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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SIMPLE SAMPLE PREPARATION FOR ANALYSIS OF MYCOTOXIN RESIDUES IN RICE

Mrs. Kunaporn Sanguankaew

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
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Thesis Title	Simple sample preparation for analysis of mycotoxin residues in rice
By	Mrs. Kunaporn Sanguankaew
Field of Study	Chemistry
Thesis Advisor	Assistant Professor Natchanun Leepipatpiboon, Dr.,rer.nat

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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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คุณาพร สงวนแก้ว : การเตรียมตัวอย่างอย่างง่ายเพื่อการวิเคราะห์สารตกค้างชีวพิษจากเชื้อราในข้าว (SIMPLE SAMPLE PREPARATION FOR ANALYSIS OF MYCOTOXIN RESIDUES IN RICE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, 150 หน้า.

การพัฒนาวิธีการเตรียมตัวอย่างซึ่งไม่อาศัยวิธีการแอนติบอดีเพื่อการตรวจวิเคราะห์สารพิษไมโคทอกซินกลุ่มอะฟลาทอกซิน ชนิด บี1, บี2, จี1 และ จี2, กลุ่มฟูโมนิซิน ชนิด บี1 และ บี 2, กลุ่มโอคราทอกซิน ชนิด เอ และ บี, ซิทรินิน, ดีออกซีนิวาลีนอล, นิวาลีนอล, เอชที-2 ทอกซิน, ที-2 ทอกซิน และ ซีราลีโนนในข้าว ร่วมกับการตรวจวัดด้วยเทคนิค UPLC-MS/MS โดยทำให้เกิดประจุไฟฟ้าด้วยเทคนิค electrospray ชนิดบวกและลบ ใช้หมวด MRM ในการวิเคราะห์ สารพิษไมโคทอกซินทั้งหมดจะถูกแยกโดยคอลัมน์ acquity UPLC BEH C18 (100 mm x 2.1 mm, 1.7 μm) ด้วย 0.5% กรดฟอร์มิกใน5 mM แอมโมเนียมฟอร์เมทและอะซิโตไนไตรล์ ต่อ เมทานอล ในอัตราส่วน 1 ต่อ 1 ภายในเวลา 11 นาที ใช้การปรับปรุงเทคนิค QuEChERS ซึ่งสกัดด้วย 10% กรดฟอร์มิกในอะซิโตไนไตรล์และกำจัดสิ่งรบกวนในสารละลายสกัดด้วยวัฏภาคของแข็งผสมระหว่าง PSA, C18 และ alumina-neutral ให้ค่าความเป็นเส้นตรงของการทำปริมาณวิเคราะห์ที่ระดับความเข้มข้นในช่วง 0.01-0.1 มิลลิกรัม ต่อกิโลกรัม และช่วง 0.05-2.5 มิลลิกรัม ต่อกิโลกรัม ให้ค่า R^2 มากกว่า 0.99 ให้ค่าร้อยละการคืนกลับอยู่ในช่วง 53-104 การศึกษาค่าความเที่ยง ณ. ระดับความเข้มข้น ต่ำ กลาง สูง ระดับละ 10 ซ้ำ ภายในวันเดียวกันและต่างวัน ให้ค่าความสัมพันธ์ของการเบี่ยงเบนน้อยกว่า 7.1% และ 11.8% ตามลำดับ ระดับความเข้มข้นต่ำสุดที่สามารถตรวจวัดได้คำนวณที่ระดับความสูง 3 เท่าของ สัญญาณต่อสัญญาณรบกวน ในการเข้าร่วมการทดสอบความชำนาญระหว่างห้องปฏิบัติการได้ค่า Z-score อยู่ในช่วงความน่าพอใจ จึงจัดเป็นวิธีการที่มีประสิทธิภาพวิเคราะห์ได้รวดเร็ว ตรงตามวัตถุประสงค์ที่จะนำวิธีการนี้มาใช้ทดแทนวิธีการเตรียมตัวอย่างโดยใช้คอลัมน์แบบแอนติบอดี ซึ่งมีราคาแพงและนำเข้าจากต่างประเทศ โดยสามารถนำมาปรับใช้กับงานประจำเพื่อการวิเคราะห์หาสารชีวพิษจากเชื้อราในตัวอย่างธัญพืชชนิดอื่นได้

ภาควิชา.....เคมี..... ลายมือชื่อนิสิต.....
สาขาวิชา.....เคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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KUNAPORN SANGUANKAEW: SIMPLE SAMPLE PREPARATION FOR ANALYSIS OF MYCOTOXIN RESIDUES IN RICE. THESIS ADVISOR: ASST. PROF. NATCHANUN LEEPIPATPIBOON, Dr.,rer.nat, 150 pp.

A new non-immunoassay based extraction method for aflatoxin B1, B2, G1, and G2, fumonisin B1 and B2, ochratoxin A and B, citrinin, deoxynivalenol, nivalenol, HT-2 toxin, T-2 toxin and zearalenone was developed for mycotoxins screening in rice. The association of ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) was used to determine the all mycotoxins via electrospray ionization in ESI+ and ES- and multiple reaction monitoring (MRM) for the operating mode of analysis. Mycotoxins were isolated by acquity UPLC BEH C18 column (100 mm x 2.1 mm, 1.7 µm) with 0.5% formic acid in 5 mM ammonium formate/(acetonitrile in methanol = 1:1) by gradient elution within 11 minutes. Modified QuEChERS method employed 10% (v/v) formic acid in acetonitrile for solvent extraction. Cleanup was done by dispersive (d-SPE) combination of PSA, C18 and neutral alumina. The linear regression was evaluated by matrix-matched calibration from 0.01-0.1 mg/L and 0.05-2.5 mg/L, acceptable linearity with all R^2 values better than 0.99 were obtained. Percentage of recovery ranged from 53 to 104 with within-day and between-day precisions at three concentration levels (low, middle, high) showed %RSD values lower than 7.1% and 11.8% (n=10), respectively. The limits of detection range were calculated by 3 times of signal to noise (3S/N). The proficiency testing was served with Z-score in the satisfactory range. This method is effective and rapid, and fit for purpose to replace expensive import immunoaffinity columns and possibly employed in routine analysis of mycotoxins residues in several grains.

Department : Chemistry Student's Signature .....
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LIST OF ABBREVIATIONS AND SYMBOLS

%CV	percent coefficient of variation
%Re	percentage recovery
%RSD	percentage of relative standard deviation
%RSDr	percent of relative standard deviation for repeatability
$\%RSD_R$	percent of relative standard deviation for reproducibility
µg/L	microgram per liter
µg/kg	microgram per kilograms
1° ion	primary product ion or quantitative ion
2° ion	secondary product ion or qualitative ion
ACN	acetonitrile
AF-B1	aflatoxin B1
AF-B2	aflatoxin B2
AF-G1	aflatoxin G1
AF-G2	aflatoxin G2
Al-N	alumina-neutral
AOAC	Association of Official Analytical Chemists
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
BEH	bridged ethylene hybrid
C18	octadecyl
°C	celsius
cal	calculation
CIT	citrinin

Conc.	concentration
crit	critical
CRMs	Certified Reference Materials
DON	deoxynivalenol
d-SPE	dispersive solid phase extraction
ELISA	Enzyme Linked Immuno Sorbent Assay
ESI	electrospray ionization
FAPAS	The Food and Environment Research Agency
Fl	florisil
FUM-B1	fumonisin B1
FUM-B2	fumonisin B2
GC-MS	gas chromatography with mass spectrometry
g	grams
HPLC-FLD	liquid chromatography with fluorescence detector
Hr	hours
HSS	high strength silica
HT-2	HT-2 toxin
IAC	immuno affinity column
insig	in-significant difference
KV	kilo-voltages
LLE	liquid -liquid extraction
LOD	limit of detection
LOQ	limit of quantitation
MeOH	methanol
mins	minutes

mL	milliliters
MLs	maximum limits
Mp	mobile phase
MRM	multiple reaction monitoring
Mtx	matrix
MW.	molecular weight
NaoAct	sodium acetate
ND	not detected
NH_2	aminopropyl
NIV	nivalenol
OTA	ochratoxin A
OTB	ochratoxin B
PBS	phosphate buffer saline
PEG	polyethylene glycol
PSA	primary secondary amine
PT	proficiency testing
QuEChERS, QuE	Quick Easy Cheap Effective Robust Safe
R^2	coefficient of determination
Rt	retention time
sig	significant difference
SD	standard deviation
SD_r	standard deviation for repeatability
SD_R	standard deviation for reproducibility
Si	silica
SPE	solid phase extraction

T-2	T-2 toxin
TLC	thin layer chromatography
UPLC-MS/MS	ultra performance liquid chromatography coupled with tandem mass spectrometry
Volts	voltages
ZEA	zearalenone

CHAPTER I

INTRODUCTION

1.1 Problem definitions.

The name mycotoxin combines the Greek word for fungus "*mykes*" and the Latin word "*toxicum*" meaning poison. The name "*mycotoxin*" is usually reserved for the toxic chemical [1] formed by different species of fungal that are growing on plants including foodstuff. Mycotoxins frequently occur such as aflatoxins trichotecene type A&B fumonisins zearalenone citrinin and ochratoxins that are mainly secreted from fungi class *Aspergillus Penicillium* and *Fusarium.* [2] Each fungi species can produce more than one type of mycotoxin, which is a very small molecule (<750). Some mycotoxins are thermo resistant, very chemically stable and need difficult detoxification procedures. Indeed, the absence of any visible mould does not guarantee the absence of mycotoxins because the mould may have died out whilst leaving the mycotoxins in the foodstuff. Therefore, they can remain in foodstuff for longer than the lifespan of the fungi that synthesized them. The contamination of mycotoxins may occur during pre/post-harvest or be generated during storage in the warehouse or during the transportation stage under conditions of high temperature and humidity which could stimulate the production of toxins. In the past, many countries in the tropical region were concerned with the problems from the contamination of mycotoxins in foodstuff because mycotoxins could produce easily and spread easily under high temperature and moisture. Nowadays, the temperatures have increased due to global warming and will directly affect global temperatures. One of the major concerns is that mould will grow and generate toxins worldwide. Therefore all countries should recognize and try to overcome the problems that may arise from these mycotoxins contamination.

Generally, if grains are stored for a few days, there is a possibility for mould to grow and mycotoxins to form. Rice (*Oryza sativa L.*) is an important grain, along with wheat barley and corn and has been a major food of human consumption in several countries. As rice is an aquatic plant that is usually harvested at very high moisture levels (35%-50%).[3] The mycotoxins producing moulds could contaminate the

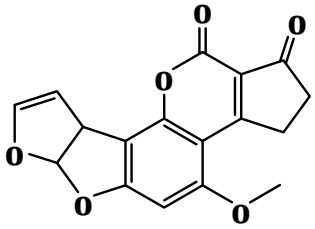
grains during storage. For instance Suprasert et al., 2000 reported that rice in Thailand was contaminated with deoxynivalenon (DON) but at acceptable levels compared with the 1 ppm limit set in USA etc [4].

As for the technique of mycotoxins extraction, the current sample preparation of mycotoxins analysis typically relies on immunological assay such as Enzyme Linked Immuno Sorbent Assay (ELISA) and Immuno Affinity Column (IAC). These methods offer improved specificity and recovery as well as providing good accuracy and precision. They are easy to employ fast, portable and involve low interference. However, the main disadvantages of using antibody-based immuno-analytical techniques are cost, storage and matrix dependency. Only single universal type cartridges/wells are available commercially, the efficiency of these cartridges/wells can be quickly destroyed by short antibody shelf life.

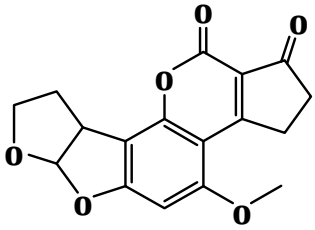
1.2 Mycotoxins and Toxicity

The chemical properties of mycotoxins have various toxic effects on the health of the consumer with varying symptoms. Generally, toxic effects are carcinogenicity genotoxicity teratogenicity nephrotoxicity hepatotoxicity and immunotoxicity etc. [3, 5, 6, 7]

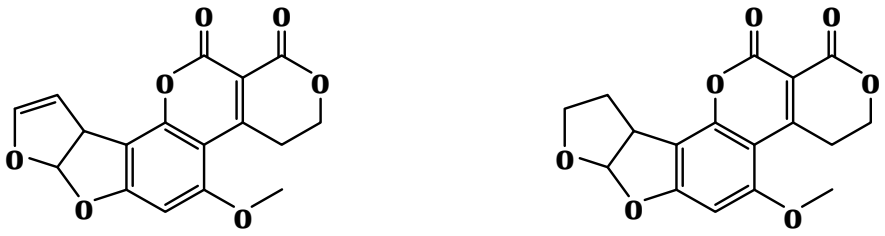
1.2.1 Aflatoxins are a family of extremely toxic substances produced by fungi *A. flavus* and *A. parasiticus* during the post-harvest. There are four major types; B1, B2, G1 and G2 which based on fluorescence under UV light (blue and green). Aflatoxins (AFs) occur widely in various commodities such as cereal, grain, specially corn, nuts, peanut, coconut, fruits, and dried fruits.



Aflatoxin B1 (AF-B1)



Aflatoxin B2 (AF-B2)

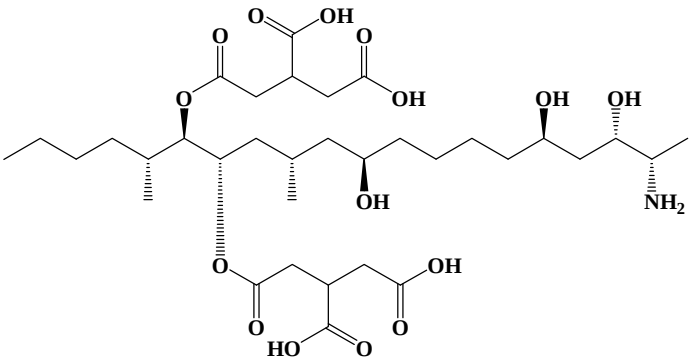


Aflatoxin G1 (AF-G1) Aflatoxin G2 (AF-G2)

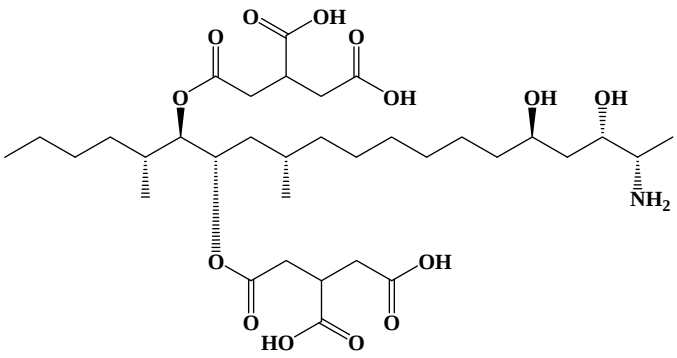
Figure 1.1 Chemical structure and category of aflatoxins.

The toxicity of aflatoxins can cause liver disease (target organ), and are immunosuppressive, carcinogenic and mutagenic in animals and human beings, especially, aflatoxin B1 (AF-B1) which is the most potent human carcinogens. The International Agency for Research on Cancer (IARC) has classified aflatoxin B1 in the group 1 as a human carcinogen and aflatoxin G1, B2 and G2 in group 2B as possible carcinogen to humans. [6]

1.2.2 Fumonisins are produced by fungi *F.moniliforme* and *F. proliferatum* during pre-harvest, mainly in corn and corn products. There are three types; B1, B2 and B3. Fumonisin B1 (FUM-B1) is the most abundant in the natural contamination, followed by fumonisin B2 (FUM-B2)



Fumonisin B1 (FUM-B1)



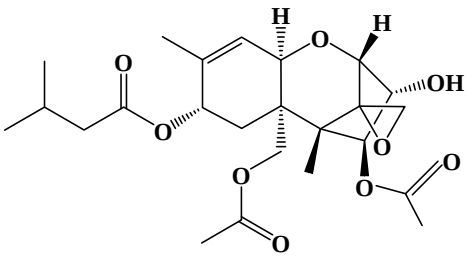
Fumonisin B2 (FUM-B2)

Figure 1.2 Chemical structure and category of fumonisins.

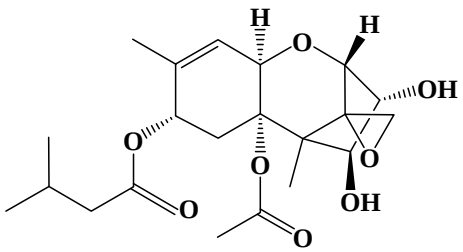
The toxicity of fumonisins can cause pulmonary edema, equine leukoencephalomacia, nephrotoxicity, hepatotoxicity and immune suppression. Fumonisins are possibly carcinogenic to humans and according to the International Agency for Research on Cancer (IARC), they are classified as group 2B carcinogens.

1.2.3 Trichothecenes are very large family of chemically related mycotoxins produce by various genus of *Fusarium*. It is divided into two groups, namely trichothecenes type A and type B.

1.2.3.1 Trichothecenes type A includes T2 toxin and HT-2 toxin. *F. sporotrichioides, F. poae, F. equiseti and F.acuminatum* is mainly mould for T-2 toxin & HT-2 toxin production during pre-harvest. Many reports have disclosed that the trichothecences type A is strained in grains such as wheat, oat, maize, barley, rice, beans and soya bean.



T-2 toxin (T-2)

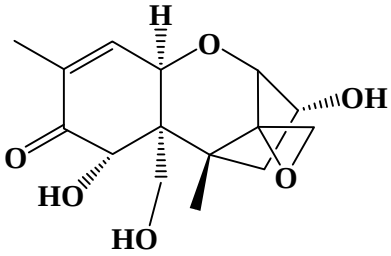


HT-2 toxin (HT-2)

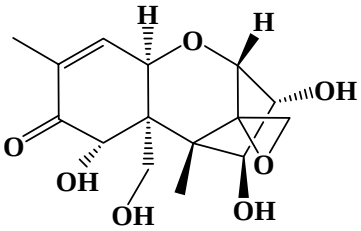
Figure 1.3 Chemical structure and Category of T-2 toxin and HT-2 toxin.

The toxicity of trichothecenes type A (T-2 toxin & HT-2 toxin) can cause antibody levels to decrease, immunoglobulins, weight loss or poor weight gain, bloody diarrhea, vomiting, dermal necrosis or beak lesions and hemorrhage. Trichothecene type A is more toxic than type B.

1.2.3.2 Trichothecenes type B composes the subdivision such as denoxynivalenol (DON, also known as vomitoxins) and nivalenol (NIV). *F. graminearum* and *F. culmorum* are mainly mould for DON and NIV production during pre-harvest. The occurrence of trichothecene type B is strained in grains such as wheat, oat, maize, rye, rice and sorghum.



Deoxynivalenol (DON)

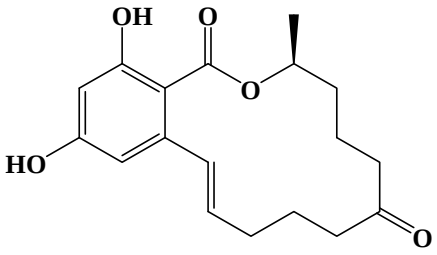


Nivalenol (NIV)

Figure 1.4 Chemical structure and category of DON and NIV.

The toxicity of trichothecenes type B (DON & NIV) can lead to loss in animal product due to reduced feed intake (anorexia) and emesis (vomiting). Although they are less toxic than other trichothecene but the most prevalent and are commonly found in barley, corn, rye, sunflower seed, wheat and mixed feed.

1.2.4 Zearalenone is a toxic substance that is generated by *F. graminearum* during pre-harvest. The commodities were mainly strains such as corn, and to a lesser extent, barley, oat, wheat, sorghum, millet and rice.

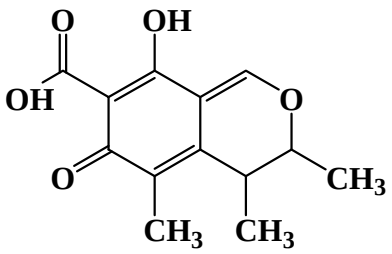


Zearalenone (ZEA)

Figure 1.5 Chemical structure of zearalenone.

The toxicity of zearalenone has a relatively low acute toxicity. The potency of zearalenone will stimulate the growth of human breast cancer cells containing estrogen response receptors, immunotoxic, genotoxic and inducible DNA fragmentation. Zearalenone was classified by the International Agency for Research on Cancer (IARC) under group 3 carcinogen (IARC, 1999).

1.2.5 Citrinin is produced by *P. verrucosum* and *P. citrinum* during post-harvest. Citrinin has mainly been found in rice, wheat, flour, barley, maize, rye, oats, peanuts and fruit and may co-occur in cereal together with ochratoxin A (OTA).

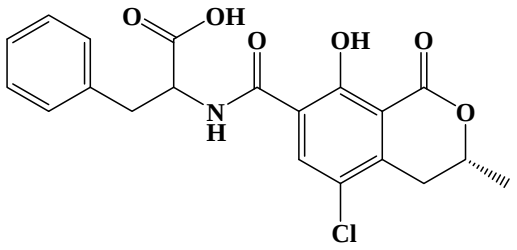


Citrinin (CIT)

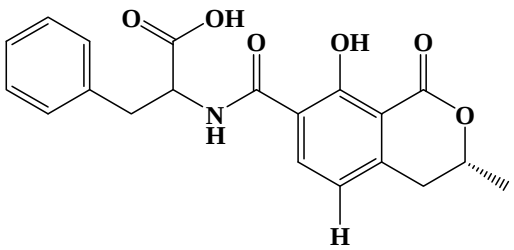
Figure 1.6 Chemical structure of citrinin.

The toxicity of citrinin may be implicated in the fatal human kidney disease, nephrotoxicity, teratogenicity and hepatotoxicity.

1.2.6 Ochratoxins has 3 types in this family such as ochratoxin A & B & C. The ochratoxins often referred to OTA because it is the most toxic one and most commonly occurring, while ochratoxin B (OTB) can occur naturally but is much less toxic. Other related mycotoxins include ochratoxin C (OTC) which rarely find in the nature. OTA is an innately exhibited blue fluorescence under UV light. The moulds responsible for OTA production are mainly *A. ochraceus, A. carbonarius, P. verrucosum, P. viridicatum* and *P. nordicum* etc during the pre/post-harvest. These moulds could be found worldwide because they are stable enough to develop under various conditions of moisture, pH and temperature. Ochratoxins may also be present in products of grain, cereal, wheat, barley, dried raisins, grapes, soy products, coffee and cocoa etc.



Ochratoxin A (OTA)



Ochratoxin B (OTB)

Figure 1.7 Chemical structure and category of ochratoxins.

The toxicity of ochratoxins can cause nephrotoxic, immunosuppressive and carcinogenic complications. In 1993, OTA was classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen under group 2B.

1.3 Regulation

Regarding the harmfulness of mycotoxins, many countries in the world recognize the hazards and toxicity from strained mycotoxins in human beings. Thus, the regulations were restricted with the maximum levels for the forbidden contaminated commodities. Consequently, the European Food Safety Authority (EFSA) announced the maximum tolerable daily intake (TWI) of EU criteria which focused on some possible toxicity of mycotoxins such as-: OTA 120 $ng.kg^{-1}.bw^{-1}.wk^{-1}$ and [8] Scientific Committee on Food (SCF) established a tolerable daily intake (TDI) for DON 1.0 $\mu g.kg^{-1}.bw^{-1}.d^{-1}$, ZEA 0.2 $\mu g.kg^{-1}.bw^{-1}.d^{-1}$**,** FUMs (B1, B2, B3) 2.0 $\mu g.kg^{-1}.bw^{-1}.d^{-1}$, NIV 0.7 $\mu g.kg^{-1}.bw^{-1}.d^{-1}$, T-2 & HT-2 toxin 0.06 $\mu g.kg^{-1}.bw^{-1}.d^{-1}$. [8,9] As regard AFs, the SCF expressed that is appropriate to limit the total AFs content in food (sum of AF-B1, B2, G1 and G2) as well as the AF-B1 content alone, AF-B1 being by far the most toxic compound. This being the case, EU and other developing countries tend to set the low maximum limits tolerant intake for the levels of these compounds in foodstuff. Along with the Commission Regulation (EC) No. 1881/2006, the maximum levels (MLs) should be set at a strict level which is reasonably achievable by following good agricultural, fishery and manufacturing practices and taking into account the risk related to the consumption of the food. In the case of contaminants which are considered to be genotoxic carcinogens or where current exposure of the population or of vulnerable groups in the population is close to or exceeds the tolerable intake, maximum levels should be set at the level which is as low as reasonably achievable (ALARA). Such an approach ensures that food business operators apply measures to prevent and reduce the contaminants as far as possible in order to protect public health. It is furthermore appropriate for the health protection of infants and young children, a vulnerable group, to establish the lowest maximum levels, which are achievable through a strict selection of the raw materials used for the manufacturing of foods for infant and young children. The strict selection of the raw materials is also appropriate for the production of some specific foodstuffs such as bran for direct human consumption. [9] Table 1.1 shows the specified maximum levels which shall apply to the edible part of grain cereal and cereal products concerned and comply with Commission regulation (EC) No. 1881/2006.

Table 1.1 Maximum levels for certain contaminants in foodstuff (concerned grain, cereal and cereal products) comply with Commission regulation (EC) No. 1881/2006.

Mycotoxins	**MLs , µg/kg**	
Aflatoxins	**B1**	**Sum B1, B2, G1 and G2**
All cereal and all products derived from cereals, including processed cereal products.	2.0	4.0
Ochratoxin A		
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption.	3.0	
Fumonisins	**Sum B1 and B2**	
Maize intended for direct human consumption, maize-based foods for direct human consumption.	4,000	
Doxynivalenon		
Unprocessed cereals other than durum wheat, oats and maize.	1250	
Zearalenone		
Cereal intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption.	75.0	
T-2 and HT-2 toxin		
Unprocessed cereals and cereal products	-	

(**Note** : The maximum limit of OTB, CIT and NIV have not been controlled.)

1.4 Literature Review

A generally used technique for isolation of mycotoxins involve liquid-liquid extraction (LLE), solid phase extraction (SPE), ion exchange, mycosep® column, thin layer chromatography (TLC), enzyme linked immuno sorbent assay (ELISA), immuno affinity column (IAC), Quick Easy Cheap Effective Rugged Safety (QuEChERS). A part of chromatographic method for determination of mycotoxins include gas chromatography (GC), high performance liquid chromatography coupled with fluorescence detector (HPLC-FLD) and high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) etc.

1.4.1 Mycotoxins analysis by Liquid-Liquid Extraction (LLE) [1, 10, 11]

Conventional LLE is a well established cleanup technique. The principle is based on the partitioning between different solubility of mycotoxins in aqueous and in immiscible organic solvents in order to isolation of mycotoxins from the matrices (see figure 1.8). For the cleanup stage, the use of solvents such as hexane or cyclohexane is removed non-polar (e.g. lipid or cholesterol). Advantages are the simplicity of the method and inexpensive apparatus. On the other hand, disadvantages include solvent consumption, long periods of extraction, loss of samples (by adsorption on glassware) and solvent approach of chemist etc.

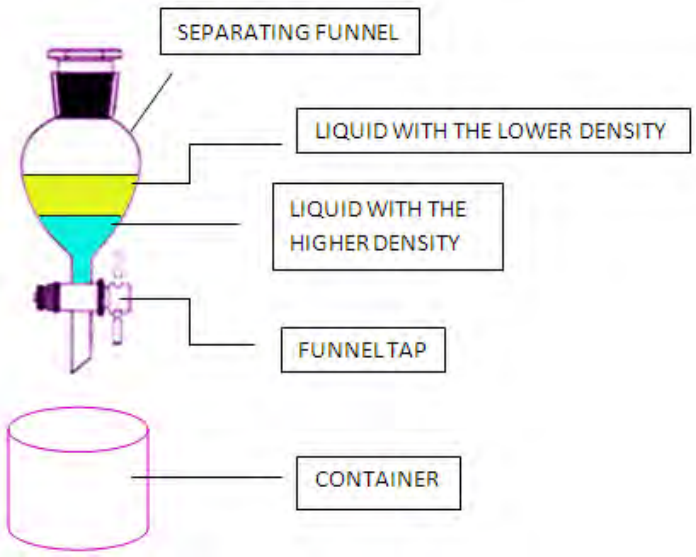


Figure 1.8 Illustration of LLE principle [12]

1.4.2 Mycotoxins analysis by Solid Phase Extraction (SPE) [1, 10, 11, 13, 14]

The SPE principle is based on the mycotoxin interaction with the sorbent being the stationary phase. SPE sequential procedure starts with conditioning of the column by activating with a solvent or aqueous solution. Secondly, extracted sample is loaded into the cartridge in order to trap mycotoxins and interference. Later on, a rinsing step was applied for isolation of the matrices from the cartridge. Finally, solvent was employed for elution of mycotoxins into the reservoir. The sorbent of SPE include C8 and C18 are most frequently used due to the pressure resistance, which gives reproducible results and operation range at pH 2-8 (which is a drawback of a limitation of pH working range). Silica gel is frequently used for OTA analysis. While florisil, cyano and C18 are responsible for trichothecene type A. Ventura M. et al., 2004 employed the HLB, polymeric SPE for isolation of AFs (B1, B2, G1 and G2) in medicinal herbs by single quadrupole mass spectrometry. [14]

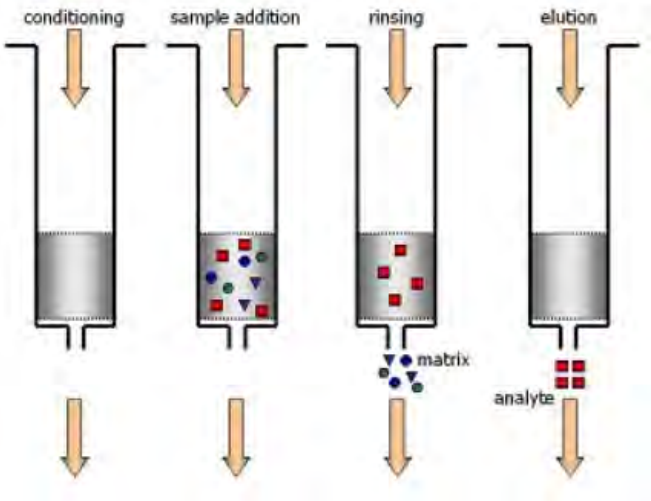


Figure 1.9 Illustration of a typical SPE sequence [10]

1.4.3 Mycotoxins analysis by Ion Exchange technique [1, 10, 5, 15]

Ion Exchange technique is used for isolation and pre-concentration of mycotoxins which are de-protronated and become charged such as FUMs, OTA, OTB and CIT etc. due to the carboxyl group contents in the structure. Consequently, Strong Anion Exchange (SAX) cartridge is used for sample preparation. Based on the principle of technique, the utility electrostatic interactions are formed between the charged analytes and functionalizes charged group on silica sorbent. Pelegri et al. offered a sensitive protocol for detection of OTA detection and quantification using SAX cartridge for the clean-up step with the limit of detection (LOD) of 0.02 ng/mL by HPLC-FLD. The limitation of this technique can be applied for the charged mycotoxins [5].

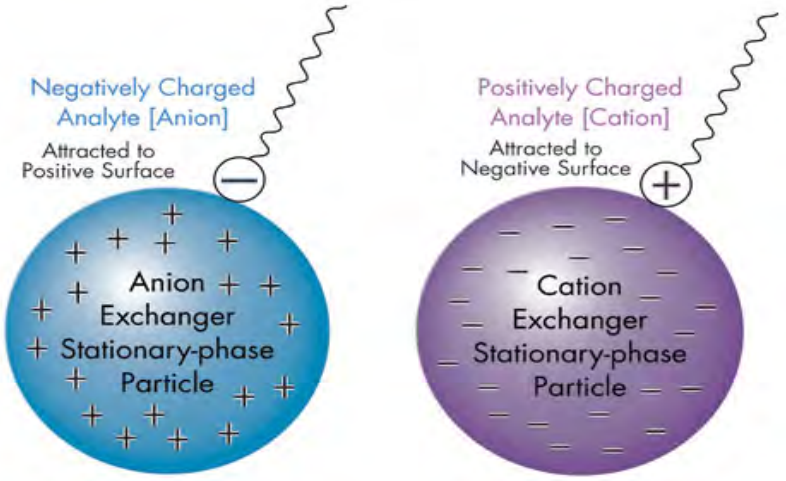


Figure 1.10 Illustration of ion exchange profile [15]

1.4.4 Mycotoxins analysis by Mycosep® column [1, 10, 16, 17, 18, 19]

The Mycosep® multifunctional clean-up columns (Romer Labs® Inc., MO, USA) consist of several adsorbents (e.g. charcoal, celite, ion exchange resins and others), which are packed in a plastic tube. A rubber flange, a porous frit and one-way valve on the lower end ensure that the extract is forced through the packing material, when the column is inserted into the culture tube. Finally, the purified extract exists on the top of the plastic tube. Almost all of the interferences are trapped in the column, while the mycotoxins do not show significant affinity in the sorbent. This method is practical, portable, quick and shows good recovery with no additional rinsing steps required. However, columns are designed for a single analysis. Therefore, it is not useful for multi-mycotoxins analysis and specified matrix. In 2007, E.M. Binder et al. used the Mycosep® #227 (trichothecenes) and #226 (ZEA) for mycotoxin analysis in commodities, feed and feed gradient. Moreover, High Performance Liquid chromatography coupled with mass spectrometry (HPLC-MS) was employed for the chromatographic. The limit of detection was 1 μg/kg and 25 μg/kg for AFs and ZEA, respectively [18].

