits and method quantitation limits in the range of 1.4-25 and 4.1-75 μg/kg, respectively. In comparison of standard calibration curves and matrix-matched calibration curves for each mycotoxin, the matrix interference of brown rice sample affects the quantitative analysis, therefore, matrix-matched calibration curves were used showing the good linearity with the experimental less than critical $F_{(0.05,13,10)}$ at the 95% confidence level and five triplicate concentration levels. Using blank black brown rice spiked with mycotoxins at known concentrations, acceptable accuracy and precision were obtained with the recoveries in a range of 81-101%, and recovery relative standard deviation of 19 down to 5% for 5.0 to 1,000 μg/kg.

This method can be applied to another two types of brown rice: red brown rice and brown brown rice with recovery of all mycotoxins in the range of 81-98% being in acceptable region. Six out of fourteen real samples of brown rice were found to be contaminated with at least one of these mycotoxins, 2.49-5.41 μg/kg of fumonisin B1, 4.33±0.04 μg/kg of fumonisin B2 and 6.10-14.88 μg/kg of zearalenone.

In future work, our developed method should be checked for interlaboratory precision in order to fulfill the validation method for a wide range of real brown rice samples. In addition, this optimized and validated QuEChERS sample preparation and UHPLC-MS/MS method may be applied for other dried samples containing complex matrices such as sesame, nutmeg, ginger, turmeric and other pigmented samples.

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APPENDICES

APPENDIX A

Table A.1 Average recovery of mycotoxins obtained from the QuEChERS sample preparation using with and without buffer for QuEChERS extraction of blank sample spiked with nine mycotoxins (50 µg/kg for AFB1, AFB2, AFG1, AFG2, FB1 and FB2, 200 µg/kg for OTA and ZON and 400 µg/kg for DON)

Analyte	% Recovery ± SD (*n* = 3)		
	Without buffer	With citrate buffer	With acetate buffer
AFB1	81 ± 2	85 ± 2	94 ± 1
AFB2	83 ± 1	82 ± 1	95 ± 1
AFG1	81 ± 2	84 ± 2	93 ± 2
AFG2	81 ± 2	87 ± 4	95 ± 2
FB1	70 ± 1	85 ± 1	49 ± 1
FB2	75 ± 1	86 ± 3	75 ± 1
OTA	82 ± 1	94 ± 2	91 ± 2
ZON	85 ± 1	88 ± 2	99 ± 1
DON	77 ± 3	82 ± 2	94 ± 2

Table A.2 Average recovery of mycotoxins obtained from the QuEChERS sample preparation using ACN with and without acid for solvent extraction of blank sample spiked with nine mycotoxins at concentration levels as shown in Table A.1

Analyte		% Recovery ± SD (*n* = 3)								
		ACN	ACN with 0.1% FA	ACN with 1% FA	ACN with 5% FA	ACN with 10% FA	ACN with 0.1% HOAC	ACN with 1% HOAC	ACN with 5% HOAC	ACN with 10% HOAC
Neutral mycotoxin	AFB1	95 ± 4	87 ± 3	91 ± 3	79 ± 2	42 ± 2	82 ± 2	90 ± 1	90 ± 3	85 ± 2
	AFB2	93 ± 4	85 ± 3	96 ± 3	96 ± 2	91 ± 3	76 ± 1	92 ± 1	93 ± 1	82 ± 1
	AFG1	95 ± 2	87 ± 3	90 ± 4	80 ± 1	43 ± 2	81 ± 2	92 ± 2	90 ± 2	84 ± 2
	AFG2	91 ± 2	89 ± 4	88 ± 3	94 ± 1	92 ± 5	77 ± 3	90 ± 2	92 ± 4	87 ± 4
	ZON	98 ± 2	86 ± 2	99 ± 3	104 ± 1	96 ± 1	75 ± 1	99 ± 2	91 ± 2	88 ± 2
	DON	72 ± 1	84 ± 2	92 ± 2	92 ± 2	88 ± 2	80 ± 2	92 ± 2	86 ± 2	82 ± 2
Acid mycotoxin	FB1	1 ± 1	1 ± 1	27 ± 2	90 ± 2	93 ± 1	1 ± 1	3 ± 1	57 ± 1	85 ± 1
	FB2	1 ± 1	2 ± 1	36 ± 1	86 ± 1	94 ± 4	2 ± 1	4 ± 1	69 ± 1	86 ± 3
	OTA	15 ± 1	21 ± 1	81 ± 1	99 ± 2	106 ± 2	24 ± 1	83 ± 2	90 ±	94 ± 2

Table A.3 Average recovery of mycotoxins obtained from the QuEChERS sample preparation using different water and extraction solvent volume ratio (mL:mL) for QuEChERS extraction of blank sample spiked with nine mycotoxins at concentration levels as shown in Table A.1

Analyte	% Recovery ± SD (*n* = 3)			
	5 : 5	5 : 10	10 : 5	10 : 10
AFB1	85 ± 2	71 ± 3	70 ± 2	68 ± 2
AFB2	82 ± 1	74 ± 3	70 ± 1	68 ± 1
AFG1	84 ± 2	69 ± 4	68 ± 3	64 ± 1
AFG2	87 ± 4	68 ± 2	65 ± 1	58 ± 3
FB1	85 ± 1	94 ± 1	98 ± 4	87 ± 3
FB2	86 ± 3	91 ± 3	88 ± 4	82 ± 3
OTA	94 ± 2	96 ± 6	102 ± 4	93 ± 2
ZON	88 ± 2	94 ± 4	100 ± 5	102 ± 1
DON	82 ± 2	76 ± 3	89 ± 5	70 ± 4

Table A.4 Average recovery of mycotoxins obtained from the QuEChERS sample preparation using different anh. $MgSO_4$ and NaCl weight ratio (g:g) for QuEChERS extraction of blank sample spiked with nine mycotoxins at concentration levels as shown in Table A.1

Analyte	% Recovery ± SD (*n* = 3)			
	0.50 : 0.125	1.0 : 0.25	2.0 : 0.50	4.0 : 1.0
AFB1	59 ± 1	65 ± 1	85 ± 2	91 ± 4
AFB2	65 ± 2	70 ± 3	82 ± 1	94 ± 5
AFG1	62 ± 2	65 ± 2	84 ± 2	96 ± 5
AFG2	71 ± 4	70 ± 1	87 ± 4	95 ± 4
FB1	73 ± 2	85 ± 2	85 ± 1	56 ± 3
FB2	73 ± 2	78 ± 2	86 ± 3	73 ± 4
OTA	68 ± 1	79 ± 1	94 ± 2	101 ± 6
ZON	72 ± 2	82 ± 1	88 ± 2	96 ± 4
DON	95 ± 2	99 ± 2	82 ± 2	90 ± 3

Table A.5 Average recovery of mycotoxins obtained from the QuEChERS sample preparation using different single sorbents in d-SPE step for QuEChERS extraction of blank sample spiked with nine mycotoxins at concentration levels as shown in Table A.1

Analyte	% Recovery ± SD (*n* = 3)				
	None	C18	PSA	GCB	Silica
AFB1	85 ± 4	81 ± 3	92 ± 3	74 ± 2	72 ± 3
AFB2	81 ± 4	87 ± 2	91 ± 3	71 ± 2	82 ± 3
AFG1	86 ± 5	80 ± 3	93 ± 4	93 ± 2	73 ± 4
AFG2	85 ± 2	88 ± 1	100 ± 3	92 ± 1	86 ± 3
FB1	94 ± 4	99 ± 3	27 ± 3	82 ± 1	93 ± 2
FB2	91 ± 3	95 ± 4	30 ± 2	82 ± 2	91 ± 3
OTA	79 ± 5	83 ± 4	69 ± 2	59 ± 2	91 ± 3
ZON	78 ± 4	80 ± 6	81 ± 6	83 ± 1	88 ± 4
DON	82 ± 3	86 ± 3	88 ± 2	87 ± 4	79 ± 5

Table A.6 Average recovery of mycotoxins obtained from the QuEChERS sample preparation using different mixed sorbents in d-SPE step for QuEChERS extraction of blank sample spiked with nine mycotoxins (50 µg/kg for AFB1, AFB2, AFG1, AFG2, FB1 and FB2, 200 µg/kg for OTA and ZON and 400 µg/kg for DON)