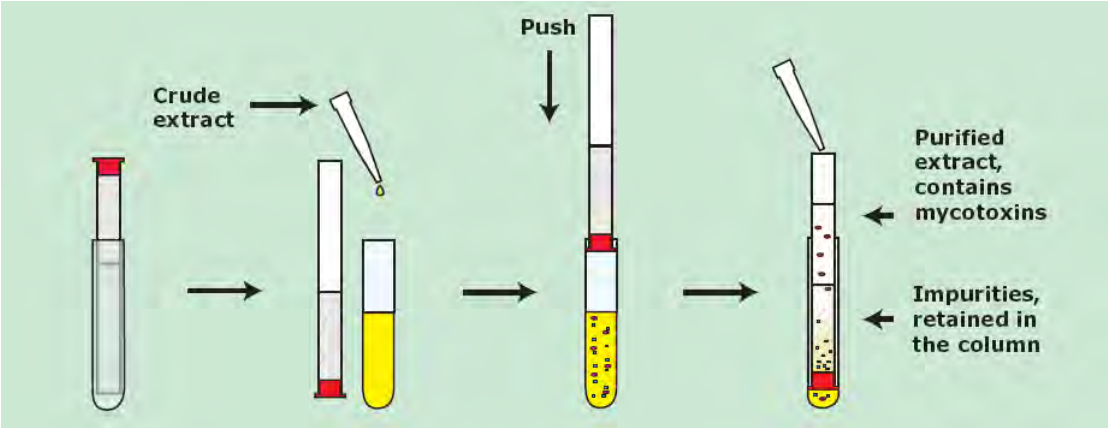


Figure 1.11 Illustration of the Mycosep® column principle [19]

1.4.5 Mycotoxins analysis by Thin-Layer Chromatography (TLC) [1, 10, 15, 18, 20, 21]

TLC technique is useful for the separation, purity assessment and ease of identification of target compound. Several methods were developed to obtain the best results with each separated class of mycotoxins and both one/two-dimensional being used. Whereas, the simplicity, rapidity and numerous samples were obtained from this technique. The basic principle is the stationary phase immobilized on glass plate or aluminium plate and solvent as mobile phase. The sample, either liquid or dissolved in volatile solvent, is deposited as a spot on the plate. The different components in matrices mixture move up on the plate at different rate due to their different partitioning behavior on stationary phase (see figure 1.12). Silica gel layer is most commonly employed with the mixture solvent. AFs can easily be applied by this technique due to they are fluorescent themselves without any further treatment. The detection limits are in the lower μg/kg levels. While for, OTA can be glittered as greenish spot by UV light. In 2007, E.M. Binder et al. used TLC for analysis of T-2 toxin in commodities, feed and feed ingredients. The results were reported as 2 positives of 338 test sample. [18]

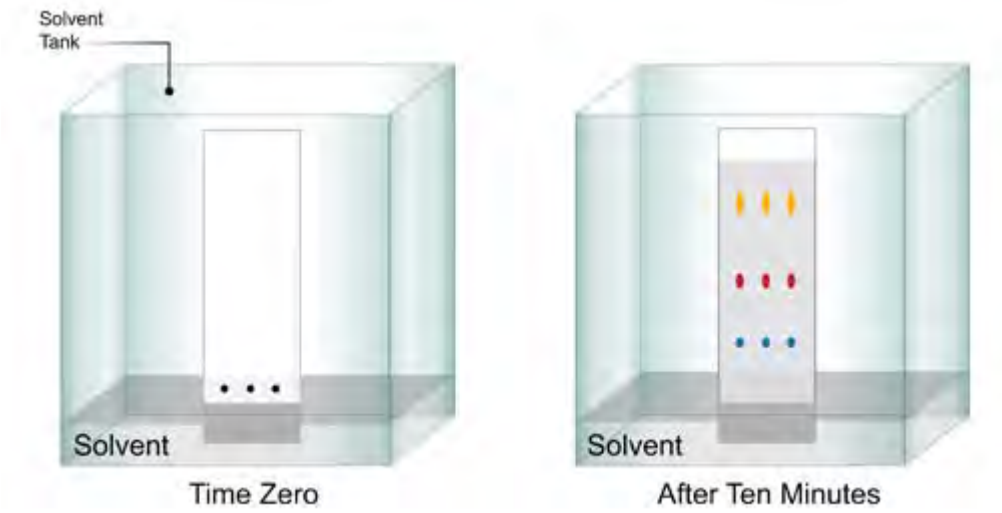


Figure 1.12 Illustration of the TLC principle [15]

1.4.6 Mycotoxins analysis by Enzyme Linked Immuno Sorbent Assay (ELISA) [1, 10, 22, 23, 24]

ELISA is generally used for screening of the presence of mycotoxins in the test sample. Typically, the basic principle of ELISA lies in immobilizing the antigen on to a solid surface, capturing the antigen by a specific antibody and specific immune conjugated to an enzyme. The positive reaction is detected by adding a suitable substrate, which is converted to a colored product on reaction with the enzyme. The ELISA kit is a competitive enzyme-labeled immunoassay. A toxin is extracted from a ground sample by blending or shaking with solvent. The extract is then diluted with water, filtered and then tested in the immunoassay. The mycotoxin-enzyme conjugate is pipetted into the test wells followed by sample extracts. A mycotoxin antibody is then pipetted into the test wells to initiate the reaction. During the 10 minute incubation period, mycotoxins from the sample and mycotoxin-enzyme conjugate competes for binding to antibody which binds to the test well. Following this 10 minute incubation, the contents of the well are removed and the wells are washed to remove any unbound toxin or enzyme-labeled toxin. A clear substrate is then added to the wells and any bound enzyme-toxin conjugate causes the conversion to color. Following 10 minutes incubation, the reaction is stopped and the amount of color in each well is read. The color of unknown samples is compared to the color of the calibrators and the mycotoxins concentration of the samples is derived. The most advantages are reasonable for usages which are numerous samples, simplicity, fast analysis and low interference from matrices etc. However, the disadvantages of the technique were discussed about the limitations of well-mixed liquids such as milk, a limited shelf-life of ELISA kits, possibility of the false positive results (colorimetric quantification), specificity, not precise and expensive.

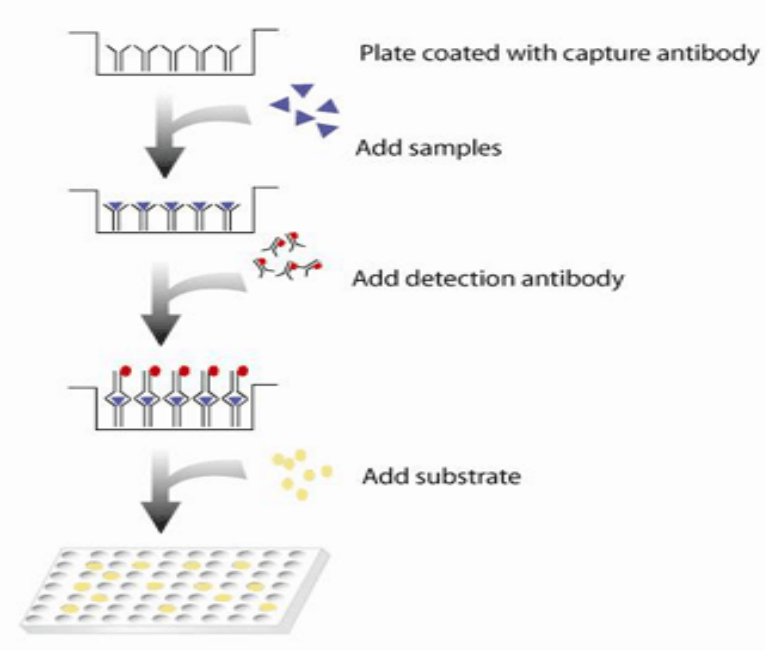


Figure 1.13 Illustration of ELISA test kit [24]

1.4.7 Mycotoxins analysis by Immuno Affinity Column (IAC) [1, 10, 25, 26]

IAC are generally used for isolation and concentration of mycotoxins from sample matrices prior to analysis by GC-MS, HPLC-FLD and HPLC-MS etc. The basic principle of IAC was determined in another chapter (section 2.3.1, chapter II). While the advantage and disadvantage regularly resembles ELISA because antigen-antibody is employed for immunogenic assay. Whilst, IAC would give a highly accurate quantification of mycotoxin concentration by comparison to ELISA. Funda et al. in 2008 used IAC for extraction of OTA in dried figs and investigation by High Performance Liquid chromatography (HPLC-FLD) with the limit of detection at 0.12 μg/kg. [26]

1.4.8 Mycotoxins analysis by QuEChERS [27, 28, 29, 30]

Recently, a new method of sample preparation technique was introduced under the name of "QuEChERS" [27, 28] (Quick, Easy, Cheap, Effective, Rugged and Safety) for analysis pesticide residue method was first published in 2003 on Journal of AOAC (Association of Official Analytical Chemist), [27] and in 2007, the same method was issued in the official standard method on Journal of AOAC International for multi-residue analysis of pesticide in fruit, vegetable and other foods. [29] QuEChERS is a sample preparation approach entailing solvent extraction with ACN and partitioning with $MgSO_4$ alone or in combination with other salts followed by clean up using d-SPE. (The extraction stage was more explained in section 2.3.2.) It is very flexible; there have been several modifications of the technique depending on the analytes, matrices instrumentation and analyst preferences. I. Sospedra et al. in 2010, the modification of QuEChERS method was developed for trichothecenes type A and B extraction and determination by LC-MS. The limit of detection was ranged 1 to 30 µg/kg in wheat flour. [30]

1.4.9 Mycotoxins analysis by Gas Chromatography coupled with mass spectrometer (GC-MS) [1, 10, 21, 31, 32]

GC-MS is currently used for quantification and qualification of the mycotoxins presence in samples in order to detect the volatile mycotoxins. Whilst, most mycotoxins are not volatile and consequently derivatived mycotoxins reaction such as silylation or polyfluoroacylation have to be employed for obtainable volatile mycotoxins in order to befit with GC-MS technique. Yoshiki et al. in 1998, eight typical mycotoxins were studied by GC-MS. This approach gave the limit of detection range from 0.1-0.5 µg/g in barley. [21]

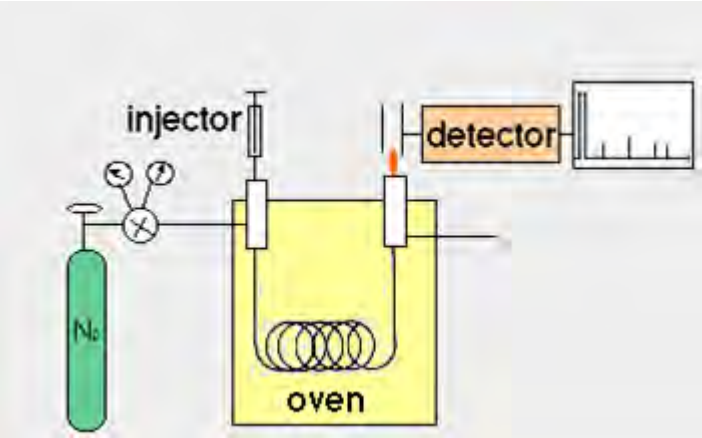


Figure 1.14 Illustration of GC instrument system. [32]

1.4.10 Mycotoxin analysis by High Performance Liquid Chromatography (HPLC) [1, 10, 15, 18, 21, 26, 33]

HPLC is a popular technique in the field of mycotoxins analysis. The diode array detector (DAD) or fluorescence detectors (FLD) either relied on the presence of chromophore or the fluorescent itself. For some mycotoxins such as FUMs produces lack a suitable chromophore, and their determination requires deravatization by o-phthaldialdehyde and 9-(fluorenylmethyl) chroroformate as the derivatizing reagent. The reversed phase mode of separation was employed by C18 or C8 columns. Moreover, sample preparation need to be prior instrument such as LLE, SPE, Mycosep and IAC etc. In 2007, E.M. Binder et al. used High Performance Liquid chromatography coupled with fluorescence detector (HPLC-FLD) for quantify of FUMs in commodities, feed and feed ingredients. In this study, pre-column derivatization was served for FUMs detection. [18]

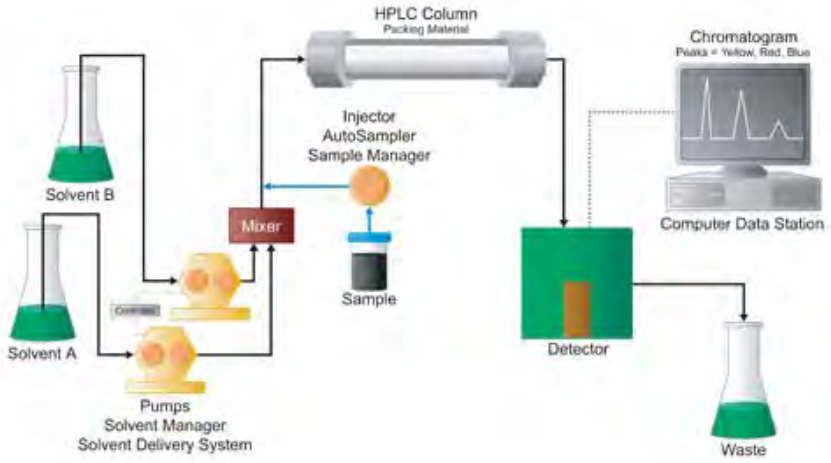


Figure 1.15 The flow diagram of HPLC system [15]

1.4.11 Mycotoxin analysis by High Performance Liquid Chromatography coupled with mass spectrometry (HPLC-MS) [1, 10, 14, 21]

One of the advanced techniques for detection of mycotoxins is HPLC-MS. Quadrupole detector is widely used for the mycotoxins analysis without prior derivatization. The use of HPLC-MS also provides strong confirmation. After the separation on the HPLC column, mycotoxins were ionized via the ionization interface e.g. electrospray or atmospheric chemical ionization etc. Ventura M. et al., 2004 isolated AFs (B1, B2, G1 and G2) in medicinal herbs by single quadrupole mass spectrometry [14].

1.5 Purpose of the study

Thailand is an agricultural country in a tropical region with both a high temperature and humidity level throughout the year. The economy still depends on the successful export of its major agricultural commodities of which rice is mainly produced for export purposes. Regarding, the problems in the export of agricultural products, one of the major factors affecting this trade has been the mycotoxins contamination. Consequently, many agricultural commodities were restricted from being exported with the maximum levels which are quite low level limits. Furthermore, the export to Europe has to comply with the EU regulation under control with the maximum limit. As the result, the exports of agricultural products are obstructed and produce strong economic losses. Thus, development and validation of more effective methods for determination of mycotoxins are urgently requested. Analytical techniques mainly include fast screening method to harmonize with the low levels and confirmatory, quantification in the same time.

In this study, the modified QuEChERS approach was used for the sample preparation of mycotoxin residues analysis in rice in order to find another alternative method which is a non-immunoassay. Fourteen mycotoxins are the representative for determination study of the efficient extraction method. The association of Ultra Performance Liquid Chromatography coupled with tandem mass spectrometry was applied for qualitative and quantitative analysis as well.

CHAPTER II

THEORY

2.1 Ultra Performance Liquid Chromatography (UPLC) [34, 35, 36, 37, 38]

High Performance Liquid Chromatography (HPLC) is a proven technique that has been used in laboratories worldwide. One of the primary drivers for the growth of this technique has been evaluation of packing materials used to effect the separation. The underlying principles of this evaluation are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). Since particle size is one of the variables, a van Deemter curve can be used to investigate chromatographic performance. According to the van Deemter equation, as the particle size decreases to less than 2.5 µm, not only is there a significant gain in efficiency, but the efficiency does not diminish at increased flow rate or linear velocity. By using smaller particles, speed and peak capacity (number of peak resolved per unit time in gradient separations) can be extended to new limits, commercialized by WATERS Corporation (Milford, Massachusetts) as ACQUITY UPLC^{TM} or Ultra Performance Liquid Chromatography. The System will eliminate significant time and cost per sample from the analytical process while improving the quality of the results. By outperforming traditional or optimized HPLC, the UPLC system allows chromatographers to work at higher efficiencies with a much wider range of linear velocities, flow rates, and backpressures. The technology takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rate for increased speed, with superior resolution and sensitivity. The instrument consists of a binary solvent manager, sample manager (including the column heater), detectors, and optional sample organizer.

2.1.1 Pump

The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient mixed under high pressure. There are built-in solvent degassing as well solvent select valves to choose from up to four solvents reservoirs.

There is a 15,000 psi pressure limit (about 1000 bar) to take full advantage of sub-2 μm particles. (Note: 1 psi = 0.069 bar)

2.1.2 Sample introduction (Injector)

Sample introduction is also important. Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure. To protect the column from experiencing extreme pressure fluctuations, the injection process must be relatively pulse-free. Injections can be performed in a full loop mode or in one of two partial loop modes: pressure-assisted or needle-overfill. For full loop injection, the loop is simply overfilled with sample. The injection valve is then switched, placing the loop in line with the column. The pressure-assisted partial loop mode uses a pressurized fluid stream to position the sample plug aspirated from the sample vial into the injection loop. In the needle-overfill partial loop mode, the syringe draws an excess of sample into the needle and through the valve while the loop remains in line with the pump. The valve is switched to bring the loop off line and the syringe then meters the appropriate volume of sample into the loop. The valve is then switched back again to complete the injection. The sample manager also controls the column heater. Column temperature up to the maximum 65 ºC can be attained. A “pivot out” design provides versatility to allow the column outlet to be placed in closer proximity to the source inlet of a MS detector to minimize excess tubing and sample dispersion.

2.1.3 Mobile phase [37]

Solvents are typically chosen based on a compound’s solubility and compatibility with various ionization techniques used in LC/MS. Volatility and the solvent’s ability to donate a proton are important in ESI and other atmospheric ionization techniques. The choice of mobile phase buffer and concentration, particularly at elevated pH, has a profound impact not only on chromatographic peak shape but also on column lifetime. In order to be successful, the method development chemist requires the flexibility to choose the appropriate buffer for selectivity and detection technique. Using of mobile phase with a mass spectrometer must be avoided to use non-volatile additive such as sodium (Na^+), potassium (K^+) or phosphate

$(PO_4)^{3-}$ in order to protect the occurred ion adduct. The volatile additives are compatible with mass spectrometers such as the additive containing ammonium $(NH_4)^+$, acetate, formate or carbonate which are recommended. Using the formic acid as the additive has obtained the low concentration of iron and other metal ions (acetic acid contains a significant amount of iron and other ions). In the ESI ionization mode, buffers and salts (Na^+, K^+, and phosphate) cause a reduction in the vapor pressure and consequently a reduced signal. The increased surface tension of the droplets, and resultant reduction of volatility, can be remedied by using relatively more volatile buffers like ammonium acetate or ammonium formate, formed by a weak acid-base pair.

Solvent considerations

- Solvent in the gas phase limits ionization by ESI to molecules more basic than the solvent. The exception is photo ionization (which is not acid/base ionization) but nonetheless mediated by solvent.
- Removing solvent and water vapor from the ionization region increases types of compounds that can be ionized at atmospheric pressure.
- Reducing liquid volume relative to the sample or analyte of interest contained in the liquid improves ESI performance (i.e. lower flow rates).
- Useful Solvents such as water, acetonitrile, methanol, ethanol, propanol and isopropanol etc.
- Acceptable additives such as acetic acid, formic acid, ammonium hydroxide and ammonium formate/acetate (conc. 10 mM or less) etc.
- Nonvolatile salts (e.g. phosphate, borate, citrate, etc.) can deposit in source and plug capillaries thus requiring more cleaning and maintenance operations.
- Tetrahydrofuran (THF) is highly flammable, so APCI and most interface techniques use nitrogen as the nebulizer gas. (Using air creates an explosion hazard). Moreover, 100% THF reacts with PEEK® tubing.

2.1.4 Stationary phase (Column)

Smaller particles provide not only increased efficiency, but also the ability to work at increased linear velocity without a loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentively as HPLC. In the fundamental resolution (Rs) equation below

$$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{k+1}\right)$$

Where : Rs = resolution

N = number of plate count

α = selectivity

k = peak retention

The relationship also is revealed from the van Deemter plot (in figure 2). As particle size decreases, the optimum flow rate F_{opt} to reach maximum N increases. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressure, and the system properly designed to capitalize on the efficiency gains. A system that can both reliably deliver the requisite pressures and that can maintain the separation efficiency of the small particles with tightly managed volumes.

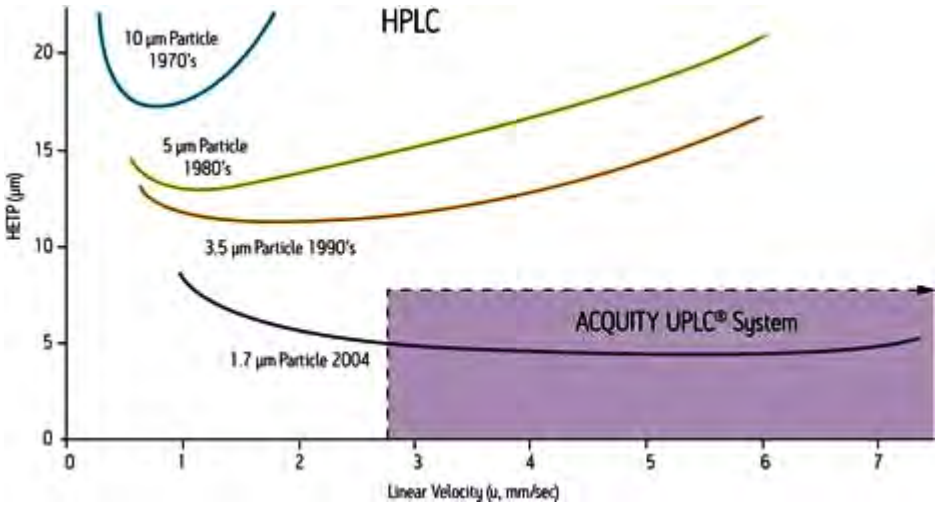


Figure 2.1 Van Deemter plot [37]

However, the analysis speed is the primary objective. Efficiency is proportional to column length and inversely proportional to the particle size. Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher due to the smaller particles and shortening the column by one third (again due to the smaller particle size), the separation is completed in 1/9 the time while maintaining resolution. So if speed, throughput, or sample capacity is a concern, theory can be further leveraged to get much higher throughput. But design and development of sub-2 µm particles is a significant challenge. Although highly efficient, nonporous 1.5 µm particles are commercially available, they suffer from poor loading capacity and retention due to low surface area. Silica-based particles have a good mechanical strength but can suffer from a number of disadvantages, which include a limited pH range and tailing of basic analytes. Polymeric columns can overcome pH limitations, but they have their own issues including low efficiencies, limited loading capacities and poor mechanical strength.

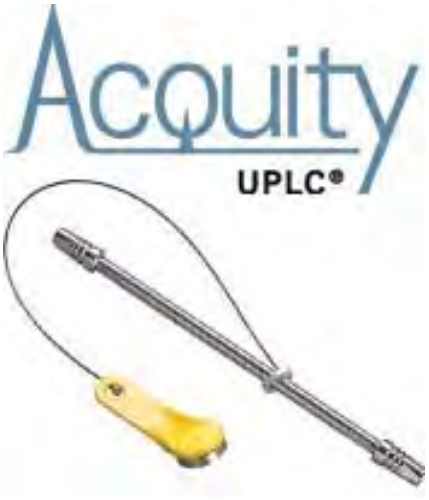


Figure 2.2 Illustration of UPLC column with eCord™ [39]

The Acquity UPLC columns have been created for widely used pH range and pressure tolerant, capable at operating at pressures up to 15,000 psi (1,000 bar). Acquity UPLC columns are available in two particle substrate Ethylene Bridged Hybrid (BEH) and High Strength Silica (HSS). The hybrid organic-inorganic particles are prepared via co-polymerizations which were designed to retain silica's mechanical strength while

overcoming pure silica's tendency to undergo column-damaging hydrolysis in alkaline environments (greater than pH 8). The column chemistries include C8, C18, phenyl and hilic etc. Moreover, eCord^{TM} technology (see figure 2.2) is installed for unique information and used record of each column type.

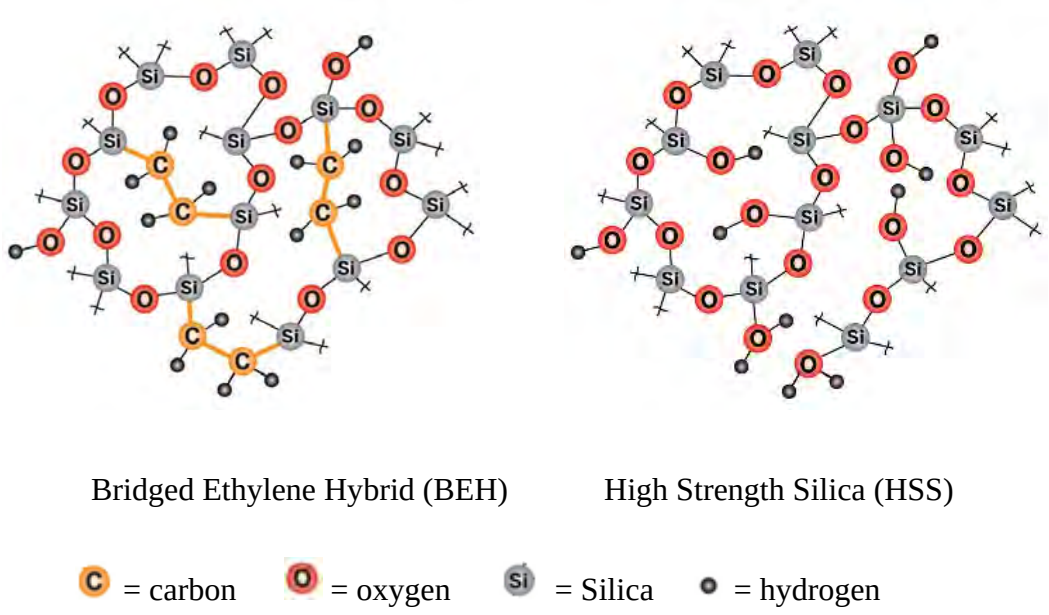


Figure 2.3 The particle profile of Acquity UPLC column [36]

The first acquity UPLC particle created was the 1.7 µm **Bridged Ethylene Hybrid (BEH)** particles which contain C-C bridges between pairs of silica atoms. (see figure 2.3) BEH's covalently bonded Si-C-C-Si units render the hybrid material chemically stable up to a pH of 12. The second acquity UPLC column type is the particle 1.8 µm **High Strength Silica (HSS)** particle which is 100% silica particle specially designed for tolerable pressure up to 15,000 psi or 1,000 bar (see figure 2.3).

2.2 Mass Spectrometry (MS/MS) [40, 41, 42]

Mass spectrometry is an analytical technique that can provide both qualitative (structure) and quantitative (molecular mass or concentration) information on analyte molecules after their conversion to ions. The molecules of interest are first introduced into the ionization source of the mass spectrometer, where they are first ionized to acquire positive or negative charges. The ions then travel through the mass analyzer and arrive at different parts of the detector according to their mass/charge (m/z) ratio.

After the ions make contact with the detector, useable signals are generated and recorded by a computer system. The computer displays the signals graphically as a mass spectrum showing the relative abundance of the signals according to their m/z ratio.

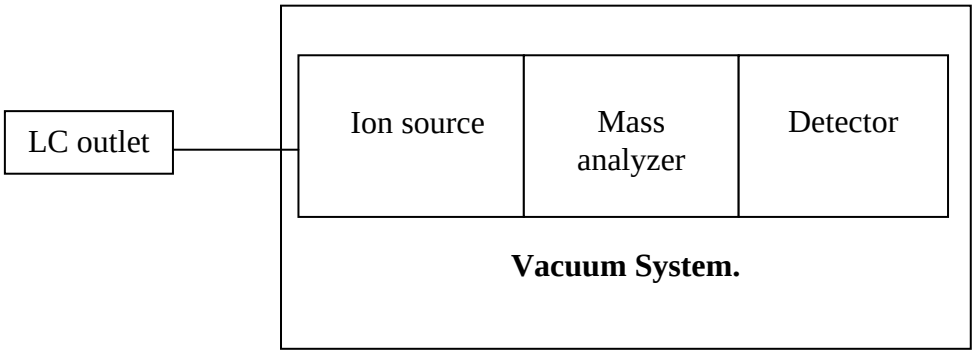


Figure 2.4 Schematic diagram of MS system.

2.2.1 Ionization Interface technique.

Compounds eluting from the LC column are then introduced to the mass spectrometer via a specialized interface. **"Atmospheric Pressure Ionization, API"** is a soft ionization technique that provides quasi-molecular information. The two most common interfaces used for ionization are the electrospray ionization (ESI) and the atmospheric pressure chemical ionization (APCI) interfaces.

2.2.1.1 Electrospray Ionization (ESI)

ESI is a technique used in mass spectrometry to overcome the propensity of macro molecules to fragment. In ESI, as in figure 3 below, a liquid is pushed through a very small charged metal capillary by a carrier gas. The liquid contains the substance and the analyte, as well as a large amount of solvent, which is usually much more volatile than the analyte. The charge contained in the capillary transfers to the liquid which charges the analyte molecule. As like charges repel, the liquid pushes itself out of the capillary and forms a mist or an aerosol of small droplets about 10 μm diameters, to increase the distance between the similarly charged molecules. A nitrogen gas is sometimes used to evaporate the neutral solvent in the small droplets; this in turn brings the charged analyte molecules closer together. The proximity of the

molecules becomes unstable, however, and as the similarly charged molecules come closer together, the droplets once again explode. This process repeats itself until the analyte is free of solvent and is a lone ion. The lone ion will then continue along to a mass analyzer.

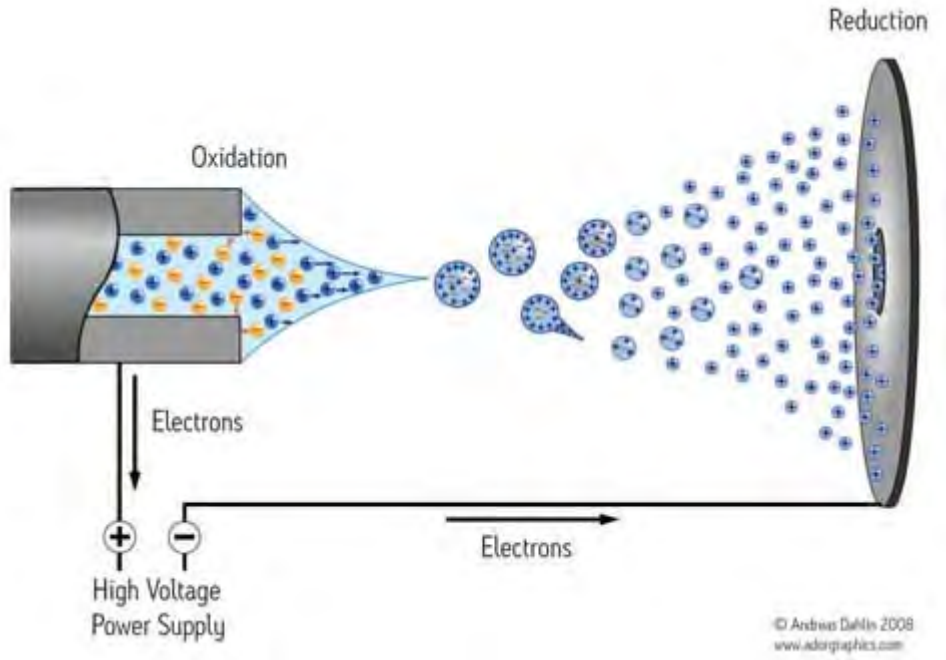


Figure 2.5 : Illustration of the mechanism of ion formation in ESI [43]

2.2.1.2 Atmospheric Pressure Chemical Ionization (APCI)

APCI is a technique which creates ions at atmospheric pressure. A sample solution flows through a heated tube where it is volatilized and sprayed into a corona discharge needle with the aid of nitrogen nebulization (see schematic illustration below). Ions are produced in the discharge and extracted into the mass spectrometer. APCI is best suited to relatively polar, semi-volatile samples. An APCI mass spectrum usually contains the quasi-molecular ion, $[M+H]^+$. This technique is used as an LCMS interface because it can accommodate very high liquid flow rate (>1 mL/min). The formation of positive ions is generally by proton transfer, while negative ions are formed by electron attachment.

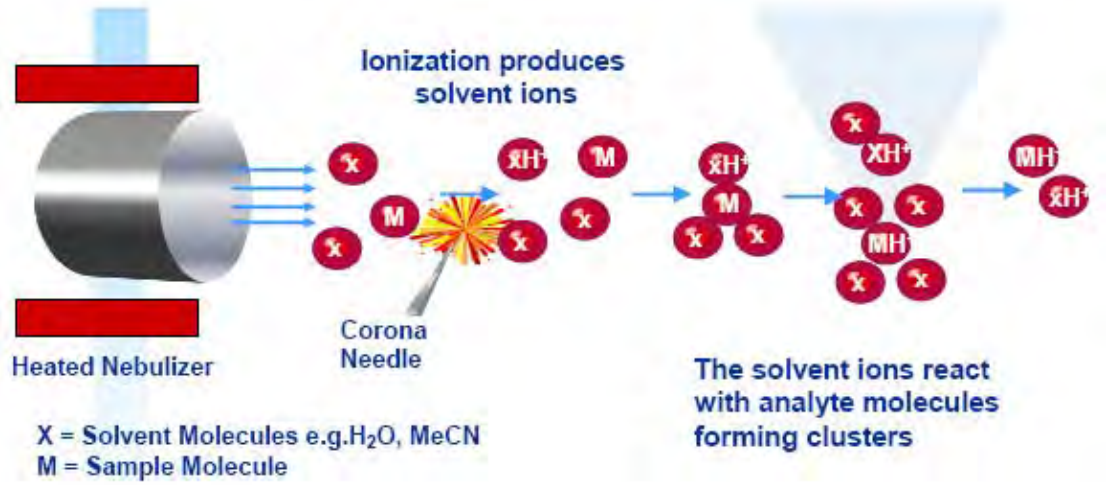


Figure 2.6 Illustration of the mechanism of ion formation in APCI [44]

2.2.2 Mass Analyzer

A quadrupole mass analyzer is most commonly used. In such a system, an assembly of 4 parallel metal rods is kept at equal distance (see figure 2.7). Each pair of opposite rods is connected electrically. An equal but opposite DC voltage superimposed with a radio frequency (RF) AC voltage is applied to the diagonally placed pair of rods. The resulting electrical field causes the ions to travel forward in the z direction with oscillatory motion in the x-y plane. The amplitude of oscillation bears a unique relationship with the m/z ratio and can be controlled by changing the DC and RF voltages simultaneously in a pre-fixed ratio. These DC and RF voltages can be set so that amplitudes of oscillation for desirable m/z ratios are “stable” with the ions traveling along the z-axis without hitting the quadrupole rods, and finally reaching the detector. On the other hand, the oscillatory amplitudes of undesirable ions are large and “unstable”; they hit the metal rods, get neutralized, and fail to reach the detector. Quadrupole mass analyzers are robust, economical, physically small, and more readily interfaced with a wide variety of inlet systems when compared with other conventional mass analyzers like the magnetic sector.

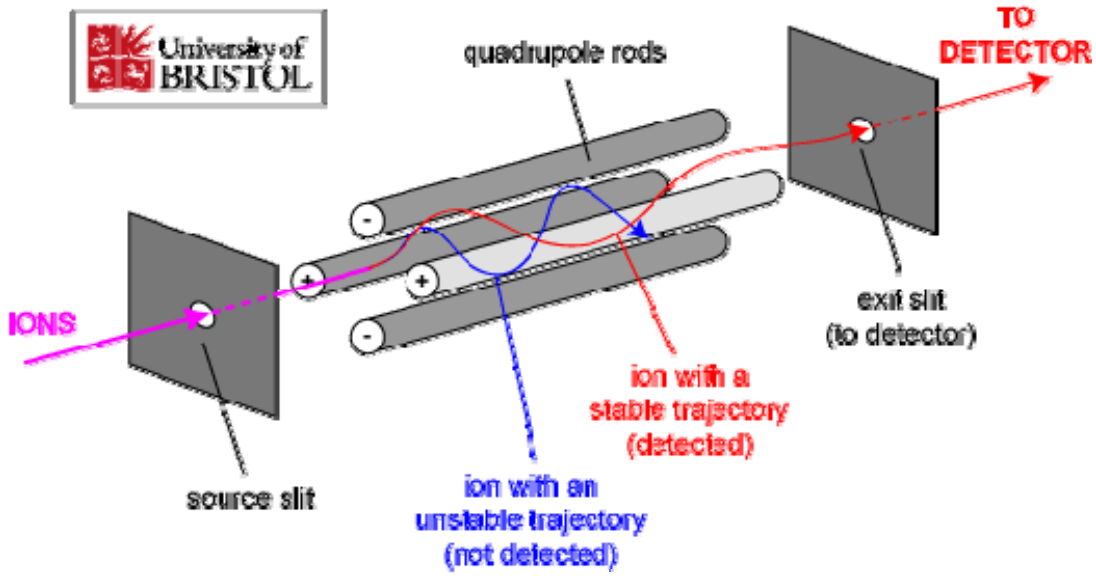


Figure 2.7 Schematic of a quadrupole mass analyzer [40]

2.2.3 MS/MS Analysis

In a typical tandem quadrupole system there are two quadrupoles set up in a linear design, often called “triple-quad” (shown in figure 2.8).

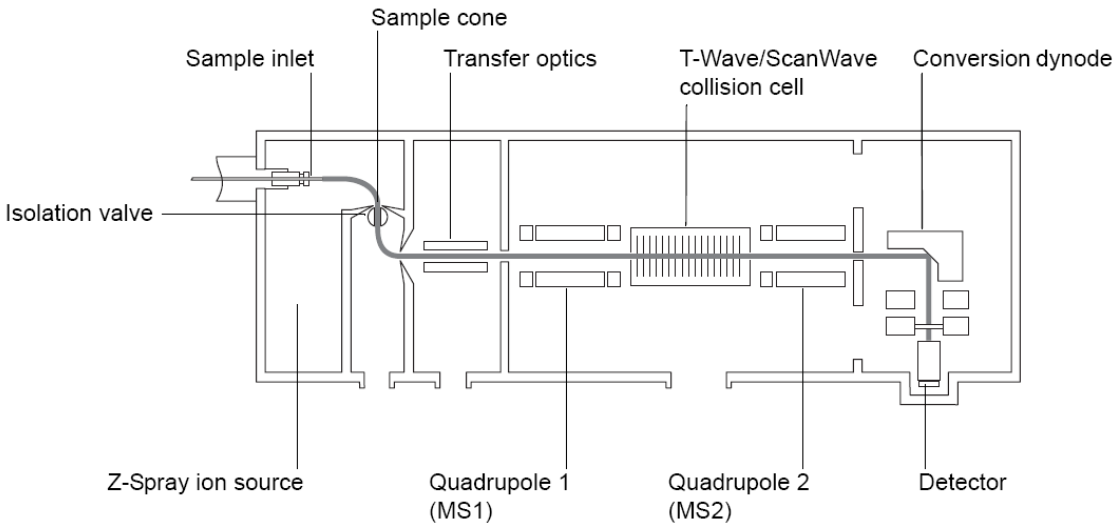


Figure 2.8 Schematic overview of triple quadrupole mass spectrometer [45]

The analyte ion of interest (usually called the precursor ion) is mass-selected by the first quadrupole (MS1) and allowed to collide with a collision gas (usually argon) in a collision cell, where the precursor ions are activated by collision and undergo further fragmentation. This process is known as collision-induced dissociation (CID). The product ions resulting from CID are related to the molecular structure of the ions and can be monitored by the second quadrupole mass analyzer (MS2) providing structural information of the molecular ions. This tandem system is commonly denoted as MS/MS in the literature. When MS1 is set to select only one specific m/z ratio, it filters out other molecular ions having different m/z ratios. This is a "purification" step inside the MS system, eliminating complicated and time-consuming sample purification procedures prior to MS analysis. There are many operating modes as described in section 2.2.3.1 to 2.2.3.4 below.

2.2.3.1 Precursor ion scan mode

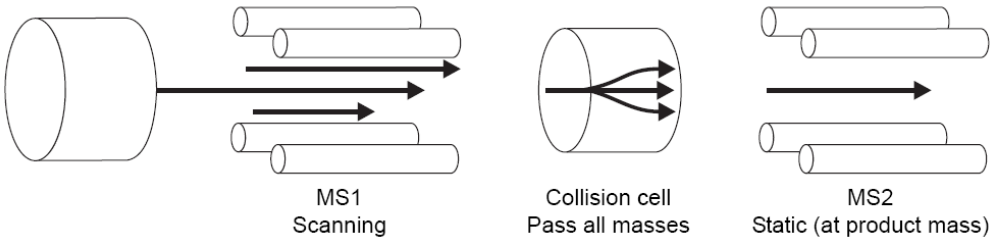


Figure 2.9 Schematic of precursor ion scan mode [45]

This mode is used to determine what higher mass has a similar fragment ion. The second quadrupole isolates a specific fragment ion and the first quadrupole is scanned to pass a wide range of ions to the collision cell for fragmentation.

2.2.3.2 Product ion scan mode

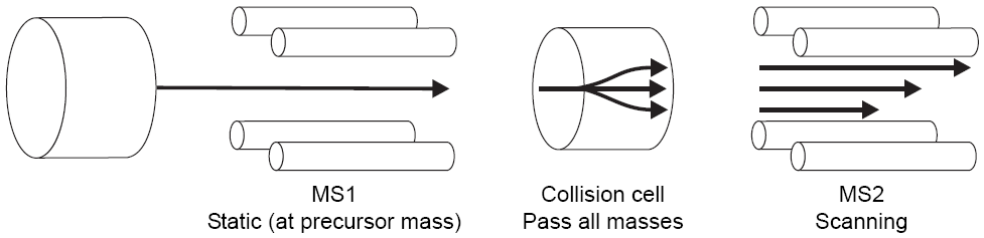


Figure 2.10 Schematic of product ion scan mode [45]

The first quadrupole is static, passing a selected m/z into the collision cell. A voltage is applied in the collision cell to accelerate the ion into the collision gas (argon); this process results in fragmentation. The ions from the fragmentation pass into the second quadrupole which is scanned to acquire all of the ions.

2.2.3.3 Constant Neutral loss

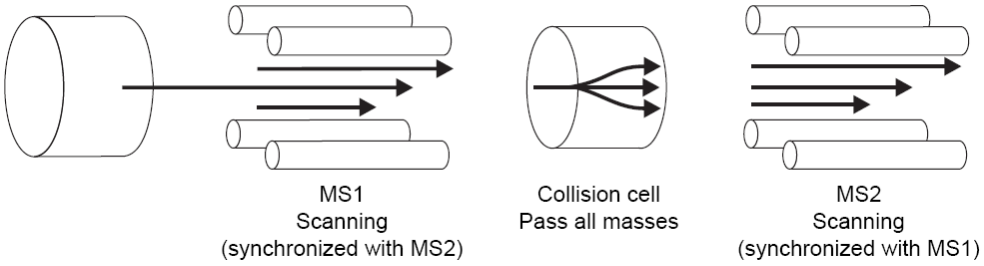


Figure 2.11 Schematic of constant Neutral loss [45]

This analysis mode detects the loss of a specific neutral fragment or functional group from an unspecified precursor. Both quadrupoles are operated in scanning mode. Constant Neutral Loss mode is useful in determining which compounds have a similar fragmentation pattern.

2.2.3.4 Multiple Reactions Monitoring (MRM)

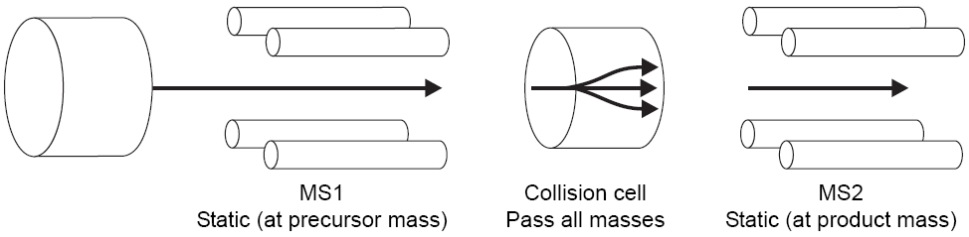


Figure 2.12 Schematic of Multiple Reactions Monitoring [45]

This mode has no scanning taking place during Multiple Reaction Monitoring (MRM). The Q1(MS1) and Q2(MS2) quadrupoles only allow transmission of a specified precursor ion which gives a specified product ion to be monitored. The data is usually viewed as a chromatogram over time, rather than a summed spectrum. This is the most selective and sensitive mode because only a specific ion which fragments to produce the specific product ion will be monitored for the whole of the scan time cycle rather than part of it. Moreover, a greater dwell time on the ions of interest is possible and therefore better sensitivity is achieved. For this reason, the sensitivity is raised up as well. MRM can eliminate the interference of matrix in the samples, reducing the amount of sample preparation required before analysis. MRM is the primary method of data recording used by triple quadrupole mass spectrometers. It is used for analysis of pharmaceutical drugs, or pesticides in environmental studies as well as many other areas of analysis.

2.2.3 Detector

The detectors are located at the exit end of the quadrupole mass filter. It receives the ions that have passed through the mass filter. The detector generates an electronic signal proportional to the number of ions striking it.

2.2.3.1 The photomultiplier detector, the Whisper Dynolite photomultiplier detector is installed for Quattro Premier^{TM} XE in order to reduce detector noise for both positive and negative ion modes, and improved sensitivity in negative ion mode. The photomultiplier detector is enclosed in its own vacuum envelope and requires minimal maintenance.

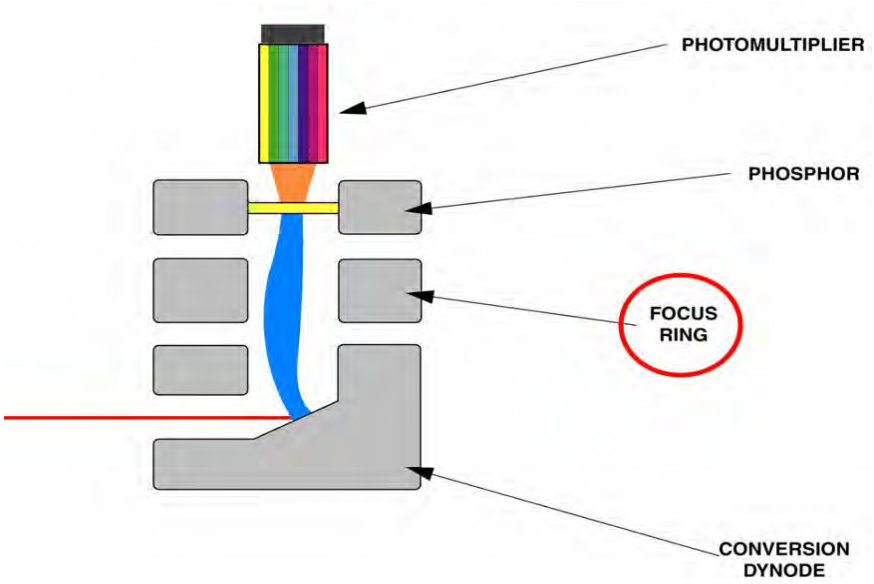


Figure 2.13 Schematic of Whisper Dynolite photomultiplier detector design on MS/MS (Quatro Premier XE, micromass by WATERS corporation) [45]

The photomultiplier tube is commonly used as a detector. It consists of a photo emissive cathode (a cathode which emits electrons when struck by photons of radiation), several dynodes (which emit several electrons for each electron striking them) and an anode. A photon of radiation entering the tube strikes the cathode, causing the emission of several electrons. These electrons are accelerated towards the first dynode. The electrons strike the first dynode, causing the emission of several electrons for each incident electron. These electrons are then accelerated towards the second dynode, to produce more electrons which are accelerated towards dynode three and so on. Eventually, the electrons are collected at the anode. By this time, each original photon has produced 10^6 - 10^7 electrons. The resulting current is amplified and measured. Photomultipliers are very sensitive to UV and visible radiation. They have fast response times. Intense light damages photomultipliers; they are limited to measuring low power radiation. Photomultiplier tube is very sensitive and has very fast response times.

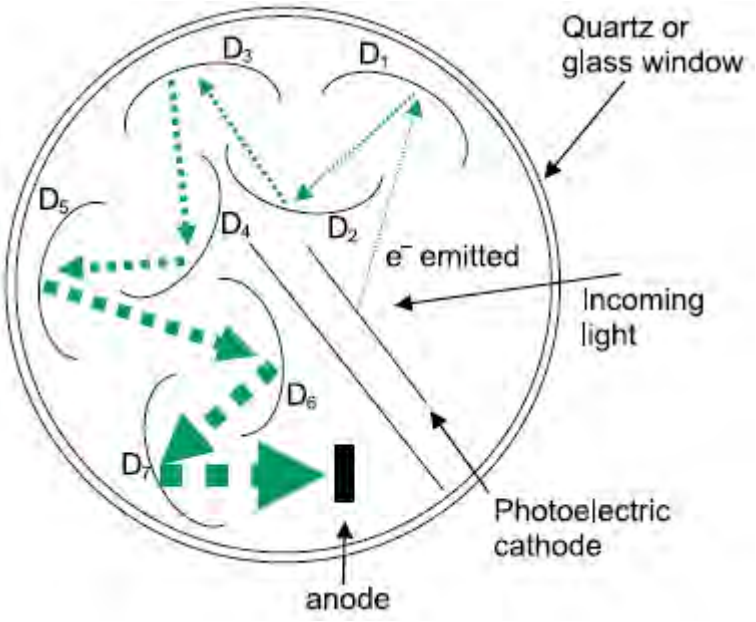


Figure 2.14 Cross section of a photomultiplier tube [46]

2.2.3.2 The detector consists of a High Energy Dynode (HED) and an electron multiplier (EM) detector. The HED is located off axis from the centre of the quadrupole to minimize signals due to photons, neutrals and electrons coming from the ion source. Positive ions are attracted to the HED from the quadrupole and cause electrons to be emitted. Electrons are then attracted into the more positive EM horn. Once the electrons hit the side of the horn more electrons are emitted from the surface, every electron impact releases even more electrons, causing a cascade. A signal current is generated by the detector proportional to the number of ions striking it. The detector and mass filter operate under high vacuum (10^{-6} Torr) to allow the ions to travel unimpeded to the detector.

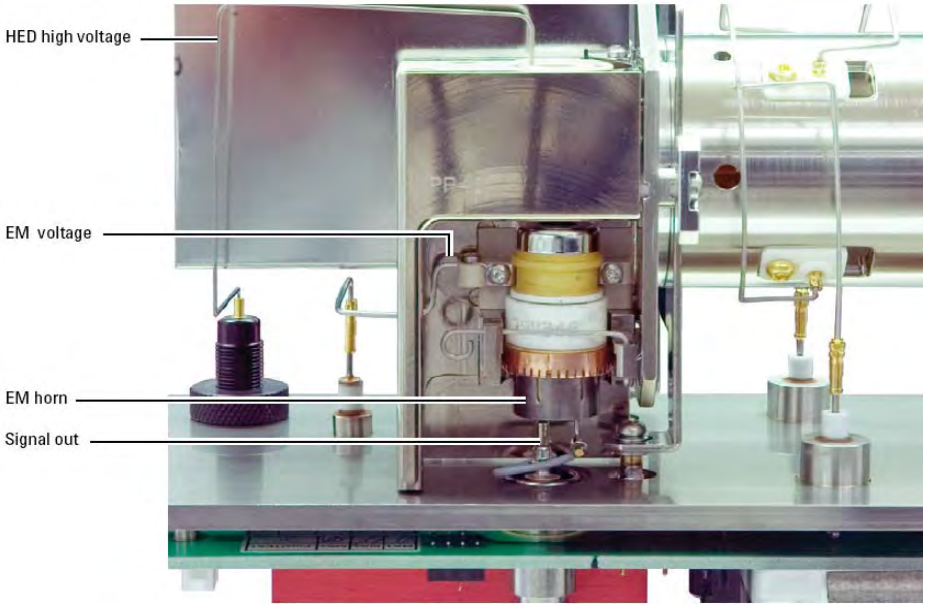


Figure 2.15 HED and EM horn by Agilent Technologies [42]

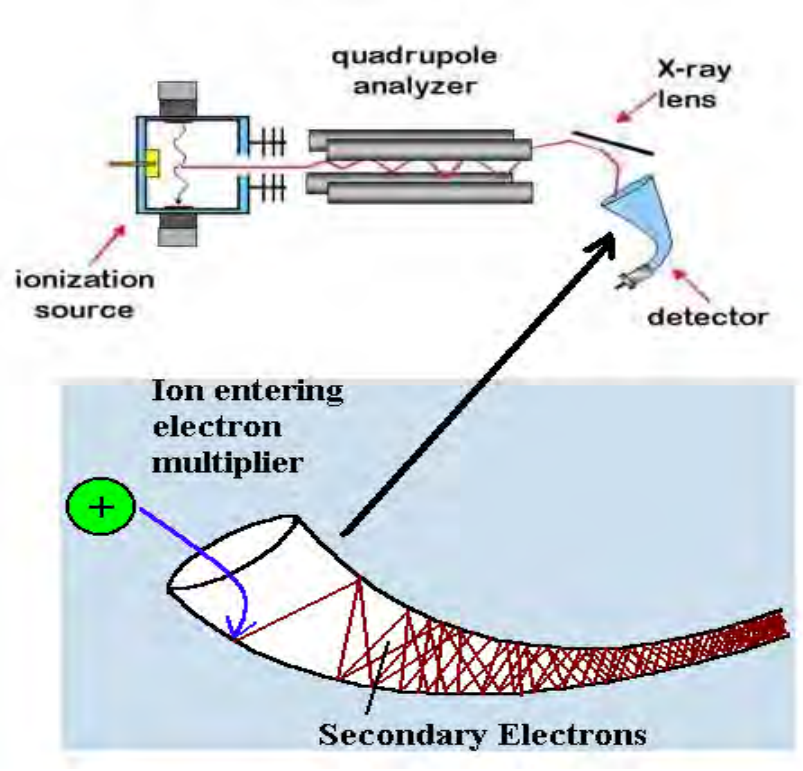


Figure 2.16 Schematic of signal generation by EM detector [47]

2.3 Sample preparation technique - clean up

Sample clean-up is the removal of substance in the sample extract which may interfere in the sample of the analysis. Usually, the target analyte is also preconcentrated by reduction of the amount of solvent in order to have enough analytes's concentration to detection.

2.3.1 Immuno Affinity columns (IAC) [10, 25, 48]

IAC have been used for many years as a method of sample purification. The principle of the IAC is relatively simple in that an antibody raised against the analyte is immobilized on a gel, and generally about 0.5-0.5 mL of gel is packed into a small plastic column. The phosphate buffered saline (PBS) is applied for condition of the column then the extract sample is loaded into the cartridge and made to slowly flow at around 1-2 mL/min. The analyte becomes bound to the antibody in the IAC gel as well. The column capacity in terms of the total quantity of antibodies site available for binding will also be important as overloading the column will induce poor recovery. For washing step, the PBS is used to completely remove any co-extractives. Finally, the breaking of antibody-antigen is employed by a small volume of methanol and acetonitrile.

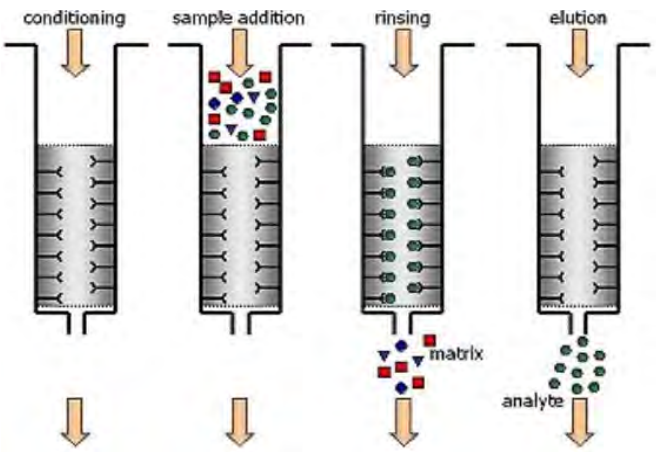


Figure 2.17 Basic principle of immuno affinity column (IAC) [10]

There are many advantages of IAC such as no interference, simplicity, good recovery and reproducibility, even though the drawbacks of technique are restricted for usage

as well. For instance, a short shelf-life of antibody, storage of the IAC (must be storage in the refrigerator), expensive, need a further instrument for mycotoxins determinations and specificity etc.

2.3.2 QuEChERS method [27, 28, 29, 49]

The extraction technique called QuEChERS (standing for quick, easy, cheap, effective, Rugged and Safe). The original method has been used for the analysis of pesticide residues in foods and agricultural products. In addition to using less solvent materials versus conventional solid phase extraction (SPE) methods, QuEChERS employs a novel and much quicker dispersive solid phase extraction (d-SPE) cleanup. There have been several modifications of the technique depending on analytes, matrices, instrumentation and analyst preferences. Basically, there are three important steps of the sample extraction which are followed according to the steps below:

Step 1: Sample preparation and extraction – Commodities are uniformly crushed and acetonitrile solvent is added for a shake extraction. Salts, acids, and buffers may be added to enhance extraction efficiency and protect sensitive analytes. Internal standards can be added to monitor extraction efficiencies.

Step 2: Extract cleanup – A subsample of solvent extract is cleaned up using d-SPE, a key improvement incorporated in the QuEChERS technique. Small polypropylene centrifuge tubes are prefilled with precise weights of magnesium sulphate ($MgSO_4$) and d-SPE adsorbents to remove excess water and unwanted contaminants from the extracted samples. After agitation and centrifugation, the cleaned extracts are ready for analysis. At this stage, the d-SPE is generally mentioned to primary secondary amine (PSA), aminopropyl (NH_2), octadecyl (C18), Alumina-neutral (Al-N), Florisil (Fl) and Silica (Si).

2.3.2.1 Primary Secondary Amine (PSA)

Ethylenediamine-N-propyl exchanged material (PSA) is a polymeric base sorbent that contains both primary and secondary amines. The structure (in figure 2.13) performs as weak anion exchanger sorbent with pKa 10.1 and 10.9. The PSA

functional group is a very good bidentate ligand, making PSA an excellent sorbent for chelation. Its higher carbon content makes it a more non-polar sorbent than NH_2 and thus a better choice for very polar compounds that retain too strongly on NH_2 sorbent.

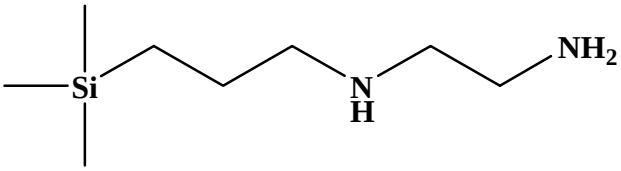


Figure 2.18 The chemical structure of primary secondary amines.

It has a strong affinity and high capacity for removing fatty acids, organic acids, some polar pigment, sugars and some other matrix co-extractives that from hydrocarbon that might act as instrumental interferences.

2.3.2.2 Aminopropyl (NH_2)

NH_2 is a very polar sorbent. It can utilize both hydrogen bonding and anion exchange. Since the pKa of the NH_2 sorbent is 9.8, at any pH below 9.8 the majority of the functional groups are positively charged (shown in figure 2.14). NH_2 is a weak anion exchanger because it is a quaternary amine sorbent that is always charged and it is therefore a better sorbent choice for retention of very strong anions, such as sulfonic acids. Because an ethyl group supports the NH_2 functionality, it can be used for non-polar isolations from polar samples, but its strong polarity is its primary characteristic like diol and silica (Si), NH_2 is excellent for separation of structural isomers.

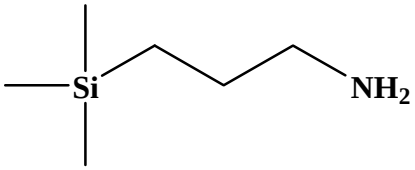


Figure 2.19 The chemical structure of aminopropyl.

PSA, NH_2 and alumina-neutral (Al-N) interact with chemicals by hydrogen bonding, and removed similar types of compounds, including fatty acids, other organic acids, and to some extent various sugars and pigments. PSA removed more matrix co-extractives than NH_2 and Al-N per given quantity because PSA has higher capacity due to the presence of amines.

2.3.2.3 Octadecyl (C18)

C18 is the most hydrophobic silica-based sorbent available (shown in figure 2.20). It is the most popular SPE sorbent because of its extremely retentive nature for non-polar compounds. C18 is generally regarded as the least selective silica based sorbent, since it retains most organic analytes from aqueous matrices. The potential for polar interactions between analytes and sorbent is less significant with C18 than with any other sorbent because of the predominant effect of the long hydrocarbon chain. C18 is suggested to use for removing of non polar interferences such as fat.

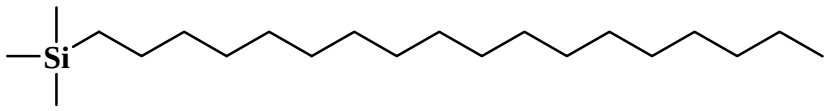


Figure 2.20 The chemical structure of octadecyl.

2.3.2.4 Alumina-neutral (Al-N)

Al-N sorbents (with 40 μm particle size) can adsorb molecules by interaction with the aluminum metal center, hydrogen bonding with the surface hydroxyl groups, or by ion exchange if the surface carries a charge. The neutralized surface allows interaction with compounds whose heteroatoms are electronegative (e.g. N, O, P, S) or with an electron-rich, the π- electrons of aromatic structure.

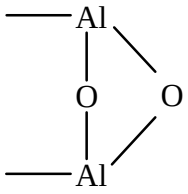


Figure 2.21 The chemical structure of Alumina-neutral

2.3.2.5 Florisil (Fl)

Fl is a selective magnesium-silica adsorbent, extremely polar in nature and ideal for the isolation of polar compounds from non-polar matrices. The larger particle size of the florisil material enables fast flow of large volume samples and thus is a potential alternative to silica when using viscous samples. Florisil is utilized for the separation of chlorinated pesticides residues prior to identification and measurement.

$$MgO_2Si$$

Figure 2.22 The chemical structure of Florisil

2.3.2.6 Silica (Si)

Si is generally regarded as the most polar (e.g. hydrogen boding) sorbent available. The silanol groups are ionizable, so they can be used as a cation exchanger at intermediate pH value). Si is one of the best sorbents available for selectively separating compounds of very similar structure. Applying the analytes in a non-polar solvent, then subtly increasing the solvent polarity at each step by adding increasing concentrations of polar modifiers, such as THF or ethyl acetate, can accomplish this separation.

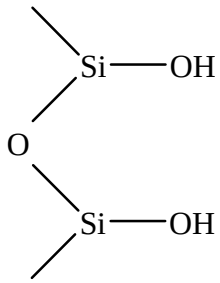


Figure 2.23 The chemical structure of Silica

Step 3: Sample analysis – Samples may be pH adjusted to protect sensitive analytes and/or solvent-exchanged to improve analysis by either GC/MS or LC/MS. Internal standards can be added.

CHAPTER III

EXPERIMENTAL

3.1 Instrument and apparatus

3.1.1 Ultra Performance Liquid Chromatography (UPLC): Acquity UPLC™ system consists of degasser, binary high pressure mixing pump, autosampler and column thermostat from WATERS Corporation, Milford, Massachusetts, U.S.A.

3.1.2 Mass spectrometry detector (MSD): Quattro Premier™ XE with electrospray ionization interface from Micromass WATERS Corporation, Milford, Massachusetts, U.S.A.

3.1.3 The Edwards XDS series Dry Vacuum Scroll Pump conversion by BOC Edwards, Wilmington, MA, U.S.A.

3.1.4 Liquid Nitrogen, pressure 100 psi from TIG, Bangplee, Samutplakarn, Thailand.

3.1.5 Argon cylinder, pressure 20 psi from Lab Gas, Thailand.

3.1.6 Column: Acquity UPLC™ BEH C18 column, 2.1 x100 mm, 1.7 µm. from WATERS Corporation, Milford, Massachusetts, U.S.A.

3.1.7 ACQUITY BEH C18 VanGuard™ Pre-column, 1.7 µm, 2.1 x 5 mm from WATERS Corporation, Milford, Massachusetts, U.S.A.

3.1.8 Milli Q, Ultrapure water system with from Millipore, Billerica, MA, U.S.A.

3.1.9 A glass filter set include 300 mL funnel, 1 L suction flask, glass base with tube cap and 47 mm spring clamp (for mobile phase filtration) from Millipore, Billerica, MA, U.S.A.