Analyte	% Recovery ± SD (*n* = 3)					
	C18+PSA	C18+GCB	C18+Silica	C18+PSA+GCB	C18+PSA+Silica	C18+GCB+Silica
AFB1	88 ± 4	57 ± 1	76 ± 3	79 ± 1	85 ± 2	68 ± 3
AFB2	90 ± 4	59 ± 3	81 ± 2	82 ± 1	82 ± 1	75 ± 3
AFG1	88 ± 4	87 ± 3	76 ± 4	90 ± 1	84 ± 2	75 ± 4
AFG2	90 ± 3	85 ± 3	85 ± 4	89 ± 2	87 ± 4	82 ± 2
FB1	23 ± 3	78 ± 3	89 ± 4	60 ± 4	85 ± 1	74 ± 3
FB2	28 ± 4	85 ± 4	91 ± 3	69 ± 3	86 ± 3	82 ± 1
OTA	74 ± 2	60 ± 3	78 ± 5	59 ± 2	94 ± 2	64 ± 4
ZON	81 ± 4	83 ± 4	74 ± 6	73 ± 3	88 ± 2	71 ± 1
DON	81 ± 5	87 ± 4	88 ± 5	93 ± 5	82 ± 2	88 ± 2

APPENDIX B

Standard calibration curve

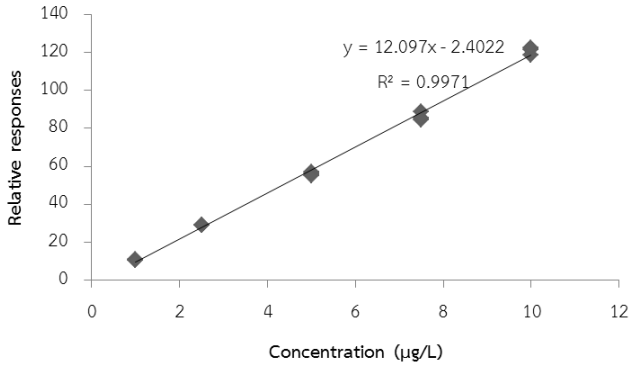

Figure B.1 Standard calibration curve of aflatoxin B1

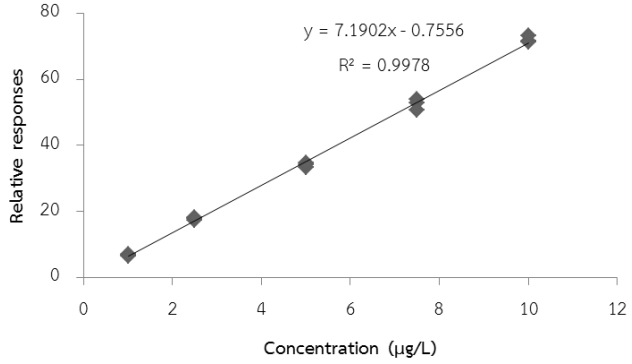

Figure B.2 Standard calibration curve of aflatoxin B2

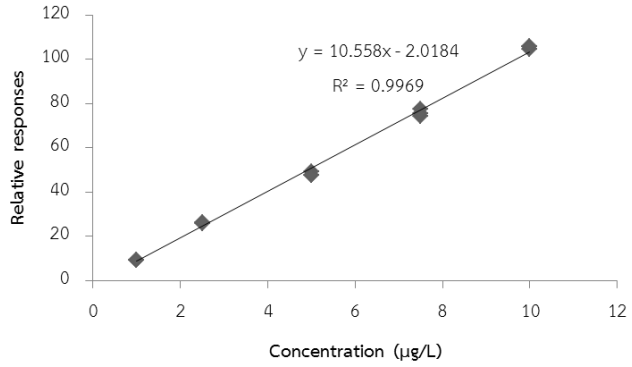

Figure B.3 Standard calibration curve of aflatoxin G1

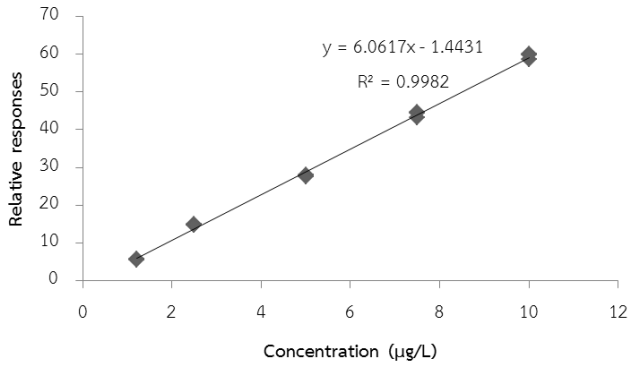

Figure B.4 Standard calibration curve of aflatoxin G2

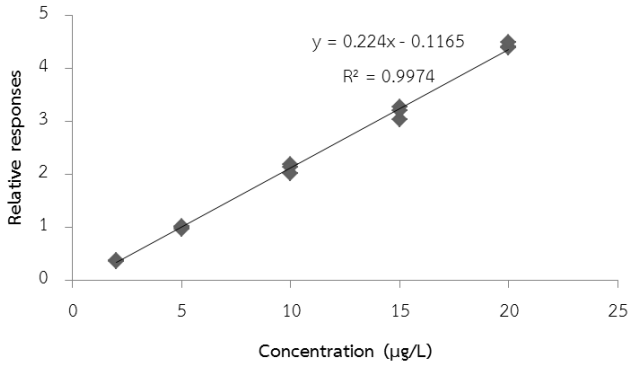

Figure B.5 Standard calibration curve of fumonisin B1