3.1.10 Nylon filters membrane 47 mm, 0.2 µm from Whatman International Ltd, Maid stone, England.

3.1.11 Nylon syringe filter 13 mm, 0.2 µm from Chrom Tech Inc., AppleValley, MN, U.S.A.

3.1.12 Filter papers Whatman No.1 with diameter 15 cm., Whatman International Ltd , maidstone, England.

3.1.13 Glasss microfiber filters 934-AH with diameter 11 cm., Whatman International Ltd , maidstone, England.

3.1.14 Vortex mixer, model GENIE 2 from Scientific Industries, Bohemia, New York, U.S.A.

3.1.15 Micropipetts : volume 0.1-10, 10-100, 25-200, 100-1000 µl and 1-5 ml with tips from Eppendorf, Hamburg, Germany.

3.1.16 Centrifuge model Allegra^{TM} X-12 from Beckman Coulter Inc., Brea, CA, U.S.A.

3.1.17 Electronic balance 2digits and 4 digits from Mettler Toledo, Prague, Czech Republic.

3.1.18 Ultra sonic bath model 8200 from Branson Ultrasonic Corporation, Danbury, CT, U.S.A.

3.1.19 Shaker from Gerhardt GmbH & Co.KG, Königswinter, Germany.

3.1.20 N_2 evaporator model N-EVAP^{TM} 12, organomation Associates, Inc., MA, U.S.A.

3.1.21 HPLC vial 2 mL with slit Teflon cap.

3.1.22 Amber bottle 75 mL with screw Teflon cap.

3.1.23 Volumetric flask volume class A 10, 50 mL

3.1.24 Teflon centrifuge tube 50 mL

3.1.25 Beaker 10, 100, 1000 mL

3.1.26 Graduated cylinder 100 mL

3.1.27 Test tube 15 mL with screw cap

3.1.28 Dispersive solid phase extraction (d-SPE)

- PSA powder and NH_2 powder, 40 µm from Varian, Oxfordshire, UK.
- C18 powder, 40 µm from Merck, Darmstadt, Germany
- Alumina-neutral (Al-N), Florisil (Fl) and Silica (Si) from WATERS Corporation, Milford, MA, U.S.A.

3.1.29 Immuno Affinity Column (IAC)

- AFLAPREP®, DONPREP® and ZEAPREP® from R-Bipharm Rhöne Ltd, Glowgow Scotland

- AflaTest P®, DonTest®, OchraTest® and ZearalaTest® from Vicam L.P., Watertown, MA, U.S.A.
- AflaStar™, DonStar ™, OchraStar ™ and ZearaStar ™ from Romer Labs® Inc, MO, U.S.A.

All glassware was rinsed with acetone before analysis in order to prevent residue contamination in the glassware.

3.2 Chemical

3.2.1 Mycotoxins standards

Aflatoxin B1 (AF-B1, CAS no.116355-83-0, purity 99.0%), Aflatoxin B2 (AF-B2, CAS no.13434-14-4, purity 98.5%), Aflatoxin G1 (AF-G1, CAS no.1165-39-5, purity 99.0%), Aflatoxin G2 (AF-G2, CAS no.7241-98-7, purity 99.5%), Fumonisin B1(FUM-B1, CAS no.116355-83-0, purity 98.0%), Fumonisin B2 (FUM-B2, CAS no.116355-84-1, purity 98.0%), Citrinin (CIT, CAS no.518-75-2, purity 99.0%), T-2 toxin (T-2, CAS no.21259-20-1, purity 99.5%), Deoxynivalenon (DON, CAS no.51481-10-8, concentration 100 μg/mL in acetonitrile), Zearalenone (ZEA, CAS no.17924-92-4, purity 99.0%) were purchased from Sigma-Aldrich (St.Louis, MO, U.S.A.). Whilst, Ochratoxin A (OTA, CAS no.303-47-9, purity 99.0%) and Ochratoxin B (OTB, CAS no.4825-86-9, concentration 50 μg/mL in benzene: acetic acid 99:1) were purchased from Supelco (Bellefonate, PA, U.S.A.). and Nivalenon (NIV, CAS no.23282-20-4 purity 97.0%) and HT-2 toxin (HT-2, CAS no.26934-87-2, concentration 100 μg/mL in acetonitrile) were purchased from WAKO (Richmond, VA, U.S.A).

3.2.2 Organic solvents

Acetronitrile (ACN) and Methanol (MeOH) LC-MS grade (JT breaker chemical company, Deven, Holland), Acetonitrile and acetone pesticide grade (Kanto, Tokyo, Japan) and 99-100% purity of formic acid and Poly Ethylene Glycol (PEG), (BDH, Briare, France).

3.2.3 Reagents

Magnesium sulphate (Panreac, Barcelona, Spain), Sodium Citrate dibasic sesquihydrate and ammonium formate (Fluka, Steinem, Germany), Sodium Citrate tribasic dehydrate (Riedel-deHaën, Austria), Sodium chloride (RFCL, New Delhi, India) and sodium acetate (Merck, Darmstadt, Germany). Phosphate Buffer Saline (PBS) in tablet (R-Biopharm Rhöne Ltd , Glasgow, Scotland).

3.3 Preparation of standard solution

3.3.1 The stock standard solutions, 200 mg/L

The primary standard of mycotoxin were prepared in different solvents (acetonitrile or methanol) by weighing 0.01 g (to nearest 0.0005 g) of the individual standard of AF-B1, AF-B2, AF-G1, AF-G2, OTA and T-2 into 50 mL volumetric flask then making up the volume with acetonitrile, whilst, the FUM-B1, FUM-B2, NIV, CIT and ZEA were prepared in methanol. Each standard solution was transferred to the amber bottle and kept in the fridge at a temperature of less than -10 °C. Particularly, OTB (section 3.2.1) was changed the solution phase to dissolve in acetonitrile by taken solution to dryness with N_2 evaporator. Add 1 mL of acetonitrile into the vial. The final concentration of OTB is 50 µg/mL in acetonitrile.

3.3.2 The individual standard for tuning, 5 mg/L

AF-B1, AF-B2, AF-G1, AF-G2, OTA, T-2, FUM-B1, FUM-B2, NIV, CIT and ZEA were individually prepared by diluting 25 µL of standard 200 mg/L (section 3.3.1) into the 2 mL vial, add 975 µL of organic solvent (the same as the dissolved stock standard solution) and vortex for a few minutes.

DON and HT-2 standard were individually prepared by diluting 50 µL of standard 100 mg/L into the 2 mL vial, add 950 µL of ACN and vortex for a few minutes.

OTB standard was prepared by diluting 100 µL of standard 50 mg/L (section 3.3.1) into the 2 mL vial, adding 900 µL of acetonitrle and vortex for a few minutes.

3.3.3 The mixed working standard solutions

The standard mycotoxins were classified into two groups due to the sensitivity of the compounds.

Group A : A mixture of AF-B1, AF-B2, AF-G1, AF-G2, FUM-B1, FUM-B2, OTA, OTB, CIT, HT-2, T-2 and ZEA at the concentration level 5 mg/L by pipette each standard 250 µL of stock standard 200 mg/L and 1000 µL of 50 mg/L, OTB in 10 mL volumetric flask and making up the volume with acetonitrile. The mixture standard solution was transferred into the amber bottle and store in the freezer at the temperature at below -10 °C.

Group B : A mixture of DON and NIV at the concentration level 50 mg/L by pipette 5000 µL of 100 mg/L, DON and 2500 µL of 200 mg/L, NIV into 10 mL volumetric flask and making up the volume with methanol. The mixture standard solution was transferred into the amber bottle and stored in the freezer at the temperature at below -10 °C.

The mixtures of working standard solution (group A and group B) were employed for preparation of the standard calibration curve and the fortified sample.

3.4 The optimization of instrumental analysis condition

The UPLC-MS/MS from WATERS Corporation was employed along with the MRM analysis via the switching of electrospray for positive and negative ionization mode.

3.4.1 UPLC conditions

The binary pump has to be installed for the UPLC system. Two lines of mobile phase was shown as line A which is an aqueous solution (5 mM ammonium formate with 0.5% formic acid) and another line was shown as a line B (organic solvents). The separation of all mycotoxins was performed by Acquity UPLC^{TM} BEH C18 (2.1 mm x 100 mm, 1.7 µm) column from WATERS Corporation. The analysis was completed by using a time gradient program as initial/(95:5), 2 mins/ (95:5), 3.5

mins/(30:70), 5.5 mins/(30:70), 6 mins/(5:95), 8 mins/(5:95), 8.2 mins/(5:95) and carried on the last gradient to 11 mins with the flow rate 0.25 mL/mins. While for the injection volume is 5 µL with needle-overfill partial loop mode of injection. Furthermore, the column and sample temperature are controlled at 40 and 20 ºC, respectively. In the first stage, the type of organic solvent (line B) was evaluated in order to optimize the conditions of UPLC. Whilst, 5 mM ammonium formate with 0.5% formic acid was fixed as line A. Three parameters varied as shown in table 3.1 below.

Table 3.1 Demonstration of organic solvent of mobile phase.

Condition No.	Organic solvent
UPLC_1	100% acetonitrile
UPLC_2	100% methanol
UPLC_3	1:1 = acetonitrile : methanol

The chromatographic of the optimization of mobile was compared and described by peak shape of mycotoxins standard at concentration levels 0.5 mg/L of group A standard and 2.5 mg/L of group B standard as shown in appendix A.

3.4.2 MS/MS conditions [17]

The Micromass Quattro Premier^{TM} XE is a triple quadrupole mass spectrometer, that is used via the ionization interface of electrospray (ESI) in the positive (ESI+) and negative mode (ESI-). Single mycotoxin standard solutions (section 3.3.2) were introduced for ion tunings via the syringe pump. Nitrogen gas was used with the cone voltage for fragmentation of the precursor ions and argon gas was also supplied into the collision cell for breaking up the precursor ions by the collision energy in order to create other product ions later. All the parameters were compromised for the best sensitivity.

After the optimized conditions, multiple reactions monitoring mode (MRM) was selected for quantification and qualification of analysis by setting up the precursor ions and product ions. The mixed standard solution at concentration level 0.1 mg/L was injected to verify the sensitivity and selectivity of mycotoxin standards.

3.5 Development of Extraction Method

The sample preparation based on the modified QuEChERS extraction method. Negative rice samples were fortified at different concentration levels. The standard solution of group A (section 3.3.3) was spiked into the sample at the first stage of extraction procedure with concentration levels 0.02 mg/kg. While the standard solution of group B (section 3.3.3) was spiked into the same sample with concentration levels at 0.1 mg/kg. These concentration levels were used for the entire investigation. Four replicates were done in each test method.

3.5.1 The acidity of extraction solvent

Indeed, the principle of QuEChERS method is based on the LLE technique to extraction of mycotoxins which acetonitrile is introduced due to it having more advantages than other solvents. In this study, the acid mycotoxins (e.g. FUMs, ochratoxin and CIT) structure consist the carboxyl group. Before the acid equilibrium, the carboxyl functional group would be deprotonated to a charge molecule which is unstable and problematic for mycotoxins residues analysis. In this study, the extraction procedure I, at stage 3.5.1.2 was varied by the parameters in table 3.2. Therefore, the percentage of acidified ACN over two acid types (i.e. formic acid and acetic acid) were compared for the optimum method and the extraction procedure complied with the extraction procedure I**.**

Table 3.2 The parameters of acidified acetonitrile study.

Method No.	Parameters
M 1	acetonitrile
M 2	1% acetic acid in acetonitrile
M 3	1% formic acid in acetonitrile
M 4	5% acetic acid in acetonitrile
M 5	5% formic acid in acetonitrile
M 6	10% acetic acid in acetonitrile
M 7	10% formic acid in acetonitrile

The extraction procedure I for the study of acidity of extraction solvent can be described as per the following method below:

3.5.1.1 Weigh 10 ± 0.05 g of the grinded rice sample in 50 mL plastic centrifuge tube, add 10 mL of water to make a slurry sample with a vortex for a few minutes. (The fortified samples were done at this stage.)

3.5.1.2 10 mL of ***acidified acetonitrile*** was added into the sample later and shaken in a shaker for 60 minutes.

3.5.1.3 4 g of $MgSO_4$, 1g NaCl, 1 g sodium citrate tribasic dehydrate and 0.5 g sodium citrate dibasic sesquihydrate were added into the solution tube and shaken vigorously by hand for a few minutes avoiding the formation of lumps. And centrifuge at 3400 rpm for 5 minutes.

3.5.1.4 After centrifuge, put the entire supernatant into another tube which contains 0.4 g PSA, 0.25 g C18, 0.25 g alumina-N and 1.2 g $MgSO_4$ then cap the tubes and shake by hand for 1-2 minutes, centrifuge at 3400 rpm for 5 minutes.

3.5.1.5 Put 5 mL of the cleaned solution into the glass tube and dry using N_2-evaporator at 40 ºC. Finally, 1 mL of mobile phase (1/1) was reconstituted and the residues were filtered through 0.2 µm nylon filter prior the UPLC-MS/MS.

3.5.1.6 The final concentration was calculated and reported as percentage recoveries of each mycotoxins.

3.5.2 The buffer effect on extraction solvent

After the partitioning with magnesium sulphate and NaCl, to induce phase separation, the buffer is added due to its property whereby the pH of the solution changes very little when an amount of acid is added into the solution as a means of controlling pH at a nearly constant value. In this study, two types of buffer (citrate and acetate buffer) were investigated in order to maintain the pH of the acidified solvent, corresponding with the extraction procedure I in which acidified acetonitrile was used for efficient extraction. In the extraction procedure II, at stage 3.5.2.3 was varied by the parameters in table 3.3 and the extraction procedure complied with the extraction procedure II.

Table 3.3 The study of buffer type.

Method No	Parameters
M 8	Without buffer
M 9	1.0 g of tri-citrate and 0.5 g of di-citrate
M 10	1.5 g sodium acetate

Where:

- tri-citrate stands for sodium citrate tribasic dihydrate.
- di-citrate stands for sodium citrate dibasic sesquihydrate.

The extraction procedure II for the study of buffer effect on extraction solvent can be described as per the following method below:

3.5.2.1 Weigh 10 ± 0.05 g of the ground rice sample in 50 mL plastic centrifuge tube, add 10 mL of water to make a slurry sample with a vortex for a few minutes. (The fortified samples were done at this stage.)

3.5.2.2 10 mL of 10% formic acid in ACN was added into the sample later and shaken up by using the shaker for 60 minutes.

3.5.2.3 4 g of $MgSO_4$, 1g NaCl, ***1.5 g buffer*** was added into the solution tube and shaken vigorously by hand for a few minutes to avoid the formation of lumps and centrifuge at 3400 rpm for 5 minutes.

3.5.2.4 After centrifuge put the entire supernatant into another tube which contains 0.4 g PSA, 0.25 g C18, 0.25 g alumina-N and 1.2 g $MgSO_4$ then cap the tubes and shake by hand for 1-2 minutes, centrifuge at 3400 rpm for 5 minutes.

3.5.2.5 Put 5 mL of the cleaned solution into the glass tube and dry using N_2-evaporator at 40 ºC. Finally, 1 mL of mobile phase (1/1) was reconstituted and the residues were filtered through 0.2 µm nylon filter prior the UPLC-MS/MS.

3.5.2.6 The final concentration was calculated and reported as percentage recoveries of each mycotoxins.

3.5.3 The optimized dispersive solid phase extraction (d-SPE) sorbent for extraction method

Regarding the acidified acetonitrile was used for extraction. The co-extractive would be presented in the extract solution. Consequently, the clean up steps to get rid of the interferences or the co-extractives are important in order to protect the instrument from becoming dirty. At this stage, dispersive solid phase extraction, d-SPE (i.e. PSA, NH_2, C18, Al-N, Si and Fl) were employed to absorb any interferences. A single type of d-SPE and a combination of d-SPE were studied for efficiency of clean up.

3.5.3.1 A single type of various d-SPE clean up

As the extraction procedure III at stage 3.5.3.3.4, a single type of d-SPE was varied by the following parameter in table 3.4.

Table 3.4 The varied single type of d-SPE for clean up

Method no.	d-SPE
M 11	Without d-SPE
M 12	0.4 g PSA
M 13	0.4 g NH_2
M 14	0.4 g C18
M 15	0.4 g Al-N
M 16	0.4 g Si
M 17	0.4 g Fl

3.5.3.2 The combination of d-SPE for clean up

As the results from method M 11 to M 17, the optimum d-SPE was further investigated by mixing three types of d-SPE. The parameters (in table 3.5) were designed for this study. Moreover, PSA and NH_2 are the anion exchange and similar functional structure and for this reason, the use of PSA and NH_2 was compared for better % recovery from usage of the mixing d-SPE.

Table 3.5 The study of three mixing d-SPE for clean up

Method no.	Mixed d-SPE
M 18	0.4 g PSA + 0.25 g C18 + 0.25 g Al-N
M 19	0.4 g NH_2 + 0.25 g C18 + 0.25 g Al-N

3.5.3.3 The extraction procedure III for the study of a single type of d-SPE can be described as per the following method below:

3.5.3.3.1 Weigh 10 ± 0.05 g of the ground rice sample in 50 mL plastic centrifuge tube, add 10 mL of water to make a slurry sample with a vortex for a few minutes. (The fortified samples were done at this stage.)

3.5.3.3.2 10 mL of 10% formic acid in ACN was added into the sample later and shaken up by using the shaker for 60 minutes.

3.5.3.3.3 4 g of $MgSO_4$, 1g NaCl, 1 g Sodium Citrate tribasic dehydrate and 0.5 g Sodium Citrate dibasic sesquihydrate were added into the solution tube and shaken vigorously by hand for a few minutes avoiding the formation of lumps, and centrifuge at 3400 rpm for 5 minutes.

3.5.3.3.4 After centrifuge put the entire supernatant into another tube which contains ***d-SPE*** and 1.2 g $MgSO_4$ then cap the tubes and shake by hand for 1-2 minutes, centrifuge at 3400 rpm for 5 minutes.

3.5.3.3.5 Put 5 mL of the cleaned solution into the glass tube and dry using N_2-evaporator at 40 ^oC. Finally, 1 mL of mobile phase (1/1) was reconstituted and the residues were filtered through 0.2 µm nylon filter prior the UPLC-MS/MS.

3.5.3.3.6 The final concentration was calculated and reported as percentage recoveries of each mycotoxins.

3.6 Method validation [50, 51, 52, 53]

The reliability of the method was performed by validation, also called performance parameters. These are described as: selectivity, linearity (also investigation of matrix effect), accuracy, precision and limit of detection (LOD) and limit of quantitation (LOQ).

3.6.1 Selectivity

An analytical method is selective, if the ability is given to measure the accurate response of an analyte in the presence of interferences. This is achieved by mass selectivity of the detector via electrospray ionization in positive and negative modes. The identification of all compounds was described by determination of retention time (t_R), and the mass ion ratio value. The ratio value is a ratio of the area of qualitative ion over the area of quantitative ion which is the most significant factors in terms of the confirmation of questioned analytes. The matrix-matched standard (in section 3.6.2.2) was performed as a chromatographic retention time and the mass ion ratio values.

3.6.2 Linearity

A measurement defines a relation between the instrumental response (peak area of quantitative ion) and the standard concentration. According to the theory, the matrix might have significant signal suppression effect on analytes in mass spectrometry technique because **"the matrix effect"** must be used for a consequent analytical (linearity) error. Thus, two types of standard calibration curves were compared for the significant difference between the dilution in mobile phase (see section 3.6.2.1) and sample extract solution (see section 3.6.2.2) by preparation of standard calibration curve with the same concentration range. Each level was done by triplicate injection.

3.6.2.1 The preparation of calibration standard (the solution of the analyte used for calibration of the determination system) in the mobile phase was prior to the UPLC-MS/MS analysis. The mixture standard solutions (section 3.3.3)

were diluted in the mobile phase (A/B = 1/1) which covered the concentration range as shown in table 3.6

3.6.2.2 The matrix-matched standards solution (calibration was intended to compensate for interference, if present) were prepared prior to UPLC-MS/MS analysis. The mixture standard solutions (section 3.3.3) were diluted in a blank extract of a matrix similar to that analyzed which covered the concentration range as shown in table 3.6

Table 3.6 The concentration levels of standard calibration curve.

Level	**concentration levels, mg/L**	
	Standard group A	**Standard group B**
1	0.01	0.05
2	0.05	0.25
3	0.10	0.50
4	0.15	0.75
5	0.20	1.00
6	0.25	1.25
7	0.30	1.50
8	0.35	1.75
9	0.40	2.00
10	0.45	2.25
11	0.50	2.50

3.6.3 Accuracy

The term **accuracy** means closeness of the test result to the “trueness” or accepted value. For the investigation of this parameter, the Certified Reference Materials (CRMs) or Reference Materials (RMs) should be measured to verify the true value. If there is no CRMs or RMs, the accuracy can be expressed by using the %recovery from the fortified negative rice sample. Three different fortification levels (low, medium and high) were repeated ten times. The concentration levels of each mycotoxin are shown in table 3.7 below.

Table 3.7 Three concentration levels of the fortified sample.

Mycotoxins	**Concentration levels of fortified sample, mg/kg**		
	low	**middle**	**high**
1. AF-B1	0.01	0.05	0.1
2. AF-B2	0.01	0.05	0.1
3. AF-G1	0.01	0.05	0.1
4. AF-G2	0.01	0.05	0.1
5. FUM-B1	0.01	0.05	0.1
6. FUM-B2	0.01	0.05	0.1
7. OTA	0.01	0.05	0.1
8. OTB	0.01	0.05	0.1
9. DON	0.1	0.5	1.0
10. NIV	0.1	0.5	1.0
11. T-2	0.01	0.05	0.1
12. HT-2	0.01	0.05	0.1
13. CIT	0.01	0.05	0.1
14. ZEA	0.01	0.05	0.1

3.6.4 Precision

The precision of a method is measured by the closeness expected between independent replicate test results conducted under specified conditions. In this study, the percentage of recovery was calculated by comparing matrix-matched standard calibration curve. Two measurements of precision, termed **repeatability** and **reproducibility** are commonly quoted.

3.6.4.1 Repeatability: Each batch was prepared by fortifying negative control of rice sample with standard group A (section 3.3.3) at 0.01, 0.05 and 0.1 mg/kg, and standard group B (section 3.3.3) at 0.1, 0.5 and 1.0 mg/kg, respectively. The fortified negative rice samples were taken through the whole extraction procedure. Fortification at each level was repeated ten times.

3.6.4.2 Reproducibility: This parameter is related to a series of measurements made under more variable conditions. Theoretically, it consists of two terms such as “single laboratory or within-laboratory” and “Inter laboratory or between-laboratory”.

3.6.4.2.1 Single laboratory or within-laboratory is commonly referred to by the term “intermediate precision” e.g. the same condition over a different period of analysis which was performed by the fortification with three different concentration levels (see table 3.7 for the fortification levels at low, medium, and high levels). Each level was repeated ten times over three days of analysis.

3.6.4.2.2 The second measuring precision is termed the inter laboratory or between-laboratory. It refers to the different conditions of testing. For the study of this parameter, the proficiency testing (PT) with “The Food and Environment Research Agency, FAPAS” provided the known sample for proving of method performance. The participation was served with the PT material as shown in table 3.8. The optimization of UPLC-MS/MS conditions was employed for quantification of mycotoxins. The measurement processes complied with the instruction such as:

(1). Treat the test material as if it was a sample for any method of analysis you wish.

(2). Determine the level of mycotoxins present in the test material, in μg/kg and correct for recovery.

Table 3.8 The proficiency test material for mycotoxins analysis in various grains.

PT material	Lab No.	Material No.	mycotoxins
Rice	25	T4151	Total AFs
Cereal (oat)	26	T1776	OTA
Breakfast cereal	58	T2257	ZEA
Maize	33	T2262	DON

3.6.5 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) of a method is the smallest amount or concentration of an analyte that can be reliably distinguished from the absence of analyte (a blank value). In this study, the LOD values were calculated at 3 times of signal to noise at the smallest fortification level.

The limit of quantitation (LOQ) of the method is often defined as the lowest concentration of analyte that can be determined with the measurable levels. Usually, the recommendation is to quote the LOQ as 10 times of signal to noise from the calculation of the smallest fortification level.

3.7 The comparison of method performance with IAC

Regarding the advantages of IAC, these are reproducibility, a good % recovery, low interference and variability of pre-concentration etc. In this study, three manufacturers of IAC were employed for evaluation of the IAC performance i.e. Romer Labs® Inc (MO, U.S.A.), R-Biopharm Rhöne Ltd (Glasgow, Scotland), Vicam L.P. (Watertown, MA, U.S.A.). The percentage recoveries were obtained from the fortified sample (spiked sample) for evaluation of the method. The standard solution (section 3.3.3) was spiked into the negative rice sample at the first stage of extraction in order to prepare the fortified sample with concentration level which is shown in table 3.11. Moreover, another parameter, the LOD values would be also assessed on the commercial method. The commercial extraction procedures were defined in table 3.9.

Table 3.9 The commercial extraction procedure of IAC column from 3 manufacturers.

Mycotoxins	**Commercial IAC**					
	Romer Labs®		**R-Biopharm Rhöne**		**Vicam**	
	Method No.	Spiked level (mg/kg)	Method No.	Spiked level (mg/kg)	Method No.	Spiked level (mg/kg)
AF-B1	IAC-1	0.4	IAC-2	0.4	IAC-3	0.1
AF-B2	IAC-1	0.4	IAC-2	0.4	IAC-3	0.1
AF-G1	IAC-1	0.4	IAC-2	0.4	IAC-3	0.1
AF-G2	IAC-1	0.4	IAC-2	0.4	IAC-3	0.1
DON	IAC-4	0.8	IAC-5	1.6	IAC-6	0.8
OTA	IAC-7	0.2	-	-	IAC-8	0.2
OTB	IAC-7	0.2	-	-	IAC-8	0.2
ZEA	IAC-9	0.4	IAC-10	0.8	IAC-11	0.25

3.7.1 AflaStar™ column from Romer Labs® Inc (MO, U.S.A.) The extraction procedure No.IAC-1 for AFs extraction was followed the step below:

3.7.1.1 Grind and weigh 25 g sample into 125 mL Teflon centrifuge tube, add 100 mL of 60/40 = MeOH/water, shake for 60 minutes.

3.7.1.2 Filter the supernatant with Whatman filter paper No.1 then mix 4 mL of extract with 12 mL of PBS solution in the beaker and stir for a few minutes.

3.7.1.3 Remove the buffer in the column provided; apply 4 mL of diluted solution to AflaStar™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.1.4 Wash column with water 10 mL x 2 times. Pass the air through.

3.7.1.5 Elute AFs residues with MeOH 1 mL x 2 times into the glass test tube and filter through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.2 AFLAPREP™ column from R-Biopharm Rhöne Ltd (Glasgow, Scotland): The extraction procedure No.IAC-2 for AFs extraction followed the steps shown below:

3.7.2.1 Grind and weigh 50 g sample into 100 mL stainless blender jar, add 4 g of NaCl, 100 mL of water and 150 mL of MeOH, blend for 1-2 mins.

3.7.2.2 Filter the supernatant with Whatman filter paper No.1, add 250 mL of water into the filtered solution and stir for a few minutes.

3.7.2.3 Remove the buffer from the column; apply 20 mL of diluted solution to AFLAPREP™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.2.4 Wash column with PBS 10 mL x 2 times. Pass the air through.

3.7.2.5 Elute AFs residues with 1 mL MeOH into the glass test tube followed by 1 mL water and filter through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.3 AflaTest P™ column from Vicam L.P. (Watertown, MA, U.S.A.): The extraction procedure No.IAC-3 for AFs extraction followed the steps below:

3.7.3.1 Grind and weigh 50 g sample into 1000 mL stainless blender jar, add 5 g of NaCl, 20 mL of water and 80 mL of MeOH, blend for 1-2 mins.

3.7.3.2 Filter the supernatant with Whatman filter paper No.1 then pipette 4 mL of extract solution dilute in 16 mL of water and stir for a few minutes.

3.7.3.3 Remove the buffer from the column; apply 10 mL of diluted solution to AflaTest P™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.3.4 Wash column with water 10 mL x 2 times. Pass the air through.

3.7.3.5 Elute AFs residues with 1 mL ACN into the glass test tube and filter through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.4 DonStar™ column from Romer Labs® Inc (MO, U.S.A.) The extraction procedure No.IAC-4 for DON extraction followed the steps below:

3.7.4.1 Grind and weigh 25 g sample into 125 mL Teflon centrifuge tube, add 200 mL of water, shake for 60 mins.

3.7.4.2 Filter the supernatant with Whatman filter paper No.1.

3.7.4.3 Remove the buffer from the column; apply 2 mL of extracted solution to DonStar™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.4.4 Wash column with PBS solution 5 mL x 2 times. Pass the air through.

3.7.4.5 Elute DON residues with MeOH 1 mL x 3 times into the glass test tube and dry using N_2-evaporator at 40 ºC. Finally, 1 mL of mobile phase (A/B = 1/1) was reconstituted the residues and filtered through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.5 DONPREP™ column from R-Biopharm Rhöne Ltd (Glasgow, Scotland): The extraction procedure No.IAC-5 for DON extraction followed the steps below:

3.7.5.1 Grind and weigh 25 g sample into 1000 mL stainless blender jar, add 200 mL of water, blend for 2-3 mins.

3.7.5.2 Filter the supernatant with Whatman glass microfiber filter paper.

3.7.5.3 Remove the buffer from the column; apply 2 mL of extracted solution to DONPREP™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.5.4 Wash column with water 5 mL. Pass the air through.

3.7.5.5 Elute DON residues with MeOH 2 mL and filtered through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.6 DonTest™ column from Vicam L.P. (Watertown, MA, U.S.A.): The extraction procedure No.IAC-6 for DON extraction followed the steps below:

3.7.6.1 Grind and weigh 50 g sample into 1000 mL stainless blender jar, add 200 mL of 0.125% (W/V) PEG, blend for 1-2 minutes.

3.7.6.2 Filter the supernatant with Whatman glass microfiber filter paper.

3.7.6.3 Remove the buffer from the column; apply 1 mL of extract solution to DonTest ™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.6.4 Wash column with water 5 mL x 2 times. Pass the air through.

3.7.6.5 Elute DON residues with 1 mL MeOH into the glass test tube and filter through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.7 OchraStar™ column from Romer Labs® Inc (MO, U.S.A.): The extraction procedure No.IAC-7 for OTA extraction followed the steps below:

3.7.7.1 Grind and weigh 50 g sample into 125 mL Teflon centrifuge tube, add 100 mL of 80% (V/V) MeOH in water, shake for 60 minutes.

3.7.7.2 Filter the supernatant with Whatman filter paper No.1, mix 2 mL of extract solution with 14 mL of PBS solution.

3.7.7.3 Remove the buffer from the column; apply 8 mL of extracted solution to OchraStar™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.7.4 Wash column with 0.2 M ammonium acetate : 10 mL x 2 times. Pass the air through.

3.7.7.5 Elute OTA residues with ACN 1 mL x 2 times into the glass test tube and filtered through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.8 OchraTest™ column from Vicam L.P. (Watertown, MA, U.S.A.): The extraction procedure No.IAC-8 for OTA extraction followed the steps below:

3.7.8.1 Grind and weigh 50 g sample into 1000 mL stainless blender jar, add 100 mL of 80% (V/V) MeOH in water, blend for 1-2 minutes.

3.7.8.2 Filter the supernatant with Whatman glass microfiber filter paper, mix 10 mL of extract solution with 40 mL of water and stir for a few minutes.

3.7.8.3 Remove the buffer from the column; apply 10 mL of extract solution to OchraTest ™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.8.4 Wash column with water 10 mL of **solution A*** then 10 mL of DI water. Pass the air through.

3.7.8.5 Elute OTA residues with 2 mL ACN into the glass test tube and filter through 0.2 µm nylon filter prior the UPLC-MS/MS.

*** Solution A** is a mixture solution of 25 g NaCl + 5 g $NaHCO_3$ in 1000 mL of water.

3.7.9 ZeaStar™ column from Romer Labs® Inc (MO, U.S.A.) The extraction procedure No.IAC-9 for ZEA extraction followed the steps below:

3.7.9.1 Grind and weigh 25 g sample into 125 mL Teflon centrifuge tube, add 100 mL of 84/16 = ACN/water, shake for 60 minutes.

3.7.9.2 Filter the supernatant with Whatman filter paper No.1 then mix 4 mL of extract with 31 mL of PBS solution in the beaker and stir for a few minutes.

3.7.9.3 Remove the buffer from the column; apply all mixed solution to ZearaStar™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.9.4 Wash column with water 10 mL x 2 times. Pass the air through.

3.7.9.5 Elute ZEA residues with MeOH 1 mL x 2 times into the glass test tube and filter through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.10 ZEARAPREP™ column from R-Biopharm Rhöne Ltd (Glasgow, Scotland): The extraction procedure No.IAC-10 for ZEA extraction followed the steps below:

3.7.10.1 Grind and weigh 25 g sample into 1000 mL stainless blender jar, 125 mL of 75% (V/V) acetonitrile in water and blend for 1-2 minutes.

3.7.10.2 Filter the supernatant with Whatman filter paper No.1, take 20 mL of extract solution to mix with 80 mL of PBS solution and stir for a few minutes.

3.7.10.3 Remove the buffer from the column; apply 25 mL of diluted solution to ZEARAPREP™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.10.4 Wash column with PBS solution 20 mL. Pass the air through.

3.7.10.5 Elute ZEA residues with 2 mL x 2 times of ACN into the glass test tube and filter through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.7.11 ZearalaTest ™ column from Vicam L.P. (Watertown, MA, U.S.A.): The extraction procedure No.IAC-11 for ZEA extraction followed the steps below:

3.7.11.1 Grind and weigh 20 g sample, into 1000 mL stainless blender jar, add 2 g of NaCl, 20 mL of water and 80 mL of MeOH, blend for 1-2 minutes.

3.7.11.2 Filter the supernatant with Whatman filter paper No.1 then pipette 20 mL of extract solution dilute in 30 mL of PBS solution and stir for a few minutes.

3.7.11.3 Remove the buffer from the column; apply 10 mL of diluted solution to ZearalaTest ™ column. Pass the solution through the column with approx. flow rate 1-3 mL/mins, and discard the solution.

3.7.11.4 Wash column with PBS solution 10 mL then follow with 10 mL of water. Pass the air through.

3.7.11.5 Elute ZEA residues with 1 mL MeOH into the glass test tube and filter through 0.2 µm nylon filter prior to the UPLC-MS/MS.

3.8 Application to mycotoxins determination in various samples

In order to evaluate the applicability of the optimized method for instrument conditions and sample preparation, the modified method was applied to feed 11 samples, corn starch 5 samples, cocoa 5 samples, maize 2 samples, rice 12 samples, and malt powder 2 samples. All samples were submitted to OMIC laboratory for the analysis of mycotoxins.

CHAPTER IV

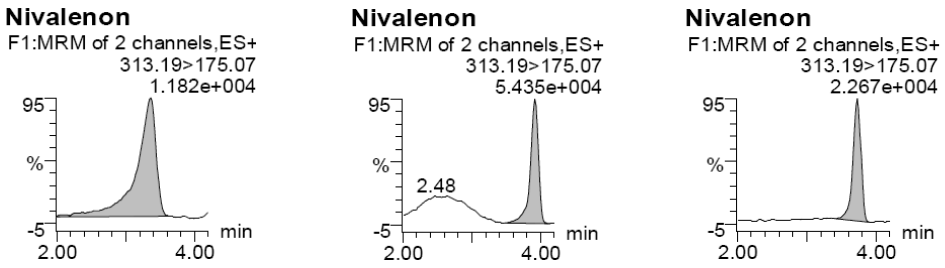
RESULTS AND DISCUSSION

This study presents the method developed for sample preparation with the simultaneous quantification of fourteen mycotoxins i.e. AFs (B1, B2, G1 and G2), FUMs (B1 and B2), OTA, OTB, DON, NIV, HT-2, T-2 and ZEA analysis in rice by UPLC-MS/MS. The validation of the method was proved by the statistical values. This new method was compared with the traditional technique e.g. IAC for fulfillment of the drawback. The applicable method was applied for routine analysis sample.

4.1 The optimization of UPLC-MS/MS conditions.

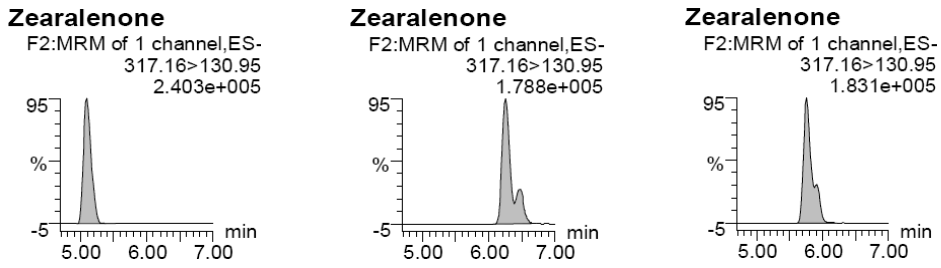
4.1.2 The selection of mobile phase for UPLC conditions.

From the optimum UPLC conditions for the separation efficiency in the chromatographic system, the choice of mobile phase concerned should be based on the consideration of ionization efficiency before entering MS/MS system in order to obtain nice resolution and high sensitivity. The selection of mobile phase was considered as two candidates (ACN and MeOH) because most of mycotoxins are easily dissolved in these solvents. The varied parameters were demonstrated in table 3.1 (chapter 3) under the employment of MS/MS condition in table 4.1 to 4.2. The results indicated that when using 100% ACN, produced excellent peak areas for some mytoxins such as AFs, FUMs, OTA, OTB, T-2 and CIT. On the other hand, DON and NIV produced excellent peak areas when using 100%MeOH because the mycotoxins were of a different polarity. Thus, the consideration of the peak shape was also important. For instance, the peak shape of NIV has a fronting when using ACN (see figure 4.1), after the addition of MeOH both the peak shape and peak area improved simultaneously. On the other hand, for ZEA, the variation of peak area was not distinguished when using ACN or MeOH or ACN/MeOH = 1/1. Whilst, the peak shape of ZEA was distorted when using of MeOH (see figure 4.2). Consequently, a mixed solvent (ACN/MeOH = 1/1) was chosen as a mobile phase for compromising the peak shape and peak area in order to optimize for 14 mycotoxins were analyzed in a single run time. Even if some mycotoxins suffered a loss of sensitivity.



(a) (b) (c)

Figure 4.1 The peak shape of NIV by using (a) 100% ACN (b) 100% MeOH (c) 1/1 = ACN/MeOH as a mobile phase



(a) (b) (c)

Figure 4.2 The peak shape of ZEA by using (a) 100% ACN (b) 100% MeOH (c) 1/1 = ACN/MeOH as a mobile phase.

As a result, the mixture of ACN and MeOH with ratio 1 to 1 was selected for the mobile phase B in this analysis. The comparison of using mobile phase B was presented by 3 chromatograms in APPENDIX A.

4.1.2 The chromatographic of mycotoxins from the MRM analysis was set up as MS/MS conditions.

After the individual standards (section 3.3.2) were tuned via the syringe pump, the primary product ion was termed a “quantitative ion” along with the secondary product ion which was termed “qualitative ion”. They were applied for the MRM transition as shown in table 4.2 under the conditions of UPLC associated with tandem mass spectrometry (triple quadrupole) conditions, table 4.1. The mixed standard at concentration 0.1 mg/L was injected for checking the effectiveness of chromatographic. (see figure 4.3)

Table 4.1 MSMS parameters for mycotoxins determinations.

Parameters	**ESI+**	**ESI-**
Capillary voltage (kv)	2.5	0.5
Extractor (Volts)	3	3
Source temperature (ºC)	120	120
Desolvation temperature (ºC)	350	350
Cone gas flow (L/Hr)	1000	1000
Low MS 1 Resolution	13.5	13.5
High MS 1 Resolution	13.5	13.5
Ion energy 1	0.3	0.6
Low MS 2 Resolution	13	12
High MS 2 Resolution	13	12
Ion energy 2	0.5	0.5
Collision gas flow (ml/min)	0.18	0.18
Multiplier	640	655

Table 4.2 The precursor ions, cone voltage, qualitative ions, quantitative ions and ionization mode for the 14 representative mycotoxins analysis by MRM mode.

Mycotoxin	Precursor m/z	Cone (Volts)	Product ion, m/z				ESI mode
			1° ion[a]	Collision (Volts)	2° ion[b]	Collision (Volts)	
AF-B1	313.12	40	285.11	25	241.09	35	ES+
AF-B2	315.12	45	259.13	30	287.14	25	ES+
AF-G1	329.12	40	243.13	25	283.15	25	ES+
AF-G2	331.21	40	313.15	25	-	-	ES+
FUM-B1	722.19	40	334.38	40	352.36	38	ES+
FUM-B2	706.21	47	336.39	36	354.39	34	ES+
OTA	404.08	24	239.07	25	358.08	14	ES+
OTB	370.12	28	205.12	25	187.09	35	ES+
DON	297.23	20	249.15	10	231.16	13	ES+
NIV	313.19	23	175.07	15	295.22	8	ES+
T-2	484.18	20	305.20	13	245.18	13	ES+
HT-2	442.14	17	263.18	13	215.16	13	ES+
CIT	251.15	25	233.14	17	191.08	25	ES+
ZEA	317.16	42	130.95	30	174.98	24	ES-

* a is the primary product ion (quantitative ions) , b is the secondary product ion (qualitative ions).

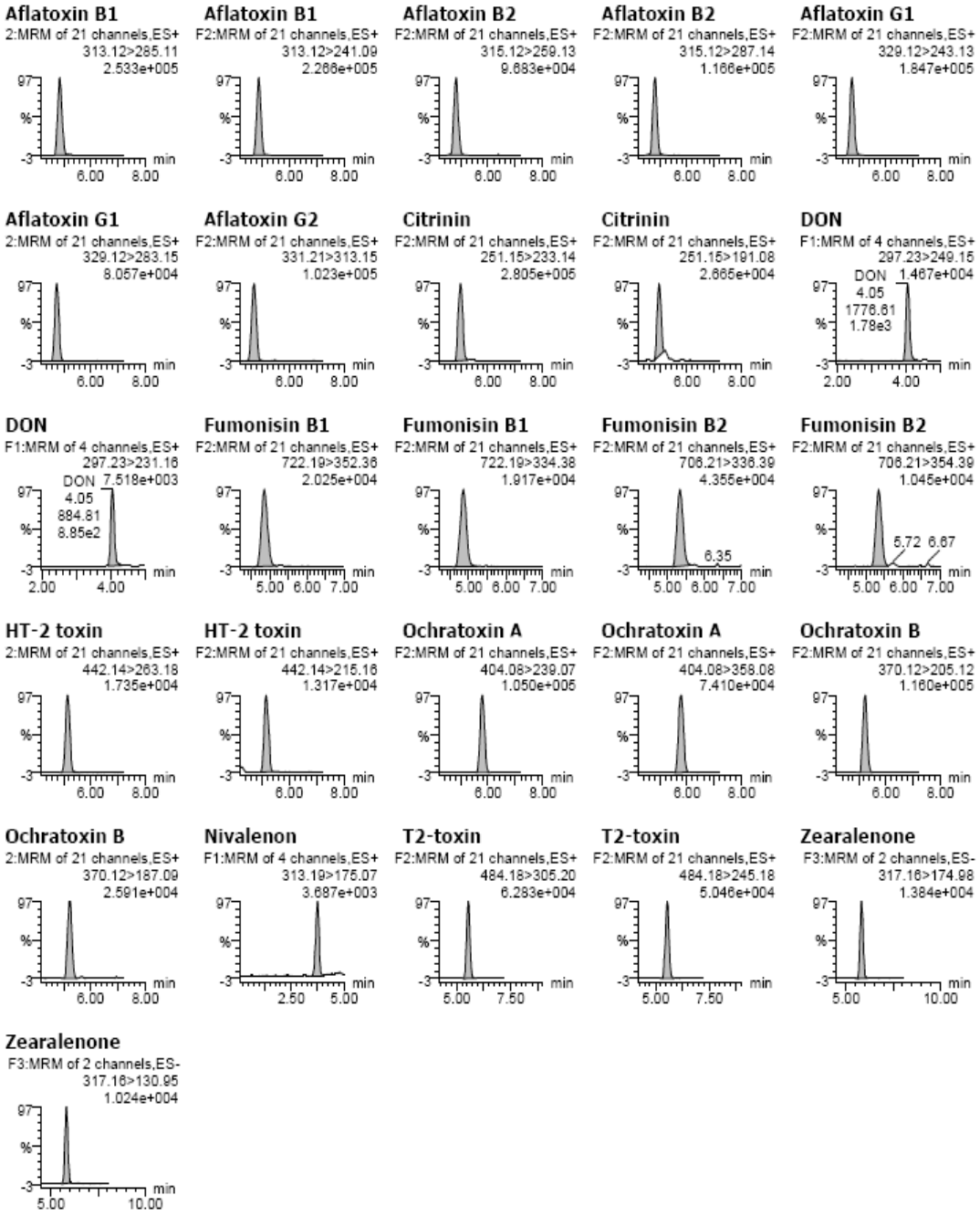


Figure 4.3 The chromatographic of mixed standard mycotoxins at concentration level 0.1 mg/L under optimum conditions.

4.2 Development of Extraction Method

Method development is the process of design and preliminary assessment of the characteristics of a method, including ruggedness. In this study, there were three critical parameters which were observed for the effective method of development. Each parameter is described as below:

4.2.1 The results of the acidity of extracted solvent

As mentioned in chapter 3, section 3.5.1, the acid mycotoxins (e.g. FUMs, OTA, OTB and CIT) and neutralized mycotoxinss were analyzed. Thus, additional acid must be served at the extraction step. The results were demonstrated in table 4.3 to 4.5 that the highest percentage recoveries from each acid mycotoxins were obtained by using 10% formic acid addition as the parameter No. M 7 by extraction procedure I, section 3.5.1 Furthermore, two weak acid types were also compared for a high efficient extraction method because formic acid is stronger than acetic acid. Consequently, the method No. M6 (10% formic acid in acetonitrile) and M7 (10% acetic acid in acetonitrile) were also described by the percentage recoveries of CIT, FUM-B1 and FUM-B2. They increased greatly when formic acid was employed, while OTA and OTB, were independent acid types. On the other hand, the variation of % acid have shown that no big difference from %acid use and acid types on the extraction of neutral mycotoxins (see figure 4.5). At this stage, 10% formic acid in ACN was selected for the best acidity of extracted solvent.

Table 4.3 The average %recovery of acidified ACN for acid mycotoxins. (n=4)

Method No.	Spiked level , mg/kg	% Recovery ± SD				
		CIT	FUM-B1	FUM-B2	OTA	OTB
no acid	0.02	8±6.8	2±2.3	1±1.6	1±0.9	0±0
1% acetic	0.02	15±7.5	0±0	1±0	16±4.2	17±4.2
1% formic	0.02	12±6.7	0±0	0±0.4	15±8.4	22±3.2
5% acetic	0.02	27±4.7	8±7.7	18±11.9	94±5.1	94±5.8
5% formic	0.02	35±4.3	46±7.5	75±8.1	89±7.0	91±2.6
10% acetic	0.02	41±2.9	52±7.8	71±2.2	95±2.6	99±2.2
10% formic	0.02	54±4.9	79±6.9	91±6.6	98±6.0	101±3.7

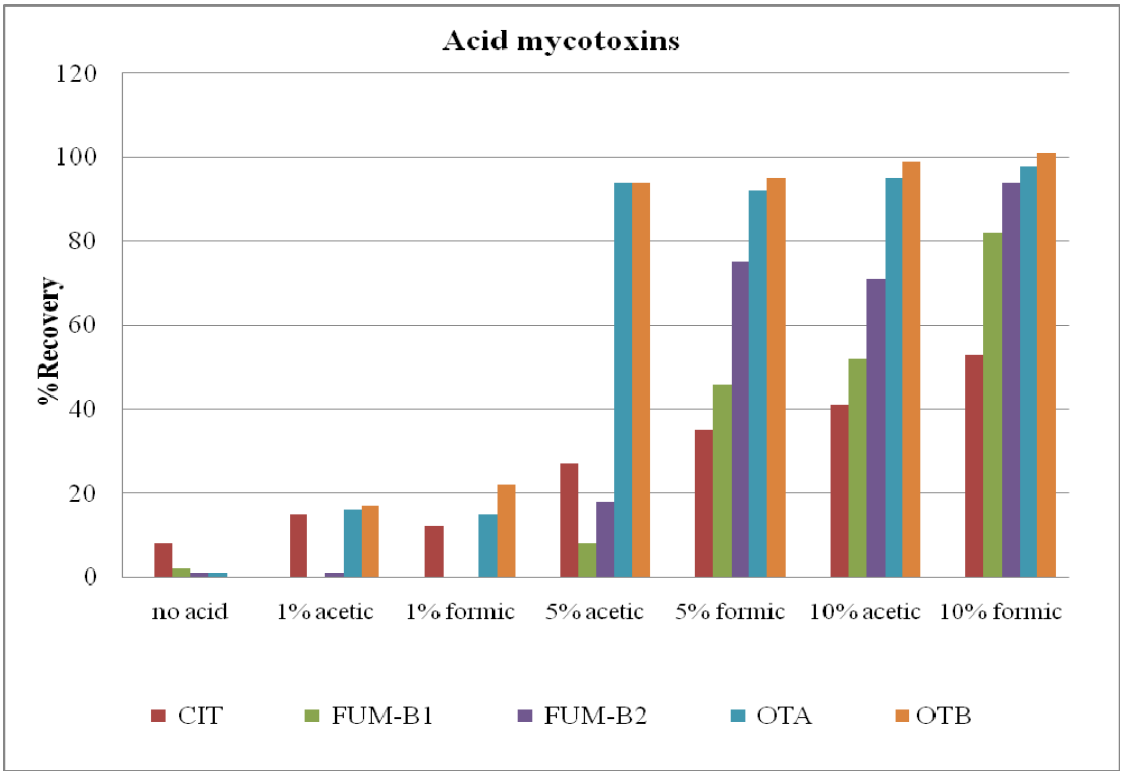


Figure 4.4 The study of acid type and the percentage of acid in acetonitrile for extraction of acid mycotoxins

Table 4.4 The average %recovery of acidified ACN for AFs and DON analysis (n=4)

Method No.	Spiked level, mg/kg	% Recovery ± SD				
		AF-B1	AF-B2	AF-G1	AF-G2	DON[a]
no acid	0.02/0.1[a]	92±4.3	95±7.1	96±3.8	99±2.6	97±3.7
1% acetic	0.02/0.1[a]	102±4.3	94±2.1	97±4.7	98±2.4	91±10
1% formic	0.02/0.1[a]	98±4.0	97±6.6	98±3.5	95±3.8	90±5.7
5% acetic	0.02/0.1[a]	97±6.6	94±6.8	87±6.6	92±4.6	90±7.2
5% formic	0.02/0.1[a]	86±2.9	101±6.0	81±5.7	97±8.00	90±2.9
10% acetic	0.02/0.1[a]	106±6.4	99±6.1	102±4.8	99±4.3	94±4.5
10% formic	0.02/0.1[a]	86±8.2	97±8.6	88±6.3	92±5.7	81±6.1

Table 4.5 The average %recovery of acidified ACN for HT-2, NIV, T-2 and ZEA analysis (n=4)

Method No.	Spiked level mg/kg	% Recovery ± SD			
		HT-2	NIV [a]	T-2	ZEA
no acid	0.02/0.1[a]	100±3.7	65±4.2	113±5.4	94±6.2
1% acetic	0.02/0.1[a]	109±3.3	51±8.9	113±1.3	102±8.7
1% formic	0.02/0.1[a]	103±6.0	55±3.0	106±4.6	87±7.5
5% acetic	0.02/0.1[a]	97±7.9	70±7.6	110±5.5	84±8.5
5% formic	0.02/0.1[a]	100±4.0	58±1.5	110±2.5	78±8.3
10% acetic	0.02/0.1[a]	106±2.3	70±4.9	106±2.9	98±7.2
10% formic	0.02/0.1[a]	102±7.2	58±5.8	106±0.6	85±8.7

[a] is spiked sample at concentration level 0.1 mg/kg of DON and NIV.

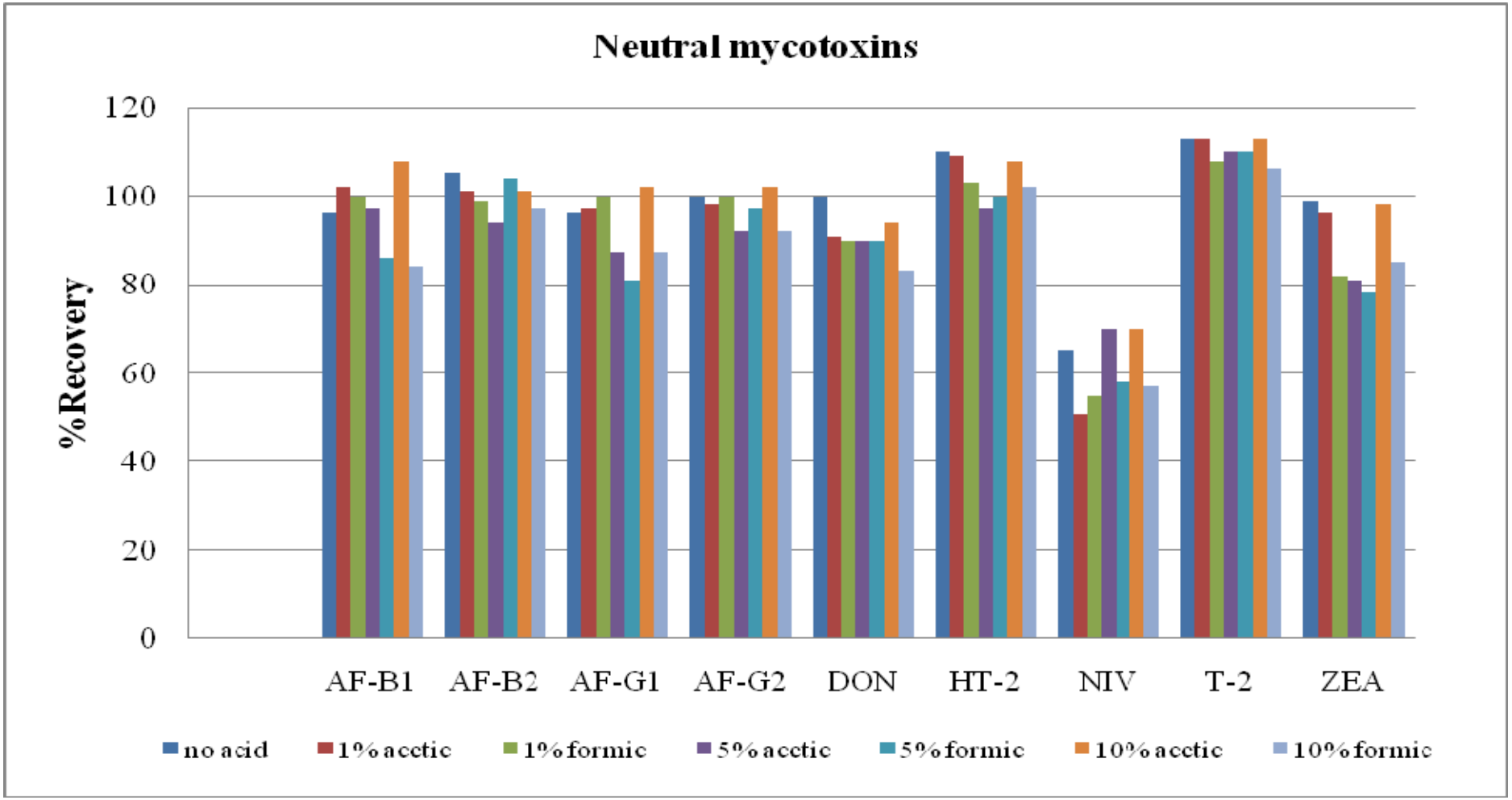


Figure 4.5 The study of acid type and the varied percentage of acid in ACN for neutral mycotoxins extraction.

4.2.2 The results of buffer types study

When the extracted solvent was acidified, the pH of solution must be controlled by a buffer addition in order to maintain the pH of the solution to change very little. In this study, method No M 9 (addition of citrate buffer) and M 10 (addition of sodium acetate buffer) were applied for the extraction procedure II and percentage recoveries of spiked sample were also compared for the effective buffer. The results were demonstrated in table 4.6 and the comparison was presented by the diagram in figure 4.6

Table 4.6 The percentage mean recovery of fortified sample for the study of buffer types effect on extraction solvent.

Mycotoxins	**Spiked level**	**%Recovery ± SD**		
	mg/kg	**No buffer**	**Citrate buffer**	**NaoAct buffer**
AF-B1	0.02	57±7.2	85±8.9	87±5.8
AF-B2	0.02	65±3.6	97±8.6	78±8.3
AF-G1	0.02	63±6.1	87±7.0	83±6.7
AF-G 2	0.02	60±7.6	92±5.7	88±4.8
CIT	0.02	44±10.2	53±5.6	35±4.5
DON	0.10	75±5.5	81±6.1	81±5.1
FUM-B1	0.02	53±9.7	79±6.9	69±6.5
FUM-B2	0.02	76±12.7	89±5.2	82±4.7
HT-2	0.02	89±8.2	99±3.3	86±10.0
OTA	0.02	73±10.9	99±4.7	85±5.2
OTB	0.02	78±9.2	100±5.2	89±4.5

Table 4.6 *(continued)*

Mycotoxins	Spiked level	%Recovery ± SD		
	mg/kg	No buffer	Citrate buffer	NaoAct buffer
NIV	0.10	60±6.6	59±4.0	60±4.6
T-2	0.02	99±5.3	108±2.7	97±5.2
ZEA	0.02	72±5.0	87±6.2	81±6.4

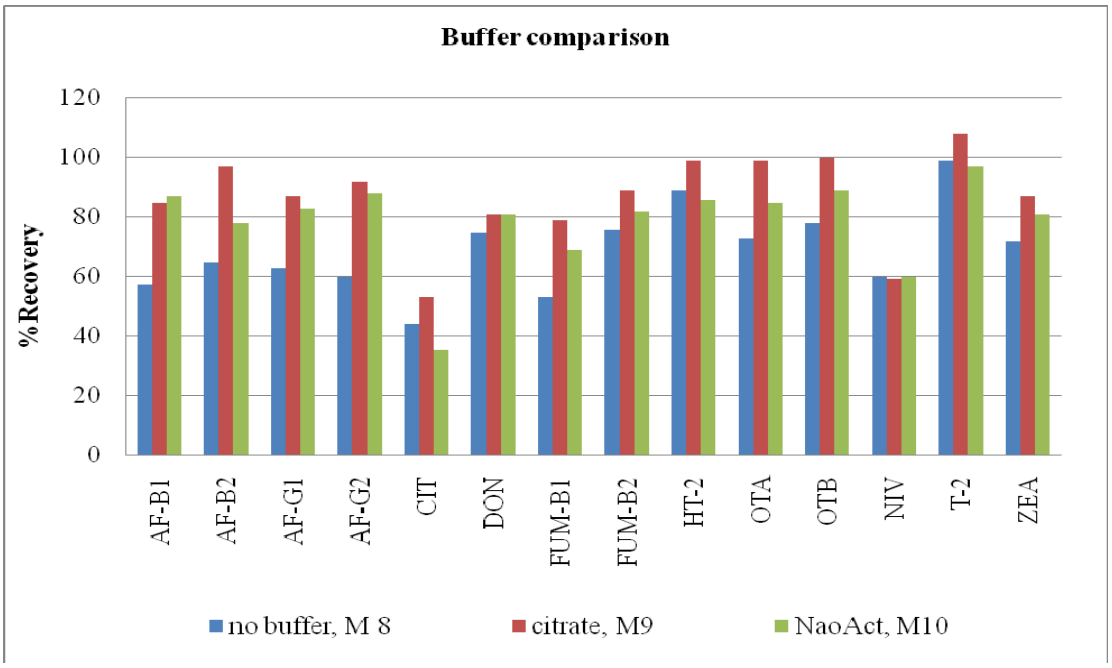


Figure 4.6 The percentage recoveries of buffer type for mycotoxins extraction.

As a comparison, the results in the diagram (in figure 4.6) indicate that the best of percentage mean recovery were obtainable when the addition of citrate buffer was employed. As the sodium citrate buffer was controlled, the pH solution over the range at 3.0 to 6.2 whilst, the sodium acetate buffer was covered at the controlled pH range, 3.6 to 5.6. Consequently, the usage of sodium citrate buffer should be suitable for the maintainable acid solution at the low pH range.

4.2.3 The study of dispersive solid phase extraction (d-SPE) sorbent

Rice is high in carbohydrates $(CH_2O)_n$ which are polar compounds and can interfere with trace analysis. Consequently, the clean up step to get rid of the interference or the co-extractives are important in order to protect the instruments from becoming dirty and reducing the background of chromatographic. There were six types of d-SPE sorbent such as PSA, NH_2, C18, Al-N, Si and Fl for the representative d-SPE in this study. All the parameters in table 3.4 and 3.5 were applied for extraction procedure III. The results were described by mean recoveries of fortified samples as below:

4.2.3.1 The results of single type d-SPE usage

Due to each d-SPE sorbent having different functions, the effectiveness of the d-SPE sorbent was compared by using six types of d-SPE before applying to the method. As a result, PSA was selected by the maximum of mean recoveries as the best for d-SPE usage for the stage of clean up (see table 4.7). The AFs especially showed aggressive % recovery when PSA was employed. However, this sorbent is restricted for the acid mycotoxins analysis because the amine group on the PSA structure could be protonated as the positive charge and interacted with the negative charge of acid mycotoxins. Consequently, the amount of PSA usage should be investigated for the optimum amount in order to prevent the lost % recoveries. In figure 4.8, trendy line was down when increasing the PSA amount. The 0.3-0.4 g amount was effective % recoveries while for the using of Si and Fl was affected on DON and NIV which are the polar compounds. They would be served with the low % recovery because the molecule themselves might interact with Si and Fl sorbent as means a retainable on these sorbents. The moderate mean recoveries were obtained by using C18 and Al-N

Table 4.7 : The results of the mean %recoveries from the comparison of single type d-SPE usage.

Mycotoxins	Spiked level,	%Recovery ± SD						
	mg/kg	no d-SPE	PSA	NH_2	C18	Al-N	Si	Fl
AF-B1	0.02	42±1.5	80±4.2	55±4.1	65±4.1	64±2.1	49±6.7	55±1.2
AF-B2	0.02	62±3.3	110±6.1	110±6.6	100±1.4	94±3.0	94±2.6	54±3.8
AF-G1	0.02	43±7.8	91±7.9	59±9.3	67±6.5	65±2.4	42±3.9	67±4.1
AF-G2	0.02	64±3.2	111±8.2	98±9.9	86±1.3	77±2.1	79±5.4	75±5.7
CIT	0.02	50±5.0	53±1.5	63±6.7	71±1.3	67±1.5	65±0.8	52±4.5
DON	0.10	62±6.8	94±6.1	92±4.8	80±4.3	75±2.9	46±5.2	54±1.2
FUM-B1	0.02	98±6.4	80±5.4	75±3.7	78±7.0	80±5.8	72±6.0	80±2.9

Table 4.7 : *(continued)*

Mycotoxins	Spiked level,	%Recovery ± SD						
	mg/kg	no d-SPE	PSA	NH_2	C18	Al-N	Si	Fl
FUM-B2	0.02	99±5.6	99±6.7	101±7.2	84±1.7	89±4.6	88±4.7	81±0.8
HT-2	0.02	70±8.9	105±9.0	108±4.2	85±3.7	87±2.1	100±6.3	93±3.6
OTA	0.02	74±6.0	112±6.5	103±8.4	86±5.2	80±4.5	82±3.5	80±1.4
OTB	0.02	84±5.3	107±3.7	110±6.6	91±2.1	91±1.9	88±2.6	87±1.4
NIV	0.10	53±5.4	58±7.4	59±9.0	59±1.7	64±3.5	38±1.5	44±0.5
T-2	0.02	105±4.8	103±5.2	105±6.4	93±2.8	106±3.0	102±2.9	101±1.7
ZEA	0.02	71±8.2	88±5.3	88±7.2	72±2.9	70±5.7	76±5.2	77±3.2

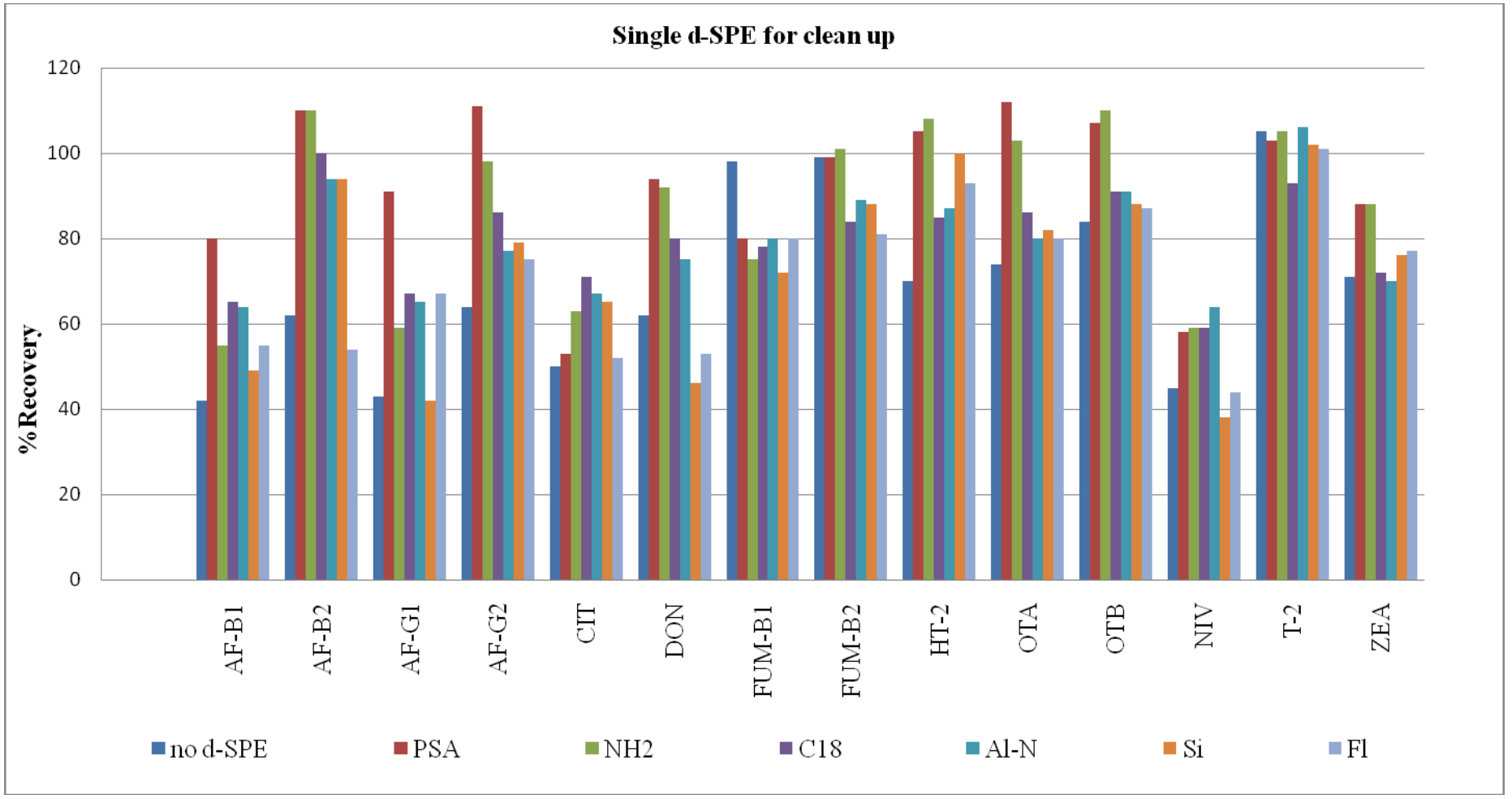


Figure 4.7 : The comparison of single d-SPE usage for mycotoxins extraction.