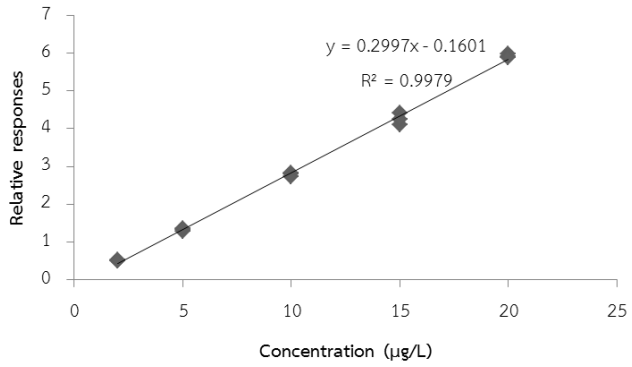

Figure B.6 Standard calibration curve of fumonisin B2

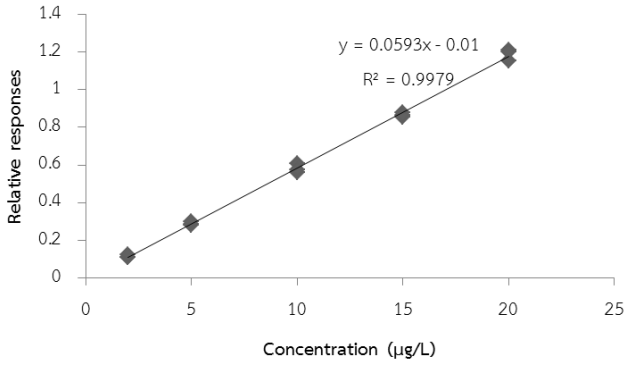

Figure B.7 Standard calibration curve of ochratoxin A

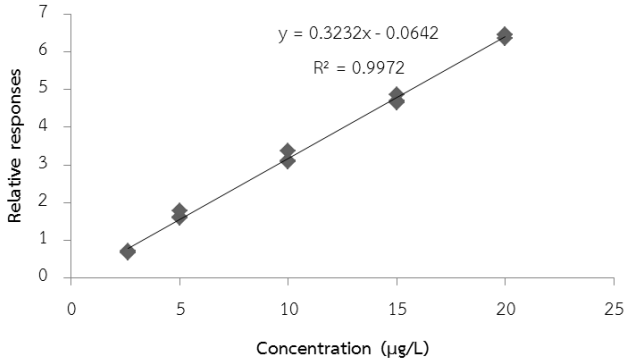

Figure B.8 Standard calibration curve of zearalenone

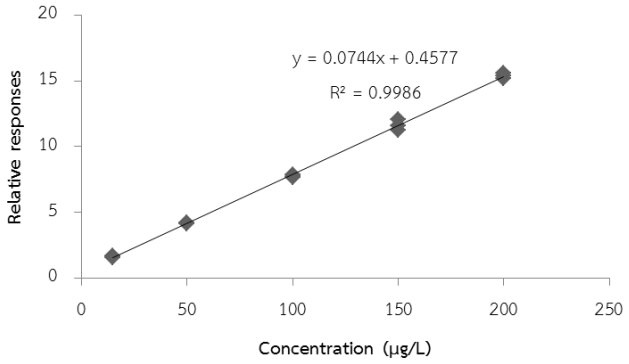

Figure B.9 Standard calibration curve of deoxynivalenol

Matrix-matched calibration curve

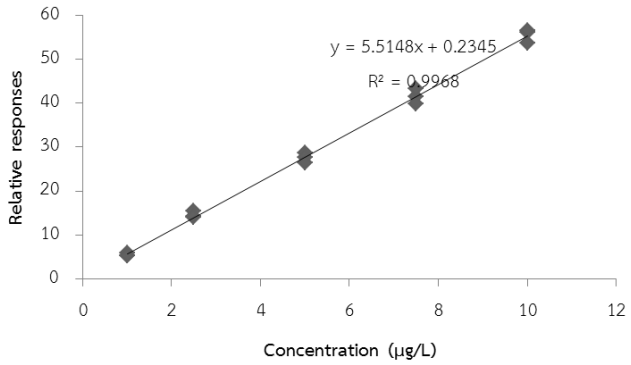

Figure B.10 Matrix-matched calibration curve of aflatoxin B1

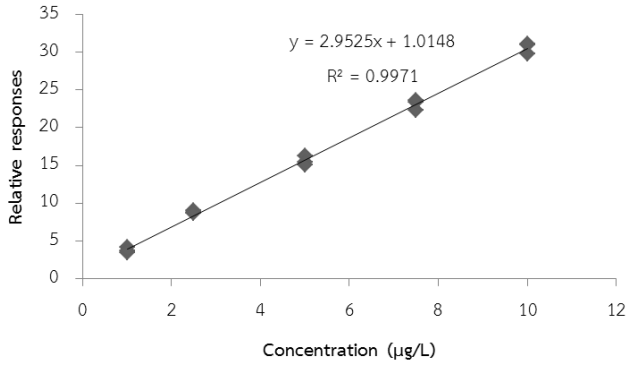

Figure B.11 Matrix-matched calibration curve of aflatoxin B2

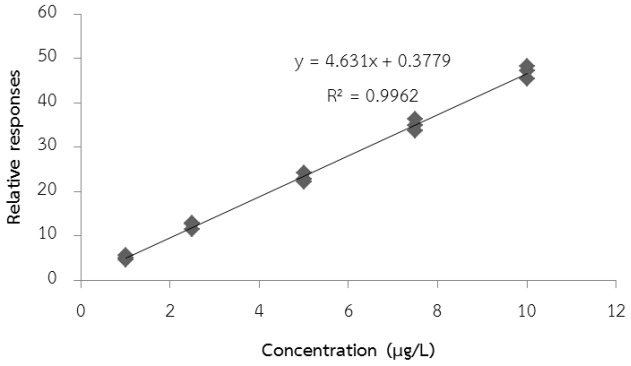

Figure B.12 Matrix-matched calibration curve of aflatoxin G1

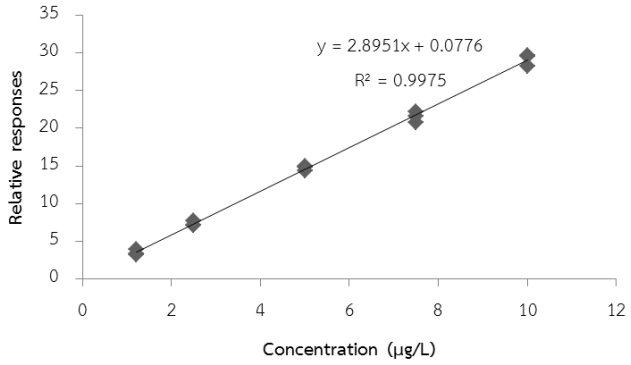

Figure B.13 Matrix-matched calibration curve of aflatoxin G2

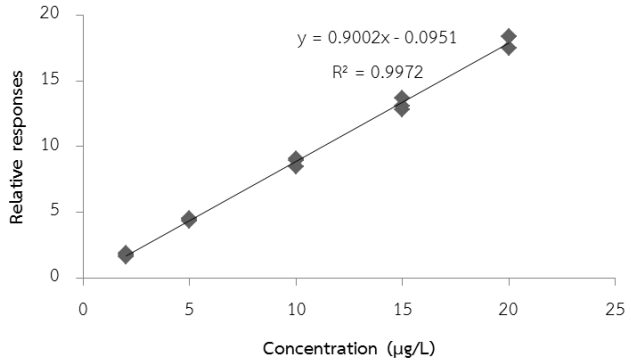

Figure B.14 Matrix-matched calibration curve of fumonisin B1

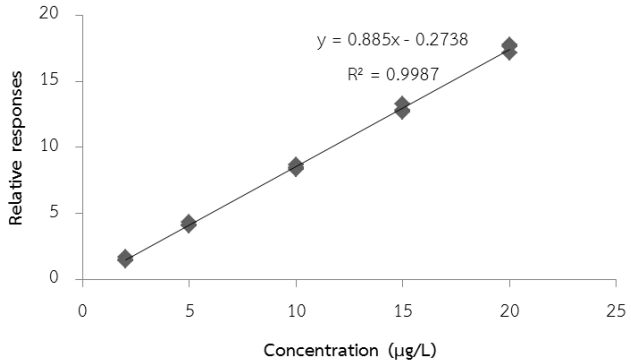

Figure B.15 Matrix-matched calibration curve of fumonisin B2

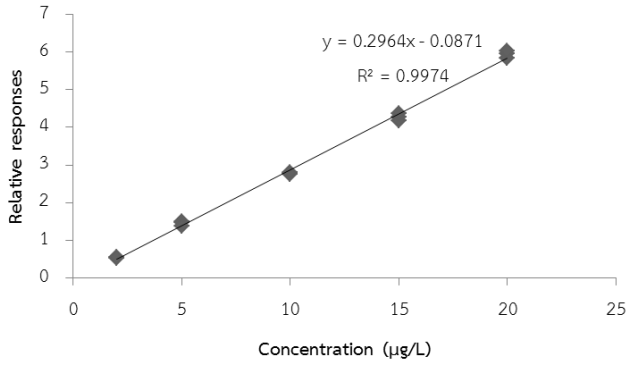

Figure B.16 Matrix-matched calibration curve of ochratoxin A

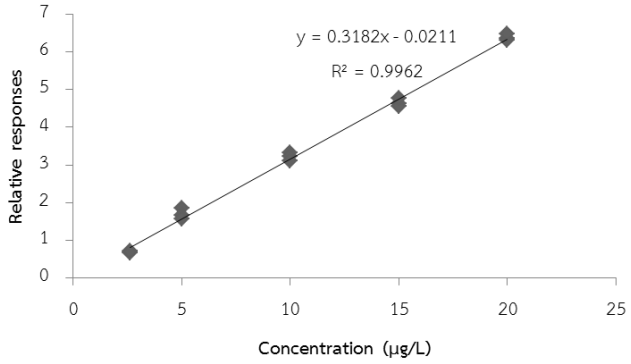

Figure B.17 Matrix-matched calibration curve of zearalenone

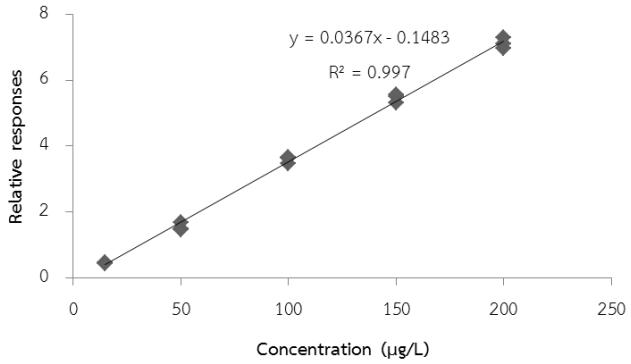

Figure B.18 Matrix-matched calibration curve of deoxynivalenol

APPENDIX C

Table C.1 ANOVA statistical analysis of aflatoxin B1 at low level (5.0 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	917.1667	91.71667	258.488
Column 2	10	826.3333	82.63333	269.4988
Column 3	10	893.6667	89.36667	281.6037

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	444.5574	2	222.2787	0.823671	0.449544	3.354131
Within Groups	7286.314	27	269.8635			
Total	7730.871	29				

Table C.2 ANOVA statistical analysis of aflatoxin B1 at medium level (25 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	907.562	90.7562	87.97046
Column 2	10	910.828	91.0828	113.7359
Column 3	10	944.412	94.4412	65.22125

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	83.21581	2	41.60791	0.467631	0.631464	3.354131
Within Groups	2402.348	27	88.97587			
Total	2485.564	29				

Table C.3 ANOVA statistical analysis of aflatoxin B1 at high level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	867.56	86.756	66.68207
Column 2	10	832.932	83.2932	25.23308
Column 3	10	905.538	90.5538	55.00396

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	263.7686	2	131.8843	2.692998	0.08582	3.354131
Within Groups	1322.272	27	48.97304			
Total	1586.041	29				

Table C.4 ANOVA statistical analysis of aflatoxin B2 at low level (5.0 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	895.4	89.54	238.8093
Column 2	10	826.4	82.64	206.1938
Column 3	10	951	95.1	118.7844

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	779.2507	2	389.6253	2.073256	0.145339	3.354131
Within Groups	5074.088	27	187.9292			
Total	5853.339	29				

Table C.5 ANOVA statistical analysis of aflatoxin B2 at medium level (25 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	906.5	90.65	84.19175
Column 2	10	923.188	92.3188	68.71789
Column 3	10	969.184	96.9184	65.78079