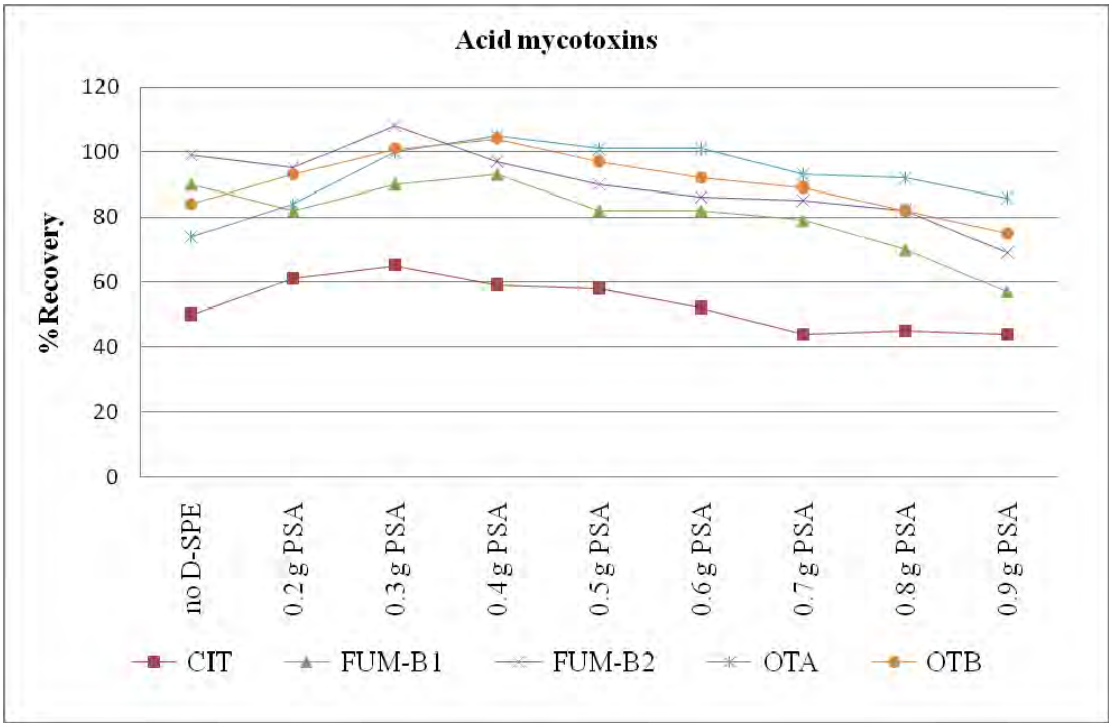


Figure 4.8 The effective of PSA amount on acid mycotoxins.

In summary of the selection of single d-SPE study, the data in table 4.7 was performed that if 80% is determined for the acceptable recovery. The using of PSA was shown that 86% of all compounds in this analysis which were achieved the criteria. On the other hands, when any d-SPE was not employed. There was just 29% of all compounds which were met the criteria. Moreover, the using of C18 and Al-N could enhance the %recovery of CIT and NIV, respectively. Consequently, the using of combination d-SPE (SPA, C18 and Al-N) could obtain the better %recovery than using of the single d-SPE.

4.2.3.2 The results of combination d-SPE usage

In this stage, three mixes of dispersive solid phase extraction (d-SPE) such as PSA, Al-N and C18 are selected for interfered elimination. For these reasons, PSA functional group is similar to NH_2 and therefore a stronger anion-exchanger than NH_2. With the PSA, there are two amine groups that offer higher ionic capacity than NH_2 and thus are a better choice for the polar compounds which sugar existed hydroxyl group could be retained on PSA sorbent. Basically, the chemical uniqueness of Al-N

(pH 7.5) is that it is an extremely polar sorbent. Most importantly, it can adsorb molecules by interaction with aluminum metal center, hydrogen bonding with the surface hydroxyl group, or by ion exchange if the surface carries a charge. The neutralized surface allows interaction with compounds whose heteroatoms are electronegative (e.g. N,O,S,P) or are electron-rich. Consequently, Al-N is another choice of dispersive solid phase extraction (d-SPE) for the elimination of sugar as a mean carbohydrate, polypeptide chain as amino acid profile and several vitamins etc in the rice sample. Furthermore, the other component in rice is saturated fatty acid that has a long chain of carbon atoms and shows the chemical property of a non polar compound. Hereby, C18 is another d-SPE for cleanup of non polar matrices because of its extreme retentive nature for non-polar compounds on the hydrocarbon long chain. In this study, a similar functional group on d-SPE sorbent was compared such as PSA and NH_2 by combination with three type d-SPE. The parameters in table 3.5 were varied on the extraction procedure III. When mixed d-SPE was employed, the effective method was performed by mean recoveries as shown in table 4.8. In summary of combined d-SPE study, the %recovery of CIT and NIV were increased for 12% and 7%, respectively (comparison with the individual PSA employment.) Consequently, the combination of PSA, C18 and Al-N were employed.

Table 4.8 The comparison results of mixed d-SPE method.

Mycotoxins	Spiked level, mg/kg	M 18*	M 19*
AF-B1	0.02	84±8.8	79±3.1
AF-B2	0.02	101±4.1	94±4.5
AF-G1	0.02	83±6.4	81±2.8
AF-G2	0.02	96±3.3	87±4.7
CIT	0.02	64±3.1	58±2.4
DON	0.10	83±2.0	76±2.2
FUM-B1	0.02	73±4.2	62±5.7
FUM-B2	0.02	88±8.2	78±1.0
HT-2	0.02	98±3.6	92±4.8
OTA	0.02	95±4.2	95±5.9
OTB	0.02	95±3.3	93±5.6
NIV	0.10	65±4.4	64±2.2
T-2	0.02	104±4.4	107±2.6
ZEA	0.02	82±6.7	77±7.5

* M18 is a mixing of 0.4 g PSA + 0.25 g Al-N + 0.25 g C18, M19 is is a mixing of 0.4 g NH_2 + 0.25 g Al-N + 0.25 g C18

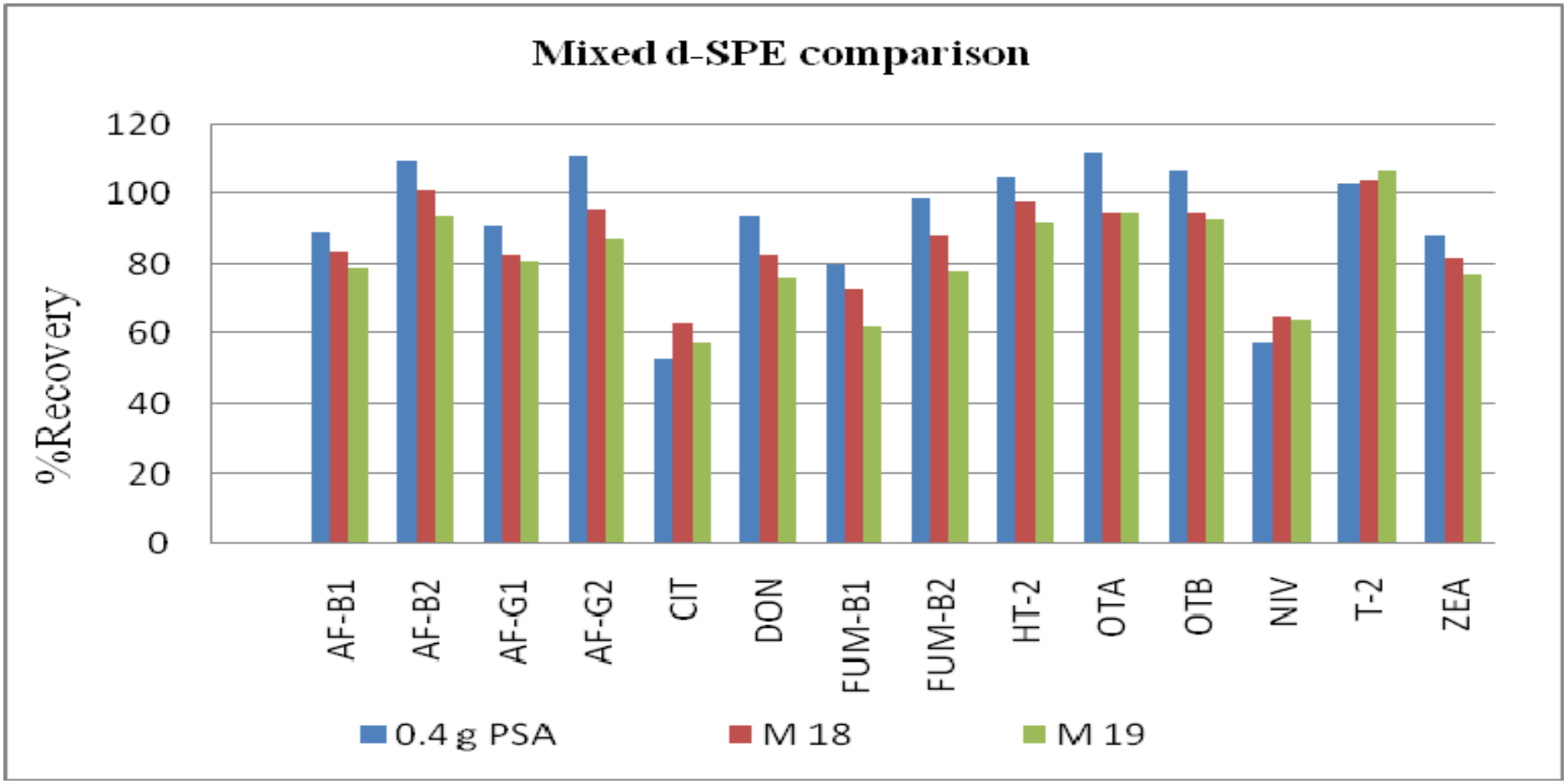


Figure 4.9 The mean recoveries comparison between the method M 18 and M 19 as Method M 18 is a mixing of 0.4 g PSA + 0.25 g Al-N + 0.25 g C18 and Method M 19 is a mixing of 0.4 g NH_2 + 0.25 g Al-N + 0.25 g C18.

4.3 Method validation

The process of characterizing the performance to be expected from a method in terms of its scope, selectivity, accuracy, repeatability, reproducibility, LOD and LOQ should be established prior to the analysis of the sample. This study described an analytical method performance to support the validity of the data.

4.3.1 The selectivity evaluation

The study of the selectivity was also shown in table 4.8 by the retention time (t_R) and the ion ratio of standard (ratio of the area of qualitative ion over the area of quantitative ion) as shown in table. 4.9. Regarding the confirmation of mycotoxins, the ion ratio was used for decisions of the accurate analytes. The ion ratio is one of the most significant factors in terms of the confirmation of questioned analytes. The acceptable range of the ion ratio values complies with 2002/657/EC concerning the performance of analytical methods and the interpretation of results. [54] Furthermore, the retention times are accepted at ± 0.15 minutes variance.

Table 4.9 The demonstration of slope, retention time and ion ratio.

Mycotoxins	t_R, (mins) Approx.	Ion ratio	Acceptable ion ratio range
AF-B1	4.86	0.91	0.73 - 1.09
AF-B2	4.76	0.83	0.66 - 1.00
AF-G1	4.70	0.48	0.36 - 0.60
AF-G2	4.65	-	-
CIT	4.93	0.08	0.04 - 0.12
DON	4.00	0.51	0.41 - 0.61
FUM-B1	4.29	0.99	0.79 - 1.19
FUM-B2	5.20	0.57	0.46 - 0.68

Table 4.9 *(continued).*

Mycotoxins	t_R, (mins) Approx.	Ion ratio	Acceptable ion ratio range
HT-2	5.08	0.91	0.73 – 1.09
OTA	5.64	0.56	0.45 – 0.67
OTB	5.15	0.31	0.23 – 0.39
NIV	3.75	0.97	0.78 – 1.16
T-2	5.45	0.76	0.61 – 0.91
ZEA	5.72	0.75	0.60 – 0.90

4.3.2 The linearity study

In this parameter, calibration curves for each mycotoxin were constructed by plotting dependence between the primary product (quantitative ion) of ion areas of standard and the actual standard concentration. Results were then fitted by the coefficient of determination (R^2). The linear regression was not forced through the origin. The results of the R^2 in table 4.10 and 4.11 demonstrate that the standard curves are linear over the investigated concentration range. The slope of linear equation determines the sensitivity of the compound. The comparison between the standard calibration curve and matrix-matched calibration curve was evaluated for the significant difference by statistical *t* values (*t*-test: Paired two samples for means) at 95% confidential level.

4.3.2.1 The standard calibration curve: The standard mixture (section 3.3.3) was prepared by dilution in mobile phase A/B = 1/1 and evenly spaced (see table 3.6) prior to UPLC-MS/MS analysis. The coefficient of determination, R^2 showed a value better than 0.99 which performed as the linearity over the concentration range. The linear regression plots were shown in APPENDIX B.

Table 4.10 The linear equation and coefficient of determination of standard calibration curve

Mycotoxins	Conc. range, mg/L	Linear equation	R^2
AF-B1	0.01-0.50	y = 291,115 x + 876	0.9972
AF-B2	0.01-0.50	y = 131,068 x + 845	0.9979
AF-G1	0.01-0.50	y = 255,024 x + 895	0.9971
AF-G2	0.01-0.50	y = 174,185 x + 1,164	0.9983
CIT	0.01-0.50	y = 423,437 x - 1,041	0.9942
DON	0.05-2.50	y = 3,036 x + 71	0.9984
FUM-B1	0.01-0.50	y = 40,069 x + 201	0.9965
FUM-B2	0.01-0.50	y = 71,731x + 353	0.9964
HT-2	0.01-0.50	y = 17,526x - 18	0.9917
OTA	0.01-0.50	y = 281,871x - 861	0.9977
OTB	0.01-0.50	y = 165,413 x - 1,614	0.9912
NIV	0.05-2.50	y = 1,623 x + 59	0.9959
T-2	0.01-0.50	y = 102,835x – 13	0.9966
ZEA	0.01-0.50	y = 30,556x – 112	0.9964

4.3.2.2 The matrix-matched calibration curve. According to the theory, the matrix might have a significant signal effect on analytes in a mass-spectrometry technique. Another issue is the potential interference of co-eluting matrix peaking at the retention time of sought analytes. As the co-eluting matrix peaks rarely interfere with compounds of interest in the MS/MS technique, we selected the matrix standard as a standard of choice for mitigation of the ion on signal effect, and consequent analytical (linearity) error. Blank sample (rice) is a representative sample of grains used for the matrix-matched standard dilution. The standard mixture (section 3.3.3) was prepared by diluting in the extract sample solution with even space prior to UPLC-MS/MS analysis. The coefficient of determination, R^2 show the value better than 0.99 which performed as the linearity in the concentrated range. The linear regression plots were shown in APPENDIX B.

Table 4.11 The linear equation and coefficient of determination of matrix-matched calibration curve

Mycotoxins	Conc. range, mg/L	Linear equation	R^2
AF-B1	0.01-0.50	y = 516,791 x + 723	0.9970
AF-B2	0.01-0.50	y = 180,140 x - 3,095	0.9920
AF-G1	0.01-0.50	y = 388,459 x - 3,680	0.9946
AF-G2	0.01-0.50	y = 262,407 x - 1,825	0.9972
CIT	0.01-0.50	y = 841,868x + 2,841	0.9949
DON	0.05-2.50	y = 3,306 x - 18	0.9971
FUM-B1	0.01-0.50	y = 59,686 x - 1,293	0.9933
FUM-B2	0.01-0.50	y = 126,110 x+ 1,261	0.9958

Table 4.11 *(continued).*

Mycotoxins	Conc. range, mg/L	Linear equation	R^2
HT-2	0.01-0.50	y = 26,206 x – 199	0.9970
OTA	0.01-0.50	y = 399,590 x + 2,453	0.9966
OTB	0.01-0.50	y = 758,260 x + 941	0.9974
NIV	0.05-2.50	y = 1,408 x – 64	0.9960
T-2	0.01-0.50	y = 152,363 x + 318	0.9978
ZEA	0.01-0.50	y = 26,424 x – 294	0.9932

4.3.2.3 The evaluation of matrix effect. The assessment of the matrix effect was investigated by the significant difference value. The t-test for paired two samples for means at 95% confident level was employed for the evaluation of significant difference. If the absolute value of t critical (the statistical *t*-test) is less than the absolute value of t calculation, the evaluation determines that there is significant difference between the dilution of standard in mobile phase and in matrix extract solution. On the other hand, if the absolute t value of calculation is less than the absolute t critical value (the statistical *t*-test), the evaluation determines that there is insignificant difference which means that there is no effect from the matrix that might prepare the standard in any solution. As a result in table 4.12, all mycotoxins showed that the absolute value of t-critical (the statistical paired *t*-test) is less than the absolute value of t-calculation (t cal) that was performed the significant difference. Moreover, the determination of the matrix effect was proved that there were the matrix effect enhancement for AF-B1, AF-B2, AF-G1, AF-G2, CIT, DON, FUM-B1, FUM-B2, HT-2, OTA, OTB and T-2. Whilst, DON and ZEA determined the matrix effect suppression. Consequently, the matrix-matched standard calibrations have to be served for this analysis because the matrix effect is almost occurred on MS/MS

technique. The results from the Excel program calculation were shown in APPENDIX C.

Table 4.12 The result of paired *t*-test for investigation of matrix effect.

Mycotoxins	**Conc.range**	**Slope**		**Paired *t*-test**	
	mg/kg	**Mp.**	**Mtx**	**| t cal |**	**| t crit |**
AF-B1	0.01-0.50	291,115	516,791	8.96	
AF-B2	0.01-0.50	131,068	180,140	5.77	
AF-G1	0.01-0.50	255,024	388,459	4.73	
AF-G2	0.01-0.50	174,185	262,407	4.23	
CIT	0.01-0.50	423,437	841,868	9.24	
DON	0.05-2.50	3,036	3,306	5.49	
FUM-B1	0.01-0.50	40,069	59,686	6.08	**2.03**
FUM-B2	0.01-0.50	71,731	126,110	9.5	**Significant difference**
HT-2	0.01-0.50	17,526	26,206	7.98	
OTA	0.01-0.50	281,871	399,590	9.81	
OTB	0.01-0.50	165,413	758,260	9.18	
NIV	0.05-2.50	1,623	1,408	11.46	
T-2	0.01-0.50	102,835	152,363	9.16	
ZEA	0.01-0.50	30,556	26,424	8.79	

4.3.3 The accuracy study

The accuracy frequently used term for trueness which described how close a test result is to the accepted reference value for the quantity measured. Lack of trueness indicates systematic error. Bias is a quantitative expression of trueness. The trueness of a result improves as bias decreases. Analytical recovery is a bias usually associated with sample preparation, extraction of the analytes. In this study, CRMs or RMs was not employed. Thus, the fortified sample was done in order to correct the bias. The fortified samples were spiked at the concentration level as in table. 3.7 by extraction procedure III. The mean recovery and standard deviation (SD) were shown in table 4.13.

Table 4.13 The mean recovery and standard deviation (SD) of the fortified sample.

Mycotoxins	**%Recovery ± SD**		
	Low	**Middle**	**High**
AF-B1	93±3.4	97±0.9	104±1.0
AF-B2	94±4.8	92±2.3	94±3.1
AF-G1	94±3.8	86±1.6	98±0.8
AF-G2	96±2.5	97±1.3	87±1.9
CIT	57±4.1	53±1.4	58±1.3
DON	75±2.9	76±1.9	79±2.7
FUM-B1	69±4.9	62±3.8	67±1.6
FUM-B2	82±3.8	82±2.5	85±2.5

Table 4.13 *(continued)*

Mycotoxins	%Recovery ± SD		
	Low	**Middle**	**High**
HT-2	85±4.0	84±4.5	84±2.5
OTA	100±2.4	93±2.3	96±1.4
OTB	94±2.4	90±0.7	96±0.8
NIV	70±2.3	73±3.5	71±4.1
T-2	97±2.5	95±1.5	98±2.1
ZEA	95±2.3	90±2.0	88±1.1

The results for the fortification at low level obtained were %recovery range 57-100%, medium level at the range 53-97% and high level at the range 58-104%, respectively. The acceptable %recovery range shown in AOAC Guidelines for single laboratory validation of chemical method for dietary supplements and botanicals said that "Acceptable recovery is a function of the concentration and the purpose of the analysis" which are as below:

Concentration	**Recovery limit**
0.01%	85-110%
10 µg/g (ppm)	85-115%
1 µg/g	75-120%
10 µg/kg	70-125%

As a result, most mycotoxins were given the %recovery within this range. There were two mycotoxins such as DON and FUM-B1 which were unacceptable as they were out of range. However, these limits may be modified as needed in view of the variability of individual results or which set of regulatory requirements are

referenced. Instances for the case of the examination of the general USDA pesticide residue proficiency study, limits of 50-150% were applied. However, recoveries of less than 60-70% should be subject to investigations leading to improvement. Refer to AOAC Guidelines for single laboratory validation of chemical method for dietary supplements and botanicals. Moreover, “Method validation and quality control procedure for pesticide residues analysis in food and feed, Document No. SANCO/2007/3131” said that the relatively poor mean recovery must be considered before taking enforcement action. Exceptionally, where recovery is low but consistent (i.e. demonstrating good precision) and the basis for this is well established, a mean recovery below 70% may be acceptable. However, a more accurate method should be used, if practicable. Intra laboratory reproducibility should be ≤ 20%.

4.3.4 The precision study.

Precision is defined as the function of **repeatability and reproducibility.** Mycotoxins were taken through the assay. Each batch was prepared by fortifying negative control of rice sample with mixed standard (section 3.3.3) at the concentration level as following table 3.7. The fortified rice samples were taken through the whole extraction procedure III. Fortification at each level was repeated ten times.

4.3.4.1 The assessment of repeatability

The percentage of recovery from ten times replicates was calculated by comparing the area of matrix-matched standard. The mean percentage recoveries obtained during the experiment are described in table 4.14 to 4.16. Indeed, the repeatability standard deviation varies with concentration. Consequently, the standard deviation (SD) of each concentration is used for calculation of the %CV. This is used for comparison to %CV target. The %CV target is the acceptance limitations for the relative standard deviation (RSD_r) of the replicate results. It is calculated from the modified Horwitz Equation.:

$$\textbf{: \%RSD}_r < 0.66 \times 2^{(1-0.5 \log C)}$$

Where: C is concentration of fortified sample (analyte /sample, g/g)

Concentration level, mg/kg	%CV or %RSDr target
0.01	21.11%
0.05	16.57%
0.10	14.93%
0.50	11.72%
1.00	10.56%

Table 4.14 The percentage recovery of the fortification at the low concentration for repeatability study. (n =10)

Replicate	%Recovery										AVG %Re	SD	%RSDr
	1	2	3	4	5	6	7	8	9	10			
AF-B1	95	87	94	90	89	93	93	97	96	97	93	3.4	3.7
AF-B2	92	90	87	88	99	100	98	98	93	97	94	4.8	5.1
AF-G1	98	92	93	91	96	91	97	93	89	101	94	3.8	4.0
AF-G2	94	98	95	96	99	98	91	98	94	97	96	2.5	2.6
CIT	51	61	55	53	54	54	62	63	57	59	57	4.1	7.3
DON	73	76	71	71	74	78	78	74	79	76	75	2.9	3.8
FUM-B1	61	69	78	64	75	69	67	67	69	68	69	4.9	7.1
FUM-B2	83	83	84	80	87	77	82	75	85	85	82	3.8	4.6
HT-2	91	89	80	88	81	79	84	87	85	85	85	4.0	4.7
OTA	102	100	103	101	101	103	100	95	101	98	100	2.4	2.4
OTB	95	93	94	96	92	92	96	95	99	91	94	2.4	2.6
NIV	68	67	71	68	75	71	70	70	71	71	70	2.3	3.2
T-2	94	94	94	97	99	99	95	100	98	100	97	2.5	2.6
ZEA	98	97	93	92	96	97	93	95	93	98	95	2.3	2.4

Table 4.15 The percentage recovery of the fortification at the middle concentration for repeatability study. (n =10)

Replicate	%Recovery										AVG %Re	SD	%RSDr
	1	2	3	4	5	6	7	8	9	10			
AF-B1	97	99	96	97	98	97	96	98	98	97	97	0.9	1.0
AF-B2	91	90	92	93	90	96	91	90	95	95	92	2.3	2.5
AF-G1	88	88	86	85	85	87	85	83	85	85	86	1.6	1.8
AF-G2	98	97	99	96	95	98	96	96	96	98	97	1.3	1.3
CIT	50	54	53	52	54	54	53	54	51	53	53	1.4	2.6
DON	74	78	74	78	77	75	77	79	74	75	76	1.9	2.5
FUM-B1	62	69	66	63	58	60	58	65	58	64	62	3.8	6.1
FUM-B2	84	82	79	87	78	82	81	82	80	82	82	2.5	3.1
HT-2	87	89	78	81	79	88	79	82	84	90	84	4.5	5.4
OTA	99	93	91	91	93	93	93	92	92	95	93	2.3	2.5
OTB	89	90	89	90	91	90	90	91	90	91	90	0.7	0.8
NIV	77	67	76	72	67	75	76	73	74	73	73	3.5	4.8
T-2	97	94	98	97	94	95	96	94	95	94	95	1.5	1.6
ZEA	89	91	90	91	87	90	93	86	90	89	90	2.0	2.2

Table 4.16 The percentage recovery of the fortification at the high concentration for repeatability study. (n =10)

Replicate	%Recovery										AVG %Re	SD	%RSDr
	1	2	3	4	5	6	7	8	9	10			
AF-B1	104	105	103	106	103	104	104	105	104	103	104	1.0	1.0
AF-B2	97	97	96	94	97	93	90	94	90	89	94	3.1	3.3
AF-G1	98	98	97	97	98	99	98	98	96	97	98	0.8	0.9
AF-G2	89	88	85	85	86	88	90	85	85	86	87	1.9	2.2
CIT	60	58	57	59	59	56	58	58	59	60	58	1.3	2.2
DON	77	83	83	74	79	78	79	80	77	78	79	2.7	3.5
FUM-B1	69	66	66	67	67	66	64	66	69	65	67	1.6	2.4
FUM-B2	83	86	85	88	87	88	84	80	85	83	85	2.5	3.0
HT-2	83	86	81	83	82	88	82	87	86	86	84	2.5	2.9
OTA	94	96	93	95	95	98	95	96	96	96	96	1.4	1.5
OTB	95	97	95	96	95	95	96	96	97	96	96	0.8	0.8
NIV	68	76	69	69	68	75	66	76	67	75	71	4.1	5.7
T-2	100	96	99	95	101	99	98	101	99	96	98	2.1	2.2
ZEA	87	89	89	87	89	88	89	87	86	87	88	1.1	1.3

Table 4.17 The summarized results of the fortification for repeatability study (n =10)

Mycotoxins	**Fortification level, mg/kg**					
	Low		**Middle**		**High**	
	%Re±SDr	**%RSDr**	**%Re±SDr**	**%RSDr**	**%Re±SDr**	**%RSDr**
AF-B1	93±3.4	3.7	97±0.9	1.0	104±1.0	1.0
AF-B2	94±4.8	5.1	92±2.3	2.5	94±3.1	3.3
AF-G1	94±3.8	4.0	86±1.6	1.8	98±0.8	0.9
AF-G2	96±2.5	2.6	97±1.3	1.3	87±1.9	2.2
CIT	57±4.1	7.3	53±1.4	2.6	58±1.3	2.2
DON	75±2.9	3.8	76±1.9	2.5	79±2.7	3.5
FUM-B1	69±4.9	7.1	62±3.8	6.1	67±1.6	2.4
FUM-B2	82±3.8	4.6	82±2.5	3.1	85±2.5	3.0
HT-2	85±4.0	4.7	84±4.5	5.4	84±2.5	2.9
OTA	100±2.4	2.4	93±2.3	2.5	96±1.4	1.5
OTB	94±2.4	2.6	90±0.7	0.8	96±0.8	0.8
NIV	70±2.3	3.2	73±3.5	4.8	71±4.1	5.7
T-2	97±2.5	2.6	95±1.5	1.6	98±2.1	2.2
ZEA	95±2.3	2.4	90±2.0	2.2	88±1.1	1.3

According to the summary results, the % RSD of repeatability precisions were calculated and compared to the AOAC values based on the manual for AOAC guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals. All % RSDr values were less than the % CV target (from Horwitz equation).

4.3.4.2 The assessment of reproducibility

Reproducibility precision refers to the degree of agreement of results when operating conditions are as different as possible. It usually refers to the standard deviation (SD_R) or the percentage of relative standard deviation ($\%RSD_R$) of results on the same test samples by single laboratory and different laboratory as terms inter laboratory.