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	210.7802	2	105.3901	1.445743	0.253232	3.354131
Within Groups	1968.214	27	72.89681			
Total	2178.994	29				

Table C.6 ANOVA statistical analysis of aflatoxin B2 at high level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	841.559	84.1559	74.79498
Column 2	10	826.893	82.6893	38.23156
Column 3	10	874.97	87.497	48.83421

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	121.4261	2	60.71307	1.125283	0.339311	3.354131
Within Groups	1456.747	27	53.95359			
Total	1578.173	29				

Table C.7 ANOVA statistical analysis of aflatoxin G1 at low level (5.0 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	807.75	80.775	163.2424
Column 2	10	945.5	94.55	302.6083
Column 3	10	887.5	88.75	244.7222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	956.6375	2	478.3187	2.019436	0.152293	3.354131
Within Groups	6395.156	27	236.8576			
Total	7351.794	29				

Table C.8 ANOVA statistical analysis of aflatoxin G1 at medium level (25 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	889.368	88.9368	66.725
Column 2	10	898.942	89.8942	97.63816
Column 3	10	910.166	91.0166	60.18552

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	21.67322	2	10.83661	0.144779	0.865881	3.354131
Within Groups	2020.938	27	74.84956			
Total	2042.611	29				

Table C.9 ANOVA statistical analysis of aflatoxin G1 at high level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	820.137	82.0137	68.33901
Column 2	10	826.505	82.6505	51.04148
Column 3	10	881.391	88.1391	53.73353

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	226.8359	2	113.4179	1.96549	0.159623	3.354131
Within Groups	1558.026	27	57.70468			
Total	1784.862	29				

Table C.10 ANOVA statistical analysis of aflatoxin G2 at low level (6.0 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	858.5	85.85	260.6694
Column 2	10	893.3333	89.33333	277.8025
Column 3	10	911.6667	91.16667	167.5988

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	145.8722	2	72.93611	0.309896	0.736098	3.354131
Within Groups	6354.636	27	235.3569			
Total	6500.508	29				

Table C.11 ANOVA statistical analysis of aflatoxin G2 at medium level (25 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	877.33	87.733	39.15323
Column 2	10	937.098	93.7098	68.55538
Column 3	10	874.7	87.47	74.54409

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	249.088	2	124.544	2.050077	0.148292	3.354131
Within Groups	1640.274	27	60.7509			
Total	1889.362	29				

Table C.12 ANOVA statistical analysis of aflatoxin G2 at high level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	837.695	83.7695	41.71109
Column 2	10	834.058	83.4058	40.84608
Column 3	10	850.715	85.0715	64.82606

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15.34013	2	7.670064	0.156125	0.856219	3.354131
Within Groups	1326.449	27	49.12774			
Total	1341.789	29				

Table C.13 ANOVA statistical analysis of fumonisin B1 at low level (10 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	951.36	95.136	120.8026
Column 2	10	979.68	97.968	239.4435
Column 3	10	974.96	97.496	181.2957

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	46.04203	2	23.02101	0.12753	0.880794	3.354131
Within Groups	4873.876	27	180.5139			
Total	4919.918	29				

Table C.14 ANOVA statistical analysis of fumonisin B1 at medium level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	906.816	90.6816	40.21309
Column 2	10	905.124	90.5124	120.1411
Column 3	10	934.241	93.4241	43.7656

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	53.42644	2	26.71322	0.392611	0.679084	3.354131
Within Groups	1837.079	27	68.03995			
Total	1890.505	29				

Table C.15 ANOVA statistical analysis of fumonisin B1 at high level (100 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	898.516	89.8516	29.04603
Column 2	10	900.559	90.0559	22.20234
Column 3	10	931.3465	93.13465	41.90982

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	67.66286	2	33.83143	1.089483	0.350725	3.354131
Within Groups	838.4238	27	31.05273			
Total	906.0866	29				

Table C.16 ANOVA statistical analysis of fumonisin B2 at low level (10 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	864.1	86.41	239.7082
Column 2	10	884.15	88.415	262.5006
Column 3	10	858.85	85.885	232.8822

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	35.65517	2	17.82758	0.072757	0.930009	3.354131
Within Groups	6615.82	27	245.0304			
Total	6651.475	29				

Table C.17 ANOVA statistical analysis of fumonisin B2 at medium level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	886.24	88.624	60.25145
Column 2	10	896.627	89.6627	102.9256
Column 3	10	899.783	89.9783	53.32821

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.0421	2	5.021049	0.069574	0.932958	3.354131
Within Groups	1948.547	27	72.16841			
Total	1958.589	29				

Table C.18 ANOVA statistical analysis of fumonisin B2 at high level (100 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	877.541	87.7541	39.68107
Column 2	10	891.2405	89.12405	31.09046
Column 3	10	920.017	92.0017	40.69196

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	93.99913	2	46.99956	1.264976	0.298445	3.354131
Within Groups	1003.171	27	37.1545			
Total	1097.171	29				

Table C.19 ANOVA statistical analysis of ochratoxin A at low level (10 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	856.2	85.62	207.9652
Column 2	10	828.8857	82.88857	108.4375
Column 3	10	845.3429	84.53429	173.8079

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	37.82618	2	18.91309	0.115745	0.891142	3.354131
Within Groups	4411.895	27	163.4035			
Total	4449.721	29				

Table C.20 ANOVA statistical analysis of ochratoxin A at medium level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	925.05	92.505	71.29026
Column 2	10	921.002	92.1002	104.2185
Column 3	10	913.055	91.3055	55.59674

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.447371	2	3.723686	0.048337	0.952894	3.354131
Within Groups	2079.95	27	77.03517			
Total	2087.397	29				

Table C.21 ANOVA statistical analysis of ochratoxin A at high level (100 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	868.069	86.8069	38.72632
Column 2	10	864.237	86.4237	40.76329
Column 3	10	901.9725	90.19725	61.62265

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	86.26998	2	43.13499	0.917036	0.411794	3.354131
Within Groups	1270.01	27	47.03742			
Total	1356.28	29				

Table C.22 ANOVA statistical analysis of zearalenone at low level (13 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	939.4667	93.94667	137.1603
Column 2	10	954.3333	95.43333	140.7568
Column 3	10	907.1333	90.71333	156.5087

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	116.4767	2	58.23837	0.402175	0.672804	3.354131
Within Groups	3909.832	27	144.8086			
Total	4026.309	29				

Table C.23 ANOVA statistical analysis of zearalenone at medium level (50 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	974.388	97.4388	56.08458
Column 2	10	978.68	97.868	98.92746
Column 3	10	958.166	95.8166	69.67944

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	23.41329	2	11.70665	0.156303	0.856068	3.354131
Within Groups	2022.223	27	74.89716			
Total	2045.637	29				

Table C.24 ANOVA statistical analysis of zearalenone at high level (100 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	996.906	99.6906	17.19471
Column 2	10	1009.226	100.9226	16.98762
Column 3	10	962.436	96.2436	67.56563

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	117.6422	2	58.82112	1.734319	0.195612	3.354131
Within Groups	915.7316	27	33.91599			
Total	1033.374	29				

Table C.25 ANOVA statistical analysis of deoxynivalenol at low level (75 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	845.7667	84.57667	86.26848
Column 2	10	852.84	85.284	60.48874
Column 3	10	864.385	86.4385	109.6999

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.66538	2	8.83269	0.103324	0.90219	3.354131
Within Groups	2308.114	27	85.48572			
Total	2325.78	29				

Table C.26 ANOVA statistical analysis of deoxynivalenol at medium level (500 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	882.8619	88.28619	53.25139
Column 2	10	916.314	91.6314	56.82901
Column 3	10	905.6212	90.56212	41.73117

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	58.37882	2	29.18941	0.576822	0.568452	3.354131
Within Groups	1366.304	27	50.60385			
Total	1424.683	29				

Table C.27 ANOVA statistical analysis of deoxynivalenol at high level (1,000 µg/kg)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	855.3914	85.53914	21.93697
Column 2	10	883.1629	88.31629	13.05676
Column 3	10	887.2649	88.72649	21.15088

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	60.1333	2	30.06665	1.606565	0.219164	3.354131
Within Groups	505.3015	27	18.71487			
Total	565.4348	29				

VITA

Mr. Adisorn Jettanajit was born on 14th August 1985, in Chainat, Thailand. He graduated with a Bachelor's of Science in Chemistry with the second class honors from Kasetsart University in 2008. After working for 4 years, he took his sabbatical leave for his study of a Master's degree program of Science in Chemistry majoring in Analytical Chemistry, in the Department of Chemistry, the Faculty of Science, Chulalongkorn University and graduated with a Master's degree in 2015. Currently, he continues his work as a scientist in the Quality Control System for Plant Product Group, Plant Standard and Certification Division, the Department of Agriculture, the Ministry of Agriculture and Cooperatives, Thailand.