4.3.4.2.1 The reproducibility precision of single laboratory or within laboratory.

The measured parameter**,** reproducibility precision is commonly termed the intermediate precision. It refers to the same test method performance (extraction procedure III) operating over a period of three days by using the same instrument and the same condition. Results of the intermediate precision experiment are shown in table 4.18 to 4.26. The ANOVA at 95% confident limitations proved significant differences by comparing the $\%RSD_R$ with $\%RSD_R$ critical values. These should be in compliance with AOAC guidelines for single laboratory validation of chemical method for dietary supplements and botanicals as below:

Concentration	**% RSD_R critical**
0.01%	8 %
10 µg/g (ppm)	11%
1 µg/g	16%
10 µg/kg (ppb)	32%

If ANOVA indicates “significant difference” among 3 days of analysis ($P < 0.05$), calculate the relative standard deviation, $\%RSD_R$ from the ANOVA using the equation below:

$$SD_R = \sqrt{S^2_{within} + S^2_{between}}$$

$$\% RSD_R = \frac{SDR}{mean} x100\%$$

If ANOVA indicates “insignificant difference” during 3 days of analysis ($P > 0.05$), calculate the relative standard deviation, $\%RSD_R$ from SD_R where you treat the results as a single data set, using the equation below:

$$SD_R = \sqrt{within \quad group \quad mean \quad square}$$

$$\% RSD_R = \frac{SDR}{mean} x100\%$$

Table 4.18 The percentage recovery of the fortification at the low concentration on 1st day (Day#1) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	95	87	94	90	89	93	93	97	96	97	93
AF-B2	92	90	87	88	99	100	98	98	93	97	94
AF-G1	98	92	93	91	96	91	97	93	89	101	94
AF-G2	94	98	95	96	99	98	91	98	94	97	96
CIT	51	61	55	53	54	54	62	63	57	59	57
DON	73	76	71	71	74	78	78	74	79	76	75
FUM-B1	61	69	78	64	75	69	67	67	69	68	69
FUM-B2	83	83	84	80	87	77	82	75	85	85	82
HT-2	91	89	80	88	81	79	84	87	85	85	85
OTA	102	100	103	101	101	103	100	95	101	98	100
OTB	95	93	94	96	92	92	96	95	99	91	94
NIV	68	67	71	68	75	71	70	70	71	71	70
T-2	94	94	94	97	99	99	95	100	98	100	97
ZEA	98	97	93	92	96	97	93	95	93	98	95

Table 4.19 The percentage recovery of the fortification at the middle concentration on 1st day (Day#1) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	97	99	96	97	98	97	96	98	98	97	97
AF-B2	91	90	92	93	90	96	91	90	95	95	92
AF-G1	88	88	86	85	85	87	85	83	85	85	86
AF-G2	98	97	99	96	95	98	96	96	96	98	97
CIT	50	54	53	52	54	54	53	54	51	53	53
DON	74	78	74	78	77	75	77	79	74	75	76
FUM-B1	62	69	66	63	58	60	58	65	58	64	62
FUM-B2	84	82	79	87	78	82	81	82	80	82	82
HT-2	87	89	78	81	79	88	79	82	84	90	84
OTA	99	93	91	91	93	93	93	92	92	95	93
OTB	89	90	89	90	91	90	90	91	90	91	90
NIV	77	67	76	72	67	75	76	73	74	73	73
T-2	97	94	98	97	94	95	96	94	95	94	95
ZEA	89	91	90	91	87	90	93	86	90	89	90

Table 4.20 The percentage recovery of the fortification at the high concentration on 1st day (Day#1) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	104	105	103	106	103	104	104	105	104	103	104
AF-B2	97	97	96	94	97	93	90	94	90	89	94
AF-G1	98	98	97	97	98	99	98	98	96	97	98
AF-G2	89	88	85	85	86	88	90	85	85	86	87
CIT	60	58	57	59	59	56	58	58	59	60	58
DON	77	83	83	74	79	78	79	80	77	78	79
FUM-B1	69	66	66	67	67	66	64	66	69	65	67
FUM-B2	83	86	85	88	87	88	84	80	85	83	85
HT-2	83	86	81	83	82	88	82	87	86	86	84
OTA	94	96	93	95	95	98	95	96	96	96	96
OTB	95	97	95	96	95	95	96	96	97	96	96
NIV	68	76	69	69	68	75	66	76	67	75	71
T-2	100	96	99	95	101	99	98	101	99	96	98
ZEA	87	89	89	87	89	88	89	87	86	87	88

Table 4.21 The percentage recovery of the fortification at the low concentration on 2nd day (Day#2) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	90	91	83	89	84	86	92	86	96	87	88
AF-B2	85	96	84	91	98	98	92	99	91	95	93
AF-G1	87	84	85	85	84	88	89	88	87	86	86
AF-G2	89	92	90	83	92	87	88	93	93	90	90
CIT	71	63	73	69	72	57	56	56	62	53	63
DON	81	89	89	86	83	83	89	83	87	82	85
FUM-B1	77	67	72	71	67	69	71	77	71	72	71
FUM-B2	81	83	84	84	87	83	83	79	85	82	83
HT-2	87	85	78	95	78	94	93	91	95	90	89
OTA	98	97	101	100	99	102	97	101	96	102	99
OTB	94	93	102	91	92	97	100	90	91	99	95
NIV	67	66	64	67	66	63	77	78	74	74	70
T-2	91	91	100	102	92	90	98	97	96	100	96
ZEA	87	90	88	92	88	86	85	88	85	89	88

Table 4.22 The percentage recovery of the fortification at the middle concentration on 2nd day (Day#2) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	80	81	81	82	84	81	82	84	82	80	82
AF-B2	96	94	99	93	93	94	91	92	92	94	94
AF-G1	97	95	90	99	95	92	99	94	98	93	95
AF-G2	84	89	89	90	92	92	94	90	93	86	90
CIT	65	65	66	62	65	62	65	66	65	64	65
DON	78	77	76	76	75	75	76	76	76	77	76
FUM-B1	72	68	65	62	62	63	66	62	64	65	65
FUM-B2	88	85	82	85	82	87	86	86	79	75	84
HT-2	84	89	81	80	81	87	88	93	95	89	87
OTA	93	91	90	90	90	91	88	90	92	91	91
OTB	100	101	102	99	100	99	101	100	102	103	101
NIV	64	66	65	64	65	64	63	65	64	65	65
T-2	93	90	95	90	91	94	89	89	91	97	92
ZEA	90	88	86	87	89	85	83	85	86	86	87

Table 4.23 The percentage recovery of the fortification at the high concentration on 2nd day (Day#2) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	108	107	108	107	107	106	105	104	105	103	106
AF-B2	114	114	112	113	114	114	114	112	113	107	113
AF-G1	104	105	105	105	101	100	99	100	97	98	101
AF-G2	83	80	82	82	79	81	83	80	83	82	82
CIT	59	58	58	59	60	59	59	60	59	60	59
DON	74	77	74	77	76	74	73	74	76	75	75
FUM-B1	59	56	67	62	64	59	61	59	59	59	61
FUM-B2	85	86	84	86	83	86	84	81	85	85	85
HT-2	94	99	95	98	95	84	82	83	83	87	90
OTA	103	104	101	101	103	102	103	102	103	96	102
OTB	96	98	98	96	96	96	98	98	95	95	97
NIV	58	60	56	57	60	60	62	59	59	60	59
T-2	91	90	91	91	95	90	93	94	92	94	92
ZEA	80	82	84	84	84	84	83	85	81	80	83

Table 4.24 The percentage recovery of the fortification at the low concentration on 3rd day (Day#3) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	79	89	94	78	78	84	82	86	83	83	84
AF-B2	84	92	99	94	99	83	80	102	102	103	94
AF-G1	75	80	84	78	78	89	79	78	79	78	80
AF-G2	101	92	97	96	96	94	98	95	91	98	96
CIT	55	55	52	55	54	51	56	56	50	53	54
DON	79	88	91	83	85	83	85	87	79	88	87
FUM-B1	75	75	78	57	63	67	72	58	52	66	66
FUM-B2	72	76	78	87	72	72	86	79	86	83	79
HT-2	91	95	98	98	88	93	90	86	98	85	92
OTA	94	92	97	96	93	91	91	91	92	92	93
OTB	92	97	93	86	91	86	87	88	88	89	90
NIV	78	76	79	79	78	75	76	76	77	75	77
T-2	101	97	101	102	100	100	98	98	95	98	99
ZEA	87	88	86	88	89	89	89	82	90	89	88

Table 4.25 The percentage recovery of the fortification at the middle concentration on 3rd day (Day#3) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	86	88	87	85	87	87	89	89	90	86	87
AF-B2	105	104	106	104	103	99	103	103	100	100	103
AF-G1	92	92	86	88	87	86	89	85	92	87	88
AF-G2	94	92	92	92	93	93	95	94	94	92	93
CIT	53	55	56	56	57	54	56	55	53	56	55
DON	73	75	73	72	72	80	72	71	82	73	74
FUM-B1	68	74	72	68	72	78	72	73	69	68	71
FUM-B2	85	87	82	84	81	80	87	85	85	80	84
HT-2	84	84	82	90	89	96	90	85	85	89	87
OTA	91	98	98	96	96	95	99	99	98	99	97
OTB	90	88	90	87	86	89	88	89	87	88	88
NIV	61	62	61	62	62	61	63	60	61	64	62
T-2	103	101	103	100	102	103	99	99	103	103	102
ZEA	85	89	83	83	86	86	83	83	83	83	84

Table 4.26 The percentage recovery of the fortification at the high concentration on 3rd day (Day#3) of analysis for the reproducibility study. (n =10)

Replicate	%Recovery										AVG %Re
	1	2	3	4	5	6	7	8	9	10	
AF-B1	84	91	85	88	87	85	89	85	86	85	87
AF-B2	103	99	104	103	105	106	106	106	103	105	104
AF-G1	85	85	88	85	86	83	89	85	85	87	86
AF-G2	85	86	87	85	93	86	94	86	87	85	87
CIT	55	51	55	58	53	53	54	58	51	54	54
DON	70	70	71	70	71	72	70	73	73	71	71
FUM-B1	60	68	63	65	60	61	60	60	60	58	62
FUM-B2	84	86	88	86	87	82	85	81	88	88	86
HT-2	81	80	87	90	88	88	84	86	88	83	86
OTA	92	93	92	91	100	99	101	101	100	99	97
OTB	88	89	88	89	88	88	90	89	89	88	89
NIV	61	60	62	61	60	60	60	60	60	61	61
T-2	95	94	92	95	93	94	92	95	99	94	94
ZEA	95	95	90	94	94	92	89	93	93	92	93

Table 4.27 The summarized mean recovery and % RSD_R results of intermediate precision collected over 3 days at each fortified level (n =10).

Mycotoxins	**%Recovery ± SD**		
	Low	**Middle**	**High**
AF-B1	88±5.2	89±8.0	99±10.9
$\%RSD_R$ cal	7.0 (sig)	9.0 (sig)	11.0 (sig)
AF-B2	94±6.5	96±6.0	103±9.8
$\%RSD_R$ cal	6.9 (insig)	6.3 (sig)	9.5 (sig)
AF-G1	87±7.8	90±5.5	95±8.4
$\%RSD_R$ cal	9.0 (sig)	6.0 (sig)	8.8 (sig)
AF-G2	94±4.5	93±4.0	85±3.9
$\%RSD_R$ cal	4.8 (sig)	4.3 (sig)	4.6 (sig)
CIT	58±6.9	57±6.3	57±3.1
$\%RSD_R$ cal	11.8 (sig)	11.0 (sig)	5.4 (sig)
DON	82±6.7	76±2.6	75±4.3
$\%RSD_R$ cal	8.2 (sig)	3.4 (sig)	5.7 (sig)
FUM-B1	69±6.1	68±6.2	62±4.1
$\%RSD_R$ cal	8.9 (insig)	9.0 (sig)	6.5 (sig)
FUM-B2	81±4.4	82±3.2	85±2.2
$\%RSD_R$ cal	5.3 (insig)	3.8 (insig)	2.6 (insig)

Table 4.27 *(continued).*

Mycotoxins	**%Recovery ± SD**		
	Low	**Middle**	**High**
HT-2	89±6.2	86±4.6	87±5.3
$\%RSD_R$ cal	7.0 (sig)	5.4 (insig)	6.1 (sig)
OTA	98±4.6	93±3.8	98±4.3
$\%RSD_R$ cal	4.8 (sig)	4.0 (sig)	4.4 (sig)
OTB	93±4.4	93±6.8	93±4.5
$\%RSD_R$ cal	4.7 (sig)	7.3 (sig)	4.8 (sig)
NIV	72±5.3	66±6.2	64±6.9
$\%RSD_R$ cal	7.3 (sig)	9.4 (sig)	10.9 (sig)
T-2	97±3.2	96±5.3	94±3.7
$\%RSD_R$ cal	3.3 (insig)	5.5 (sig)	3.9 (sig)
ZEA	90±4.8	86±3.3	87±5.3
$\%RSD_R$ cal	5.3 (sig)	3.8 (sig)	6.0 (sig)

(sig) stands for significant difference, (insig) stands for insignificant difference

During the experiment, ANOVA indicated significance between 3 days as shown in table 4.27 above. Thus $\%RSD_R$ of intermediate precisions were calculated and compared to the AOAC values based on the manual for AOAC guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals. The $\%RSD_R$ of each fortification level was demonstrated in the table 4.27. All results were less than the acceptance value ($\%RSD_R$ critical).

4.3.4.2.1 The reproducibility precision of inter laboratory or between laboratories.

Another parameter, measurement of the trueness is performed by the proficiency testing (PT) that participated with “Food and Environment Research Agency, FAPAS” which provided the analysis of an external quality check sample and was dispatched to the participants. The majority of results will be centered on the mean value (median). Whilst, the satisfactory factor (z-score) was calculated to assess the competence for the laboratory that will lie between the z-score of -2 and +2. In this study, there were 4 test materials; rice, cereal (oat), breakfast cereal and maize (see table 3.8) for the determination of mycotoxins. As the results in table 4.28 were demonstrated the comparison with the assigned value, results, % recovery and z-score.

Table 4.28 The summary results and z-scores of PT

Test Material	**Mycotoxins**	**%Re[a]**	**Assigned value, µg/kg**	**Results, µg/kg**	**z-score**
Rice	Total AFs	94	5.09	5.76	0.6
Oat	OTA	95	5.92	3.58	-1.8
Breakfast cereal	ZEA	100	69.5	76.07	0.4
Maize	DON	88	1714	1513	-0.8

a is the %recovery form lab’s participants.

4.3.5 The limit of detection and the limit of quantitation.

The limit of detection is a very simple concept. It is the smallest amount or concentration of an analyte that can be estimated with acceptable reliability. This value is used for the control of undesirable impurities that are specified as “not more than” a specified low level and for low level contaminants. The calculation of the LOD value is 3 times signal to noise of the smallest fortification level. Whilst, the limit of quantification is that means the concentration level of an analyte that can be determined as the absolute amounts which was calculated by 10 times signal to noise of the smallest fortification level. The summary of LOD and LOQ level are shown in table 4.29 below:

Table 4.29 The LOD and LOQ values of mycotoxins in rice.

Mycotoxins	LOD, µg/kg	LOQ, µg/kg
AF-B1	0.5	1.7
AF-B2	0.5	1.7
AF-G1	0.5	1.7
AF-G2	1.0	3.3
CIT	1.0	3.3
DON	5.0	16.7
FUM-B1	1.0	3.3
FUM-B2	0.5	1.7
HT-2	5.0	16.7
OTA	0.5	1.7
OTB	0.5	1.7
NIV	15.0	50
T-2	1.0	3.3
ZEA	0.5	1.7

4.4 The comparison results of method performance with IAC

For the extraction of mycotoxins by IAC, three manufacturers i.e. Romer Labs® Inc (MO, U.S.A.), R-Biopharm Rhöne Ltd (Glasgow, Scotland) and Vicam L.P. (Watertown, MA, U.S.A.) provided the IAC for this study. The % recoveries from duplicates of the fortified samples and LOD of IAC method were compared for the method performance. The fortified samples were prepared at the concentration level following the table 3.9 by the individual commercial extraction procedure (method no. IAC-1 to IAC-11). The results were demonstrated in table 4.30 below:

Table 4.30 The % recovery and LOD of IAC from three manufacturers.

Mycotoxins	Manufacturer	Method No.	%Recovery	LOD, µg/kg
Total AFs	Romer	IAC-1	108	112
Total AFs	R-Biopharm	IAC-2	95	72
AFs	Vicam	IAC-3	89-118	(0.3, 2, 0.05, 3)[a]
DON	Romer	IAC-4	74	75.0
DON	R-Biopharm	IAC-5	103	30
DON	Vicam	IAC-6	101	20
Total OTA & OTB	Romer	IAC-7	82	3.0
OTA	Vicam	IAC-8	71	0.3
ZEA	Romer	IAC-9	89	1.0
ZEA	R-Biopharm	IAC-10	103	3.0
ZEA	Vicam	IAC-11	108	0.3

a is the LOD of AF-B1, AF-B2, AF-G1 and AF-G2, respectively.

To summarize the results of IAC usage, the good % recoveries and low LOD were obtained. However, the individual extractions, specification of the IAC and consumable times must be served for this technique.

4.5 The results of application to mycotoxin determination in various samples.

In order to evaluate the applicability of the optimized method, various commodities such as feed, corn starch, cocoa, maize, rice, and malt powder were the representatives of test sample for the determination of mycotoxins in the contaminated sample. The optimization of the modified QuEChERS method was applied with the UPLC-MS/MS condition. As shown in table 4.31, ZEA is often detectable but at a low level. While for, FUM-B1 and DON were detected in feed and maize at high levels. However, it was hardly any mycotoxins were detected in corn starch. For the study of mycotoxins analysis in real sample could be supported for the monitoring of mycotoxins with trace analysis.

Table 4.31 The occurrence of mycotoxins in various commodities by modified QuEChERS and determination with UPLC-MS/MS.

Commodities	**Detectable Mycotoxins**	**No. of positive sample**	**Positive range (min-max), µg/kg**
Feed 11 sample	ZEA	5	3 - 15
	DON	3	48 - 62
	FUM-B1	3	27 - 32
Corn starch 5 sample	ND [a]	-	-
Cocoa 5 sample	ZEA	5	5 - 10

ND [a] stands for Not detected.

Table 4.31 *(continued).*

Commodities	Detectable Mycotoxins	No. of positive sample	Positive range (min-max), µg/kg
Maize flour 2 sample	FUM-B1	2	25 - 50
Rice 12 sample	AF-B1	6	2 - 6
	ZEA	2	2 - 3
	ND [a]	4	-
Malt powder 2 sample	ZEA	1	2
	ND [a]	1	-

ND [a] stands for Not detected.

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

Finally, a new method was applied for the identification and determination of fourteen mycotoxins i.e. aflatoxin B1 (AF-B1), aflatoxin B2 (AF-B2), aflatoxin G1 (AF-G1), aflatoxin G2 (AF-G2), citrinin (CIT), deoxynivalenol (DON), fumonisin B1 (FUM-B1), fumonisin B2 (FUM-B2), HT-2 toxin (HT-2), ochratoxin A (OTA), ochratoxin B (OTB), nivalenol (NIV), T-2 toxin (T-2) and zearalenone (ZEA) in rice. This analysis was developed for the isolation of these residues from the matrix which contains high carbohydrates (sugar) such as rice. Moreover, ultra performance liquid chromatography (UPLC) coupled with the Micromass Quattro Premier XE (tandem quadrupole mass spectrometer) is used as a suitable technique and instrument of choice for the analysis of these mycotoxins residues in rice matrixes.

For the optimization of the resolution and sensitivity, ultra performance liquid chromatography (UPLC) was employed under the time/gradient program (in table 5.1) and the conditions of UPLC as below:

Column	:	Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm) with guard column (2.1 x 5 mm, 1.7 µm)
Flow rate	:	0.25 mL/min
Mobile phase	:	A is 5 mM ammonium formate with 0.5% formic acid B is ACN / MeOH = 1/1 (V/V)
Total run time	:	11 minutes
Post time	:	2.5 minutes
Injection volume	:	5 µL
Injection mode	:	Needle-overfill partial loop

Table 5.1 The time/gradient program for isolation of 14 mycotoxins

Times	% A	% B
0	95	5
2	95	5
3	30	70
5.5	30	70
6	5	95
8	5	95
8.5	95	5

Under the optimization of MS/MS conditions (in table 5.2), the precursor ions and product ions were employed for multiple reaction monitoring (MRM) of operating mode (as shown in Table 5.3) via electrospray ionization in positive and negative mode.

Table 5.2 The optimized parameters of MS/MS conditions.

Parameters	ESI+	ESI-
Capillary voltage (kv)	2.5	0.5
Extractor (Volts)	3	3
Source temperature (ºC)	120	120
Desolvation temperature (ºC)	350	350
Cone gas flow (L/Hr)	1000	1000
Low MS 1 Resolution	13.5	13.5
High MS 1 Resolution	13.5	13.5
Ion energy 1	0.3	0.6

Table 5.2 *(continued)*

Parameters	**ESI+**	**ESI-**
Low MS 2 Resolution	13	12
High MS 2 Resolution	13	12
Ion energy 2	0.5	0.5
Collision gas flow (mL/min)	0.18	0.18
Multiplier	640	655

Table 5.3 The molecular weight, ionization mode and MRM transition

Mycotoxins	**MW.**	**Transitions**	**Ionization mode**
AF-B1	312.27	313.12 > 285.11	ESI +
		313.12 > 241.09	
AF-B2	314.29	315.12 > 259.13	ESI +
		315.12 > 287.14	
AF-G1	328.27	329.12 > 243.13	ESI +
		329.12 > 283.15	
AF-G2	330.29	331.21 > 313.15	ESI +
CIT	250.25	251.15 > 233.14	ESI +
		251.15 > 191.08	
DON	231.27	297.23 > 249.15	ESI +
		297.23 > 231.16	
FUM-B1	721.83	722.19 > 334.38	ESI +
		722.19 > 352.36	

Table 5.3 *(continued)*

Mycotoxins	MW.	Transitions	Ionization mode
FUM-B2	705.83	706.21 > 336.39	ESI +
		706.21 > 354.39	
HT-2	424.48	442.14 > 263.18	ESI +
		442.14 > 215.16	
OTA	403.81	404.08 > 239.07	ESI +
		404.08 > 358.08	
OTB	369.37	370.12 > 205.12	ESI +
		370.12 > 187.09	
NIV	312.32	313.19 > 175.07	ESI +
		313.19 > 295.22	
T-2	466.52	484.18 > 305.20	ESI +
		484.18 > 245.18	
ZEA	318.36	317.16 >130.95	ESI -
		317.16 > 174.98	

For the stage of sample preparation, the QuEChERS was modified for the determination of these mycotoxin residues referring to the “Official Method of Analysis of AOAC International” 18th Edition, AOAC International, revision 2 (2007)” and “Fast and easy multiresidue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residues in produce, Journal of AOAC International, Vol. 86 (2003)”. Rice is a representative of grain in this study by following the extraction procedure as per the flow chart below:

Stage I : Weigh 10 ± 0.05 g of the grinded rice sample in 50 mL plastic centrifuge tube, add 10 mL of water to make a slurry sample with a vortex for a few minutes. (The fortified samples were done at this stage.)

Stage II : 10 mL of ***10% formic acid in ACN*** was added into the sample later and shaken up by using the shaker for 60 minutes.

Stage III : 4 g of $MgSO_4$, 1g NaCl, ***1 g Sodium Citrate tribasic dehydrate and 0.5 g Sodium Citrate dibasic sesquihydrate*** were added into the solution tube and shaken vigorously by hand for a few minutes avoiding the formation of lumps. And centrifuge at 3400 rpm for 5 minutes.

Stage IV : After centrifuge take the entire supernatant into another tube which contains ***0.4 g PSA, 0.25 g C18, 0.25 g alumina-N*** and 1.2 g $MgSO_4$ then cap the tubes and shake by hand for 1-2 minutes, centrifuge at 3400 rpm for 5 minutes

Stage V : Put 5 mL of the cleaned solution into the glass tube and dry using N_2-evaporator at 40 ºC. Finally, 1 mL of mobile phase (1/1) was reconstituted and the residues were filtered through 0.2 µm nylon filter prior the UPLC-MS/MS.

Figure 5.1 The schematic diagram of extraction procedure with optimized condition.

For a validity of the test method it should be ensured that the parameters are appropriate for the method's intended use and reliability. Thus, the method validation was done in order to prove the method performances. The R^2 (in table 5.4) demonstrated that the R^2 values are better than 0.99 as the linearity over the calibration range.

Table 5.4 The coefficient of determination (R^2) for the linear regression study.

Mycotoxin	**R^2**	**Mycotoxin**	**R^2**
AF-B1	0.9972	FUM-B2	0.9964
AF-B2	0.9979	HT-2	0.9917
AF-G1	0.9971	OTA	0.9977
AF-G2	0.9983	OTB	0.9912
CIT	0.9942	NIV	0.9959
DON	0.9984	T-2	0.9966
FUM-B1	0.9965	ZEA	0.9964

The study of accuracy and precision were evaluated at 3 concentration levels (low, middle, high). The percentages of recoveries were accepted over the range 53-104%. Consequently, the % RSD of within-day reproducibility was obtained at ≤ 7.3% by calculating from Horwitz equation. Thus, the low recovery (lower than 70%) was acceptable, according to the reference document as mentioned in section 4.3.3. Whilst, the % RSD of between-day (3 day) also demonstrated good precision with % RSD_R ≤ 12.5% by ANOVA single factor of data analysis at 95% confident levels. Furthermore, the PT results were served for assessment of the effective method of the contamination in trace analysis by FAPAS with rice, oat, breakfast cereal and maize as the test material. All results of z-score were within the satisfactory range (z-score ≤

|2|). The LOQ were mostly lower than the maximum limits (MLs) as shown in table 5.5. The LOQ was evaluated and showed that the effective method could be applied for the monitoring of mycotoxins analysis in rice.

Table 5.5 The comparison between the LOQ of the method and MLs.

Mycotoxins	**LOQ, µg/kg**	**MLs,**
	Modified QuE.	**µg/kg**
AF-B1	1.7	AF-B1
AF-B2	1.7	2.0
AF-G1	1.7	Total AFs
AF-G2	3.3	4.0
CIT	3.3	-
DON	16.7	1250
FUM-B1	3.3	Total FUMs
FUM-B2	1.7	4,000
HT-2	16.7	-
OTA	1.7	3.0
OTB	1.7	-
NIV	50	-
T-2	3.3	-
ZEA	1.7	75.0

Finally, a newly modified QuEChERS method was authorized as the effective approach for the determination of 14 mycotoxins in rice. This method may be applied for several types of grains extraction including tapioca starch and corn starch. Nevertheless, LOQ of the method for total AFs is not satisfactory. However, the LOQ values of the mycotoxins in this group were almost achieved the MLs. Unless, only the AFs were more developed by the new modified QuEChERS method dependency in order to achieve the MLs of the EU regulation.

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APPENDICES

APPENDIX A

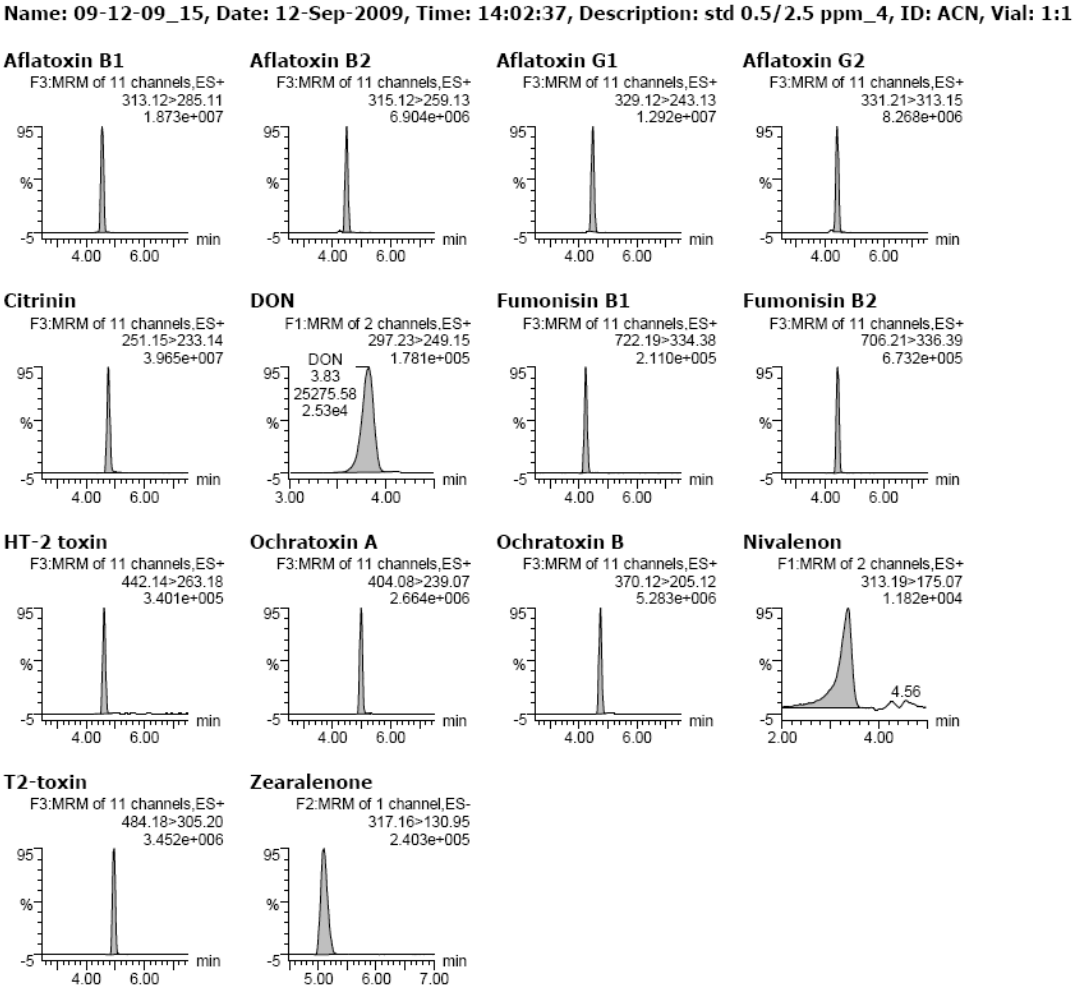

Figure A-1 The chromatographic of mixture standard mycotoxins by using the mobile phase A (5 mM ammonium formate with 0.5% formic acid) and B (100% acetonitrile).

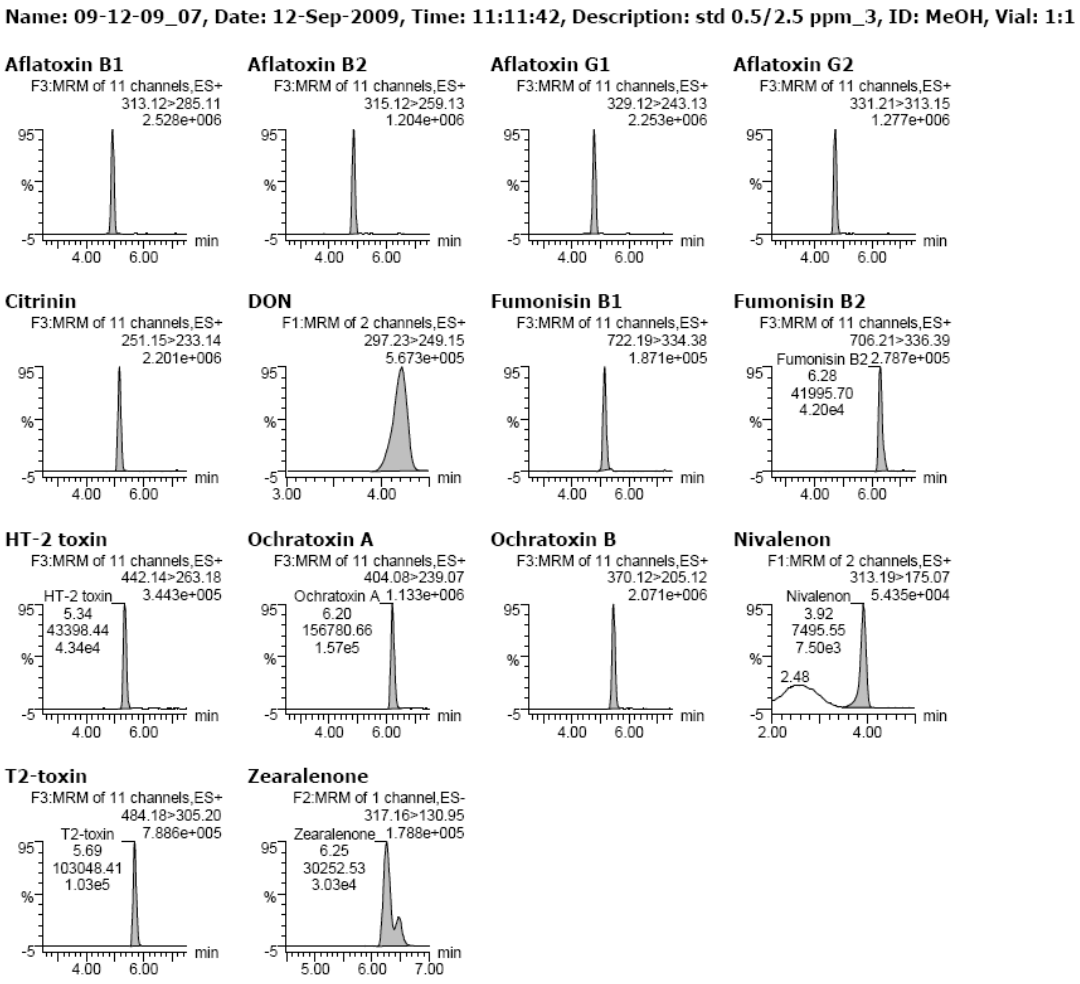

Figure A-2 The chromatographic of mixture standard mycotoxins by using the mobile phase A (5 mM ammonium formate with 0.5% formic acid) and B (100% methanol).

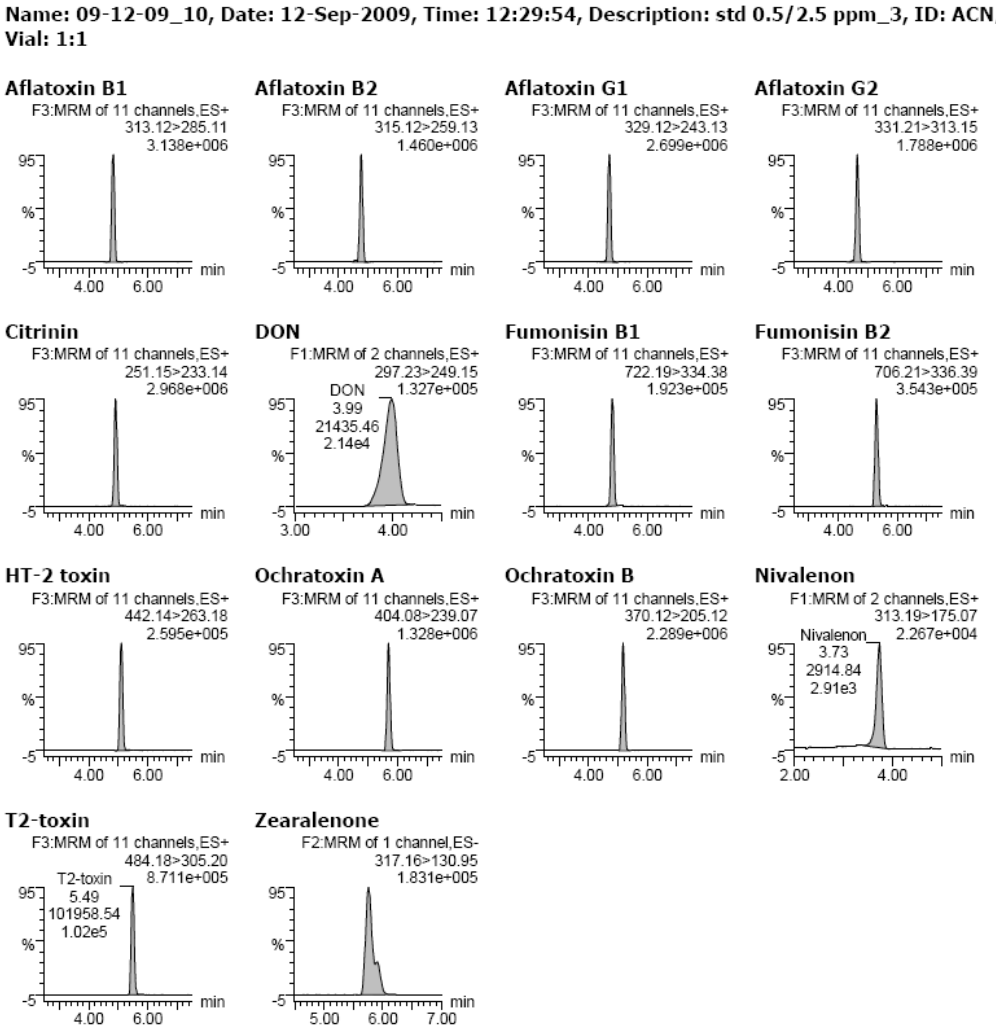

Figure A-3 The chromatographic of mixture standard mycotoxins by using the mobile phase A (5 mM ammonium formate with 0.5% formic acid) and B (1:1 acetonitrile : methanol).

APPENDIX B

B. Standard calibration and matrix-matched standard calibration curve

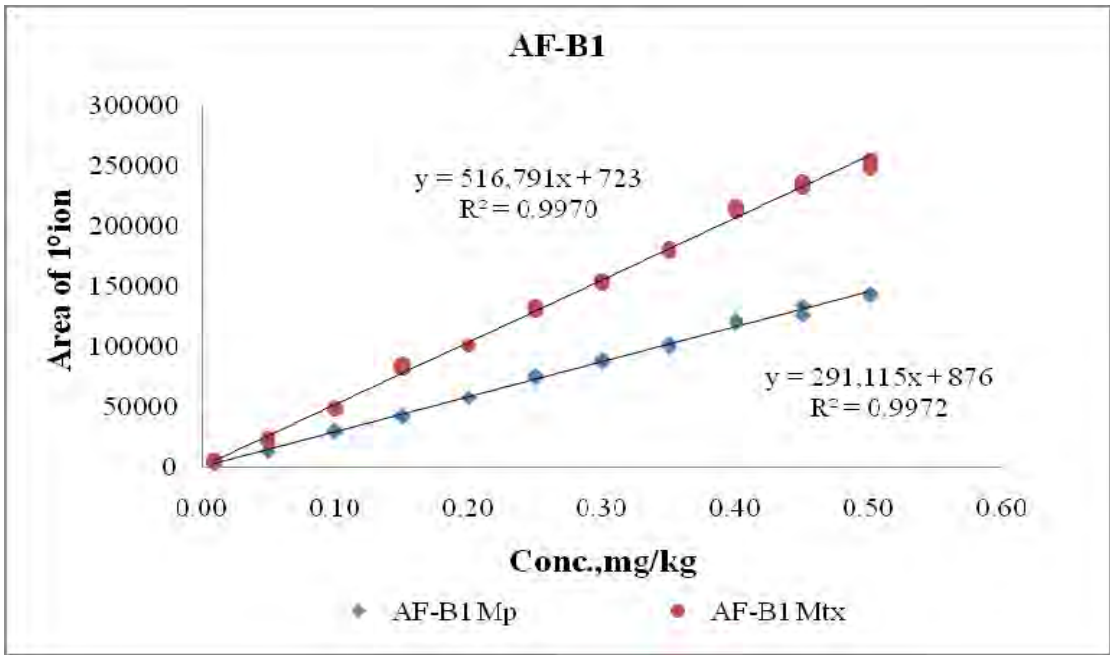

Figure B-1 The standard calibration curve of aflatoxin B1 (AF-B1)

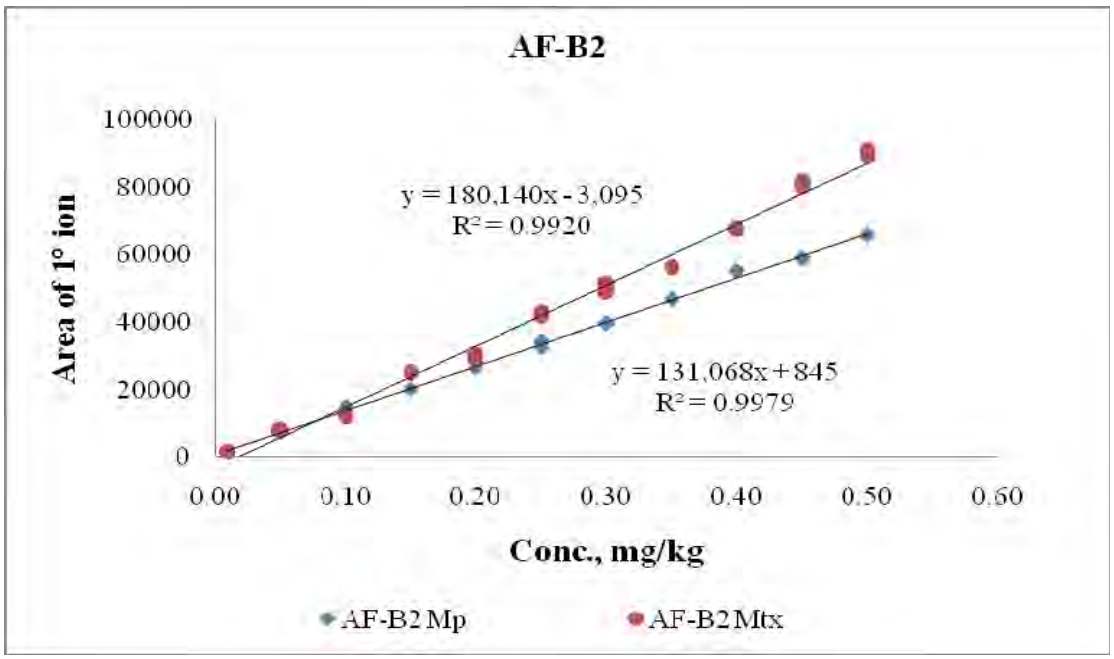

Figure B-2 The standard calibration curve of aflatoxin B2 (AF-B2).

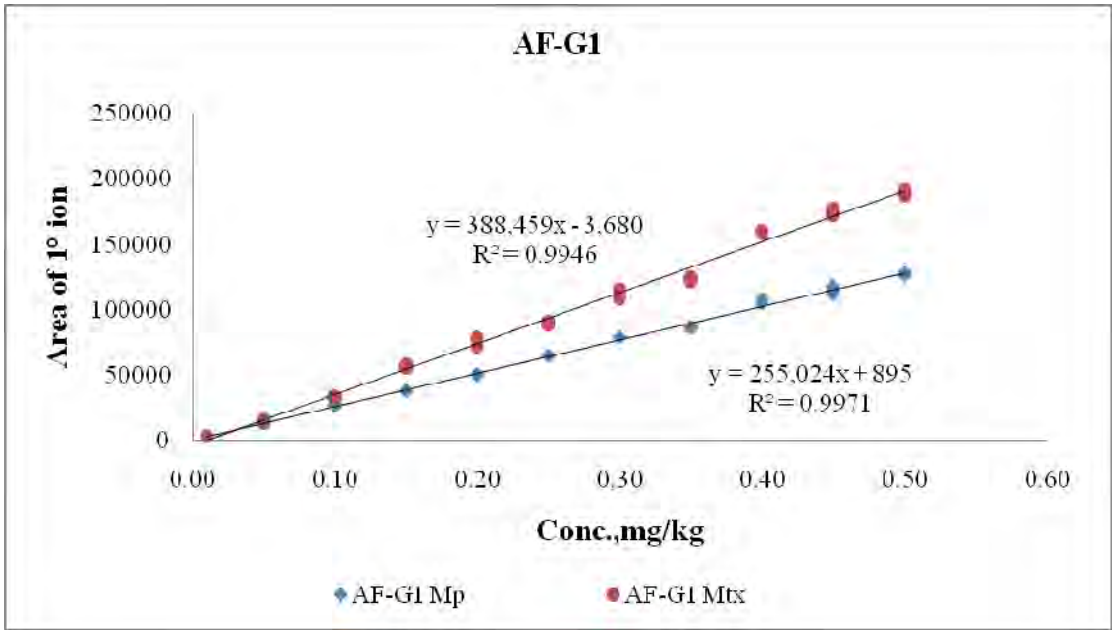

Figure B-3 The standard calibration curve of aflatoxin G1 (AF-G1).

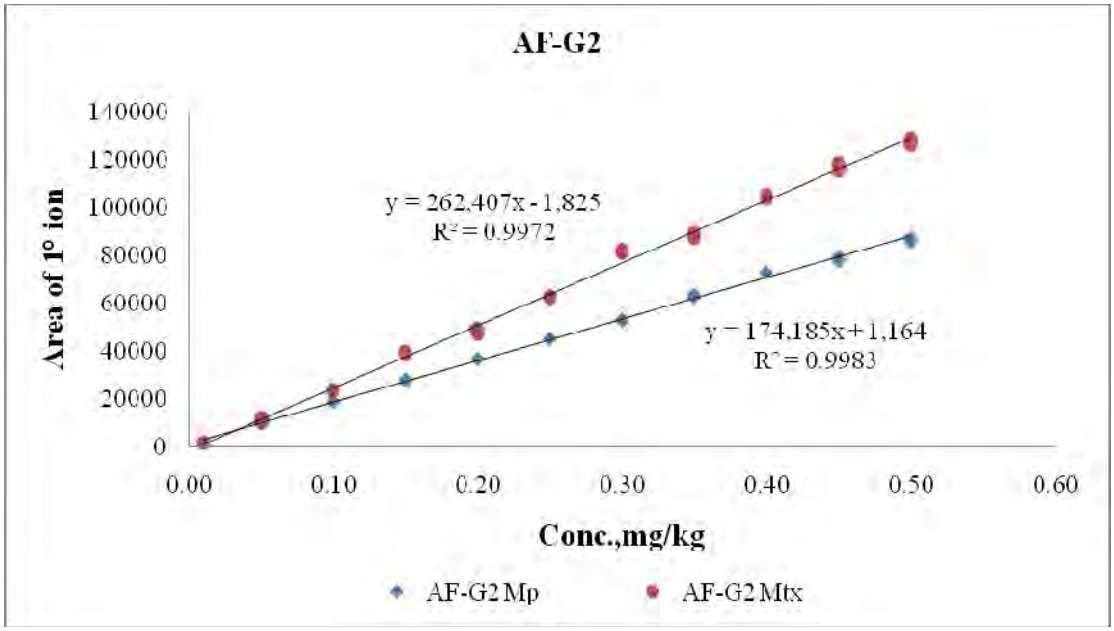

Figure B-4 The standard calibration curve of aflatoxin G2 (AF-G2).

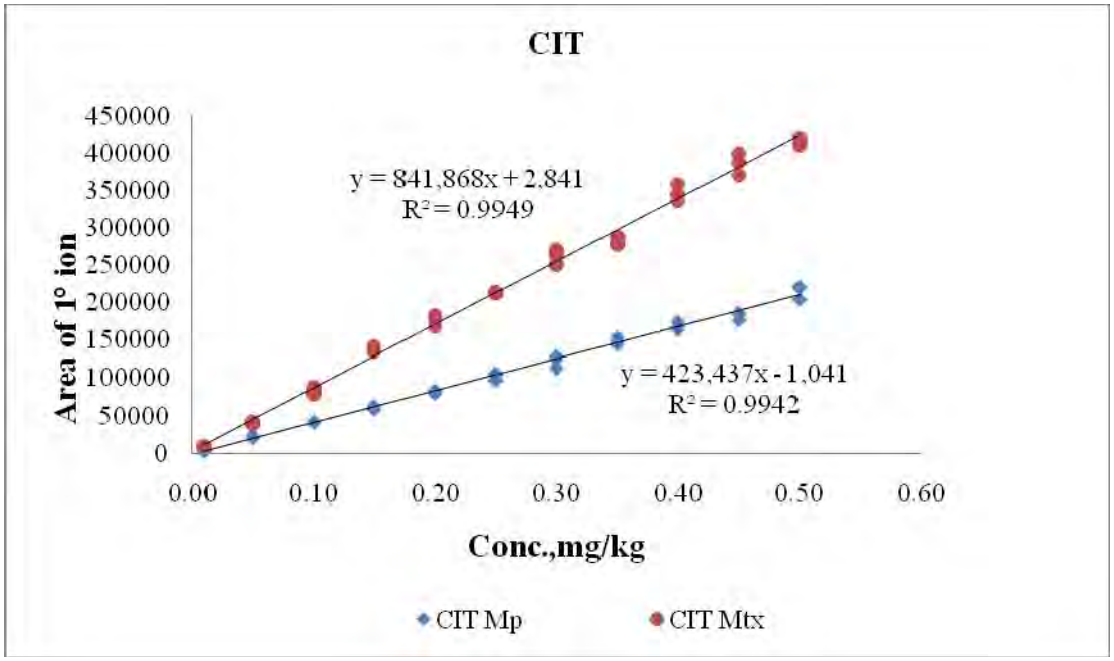

Figure B-5 The standard calibration curve of citrinin (CIT).

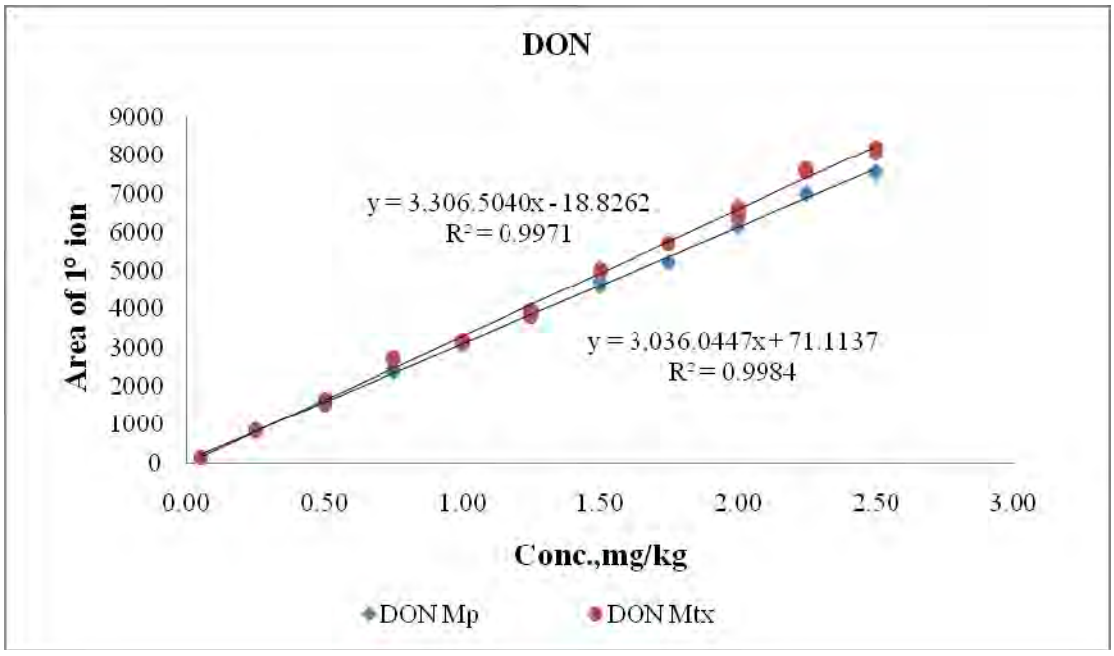

Figure B-6 The standard calibration curve of deoxynivalenol (DON).

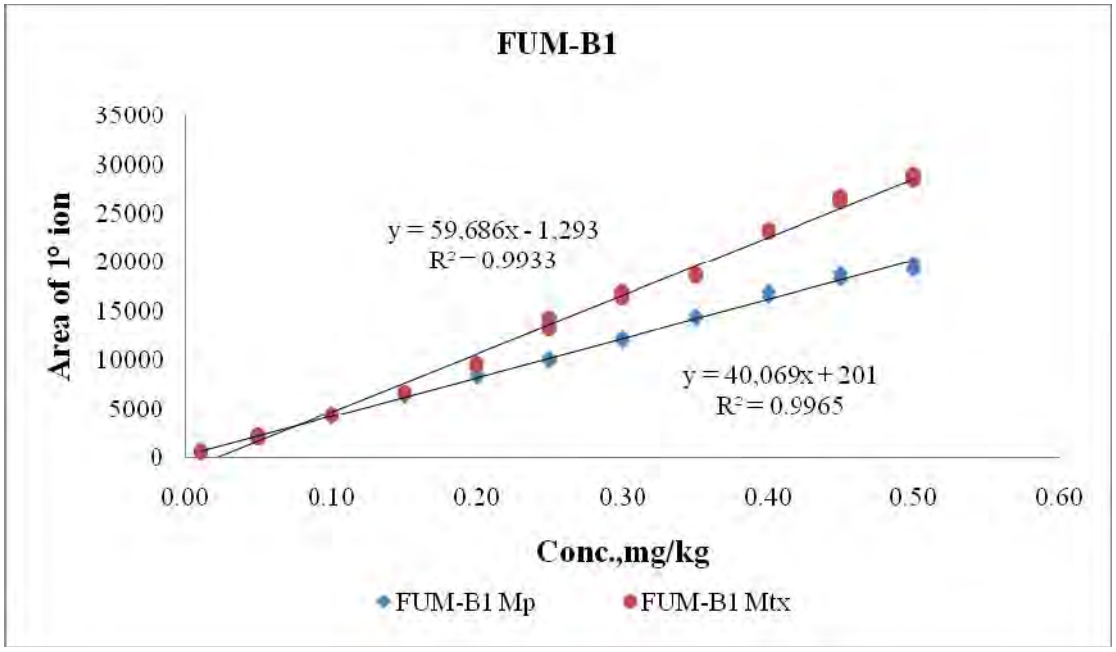

Figure B-7 The standard calibration curve of fumonisin B1 (FUM-B1)

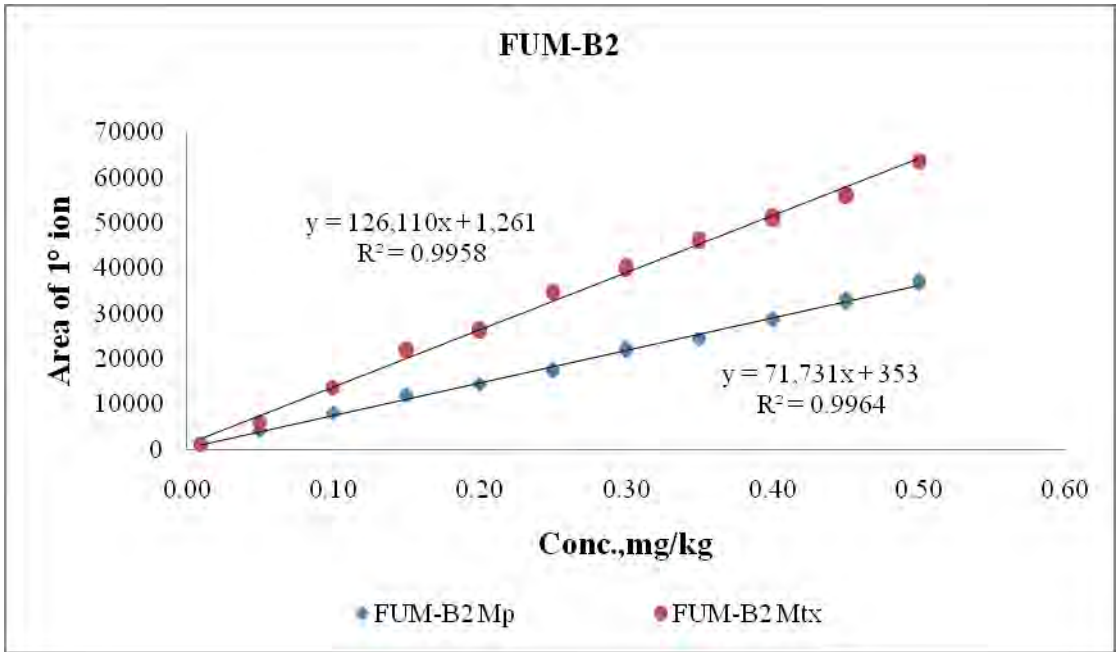

Figure B-8 The standard calibration curve of fumonisin B2 (FUM-B2)

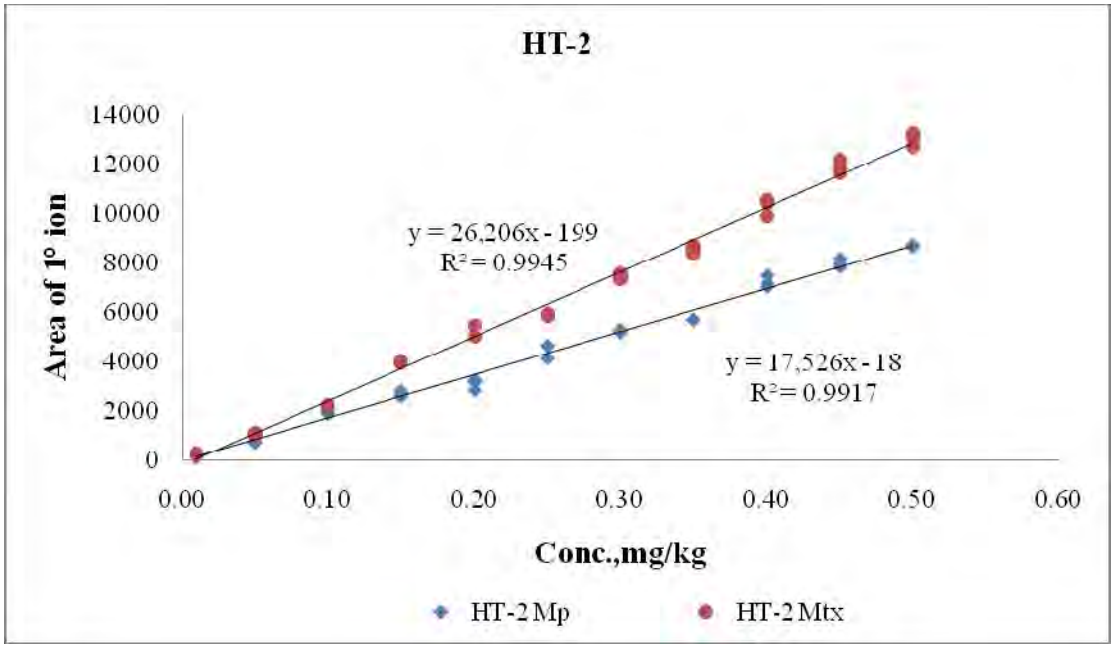

Figure B-9 The standard calibration curve of HT-2 toxin (HT-2)

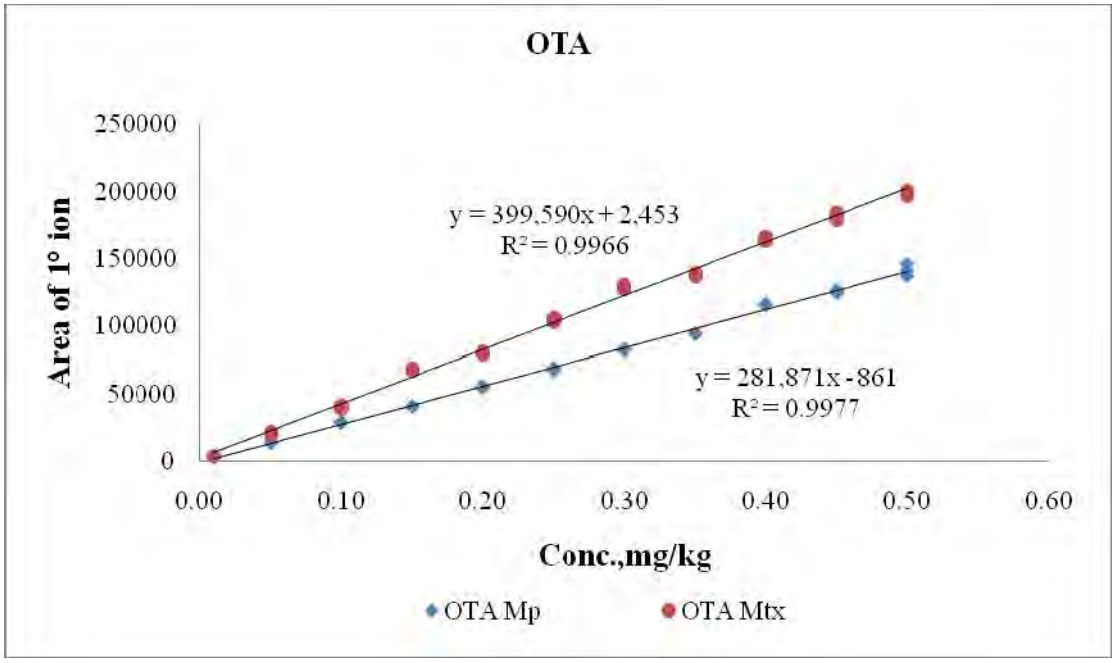

Figure B-10 The standard calibration curve of ochratoxin A (OTA)

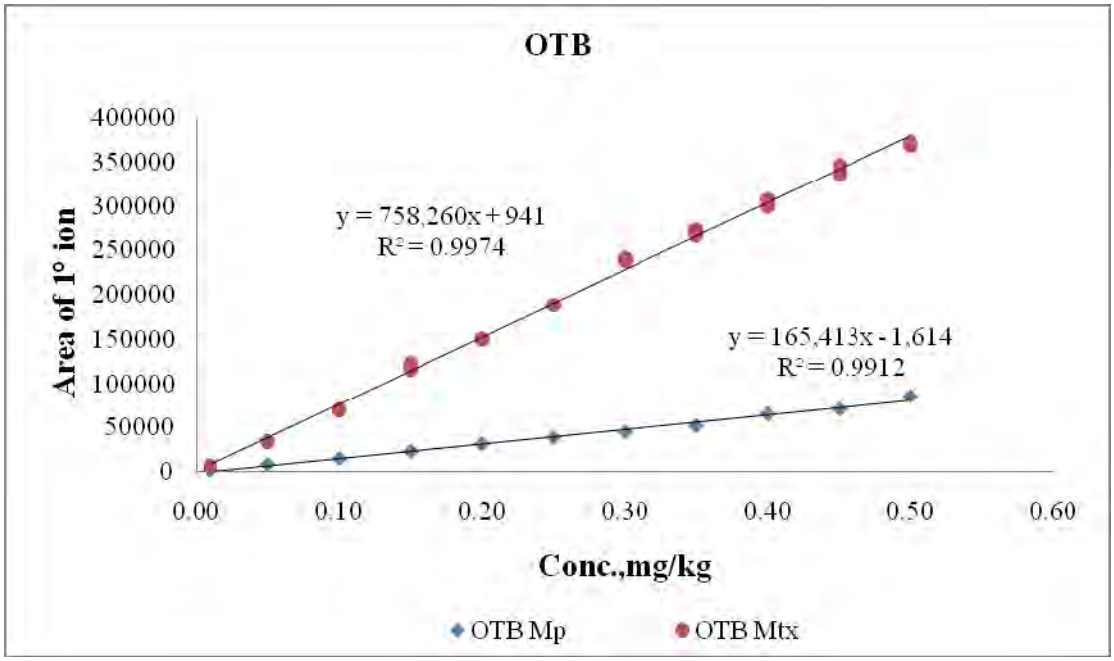

Figure B-11 The standard calibration curve of ochratoxin B (OTB)

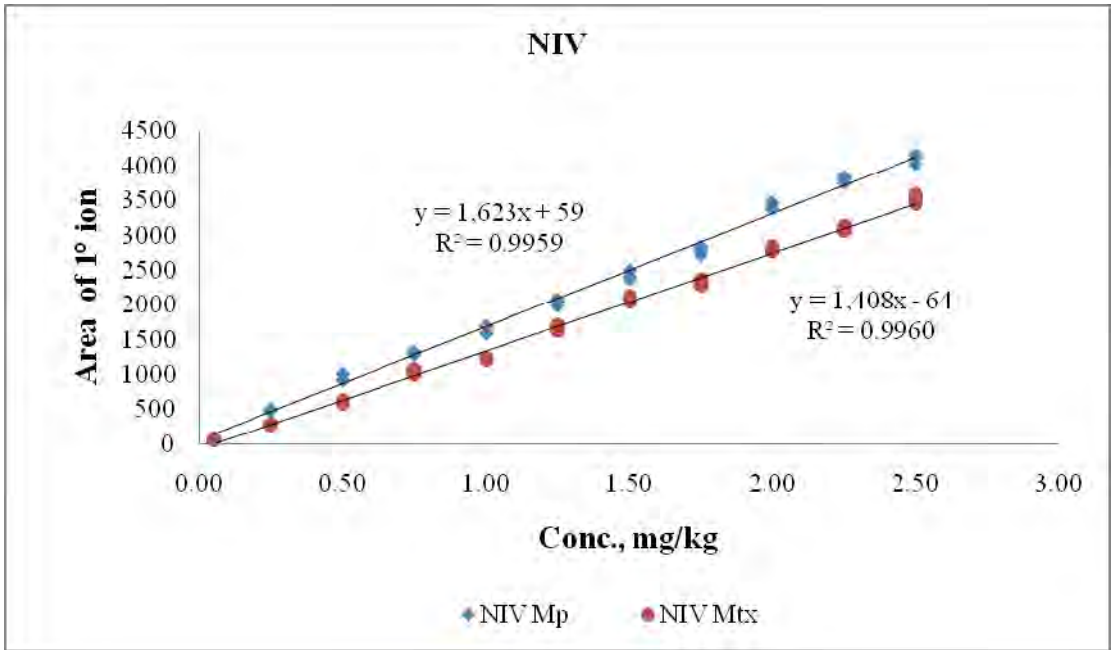

Figure B-12 The standard calibration curve of nivalenol (NIV)

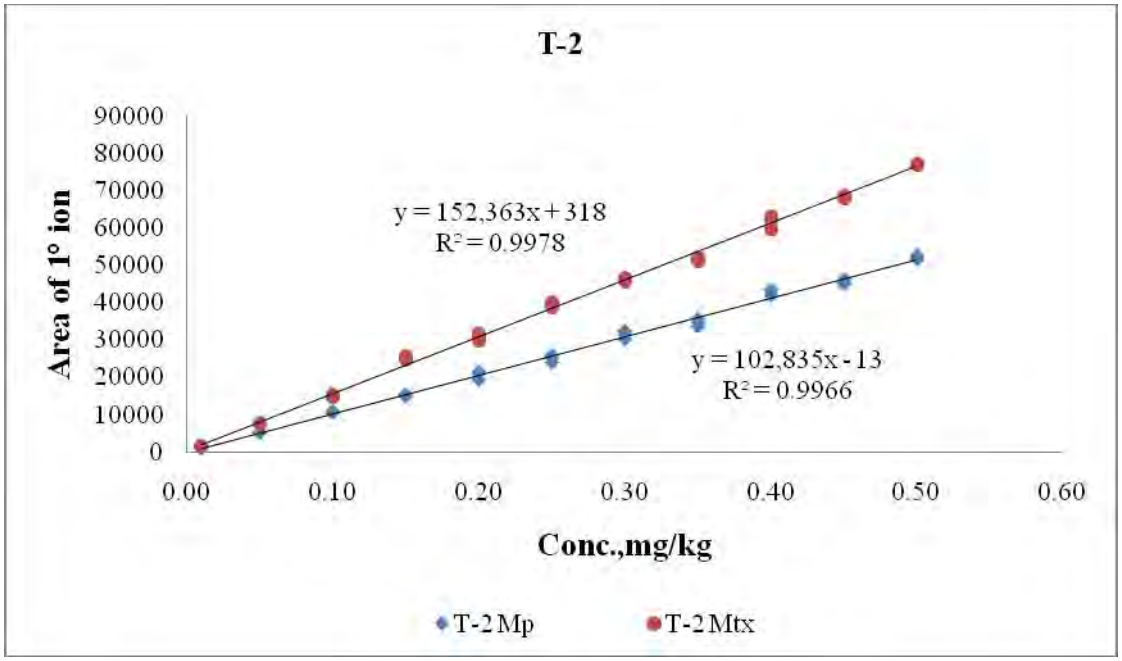

Figure B-13 The standard calibration curve of T-2 toxin (T-2)

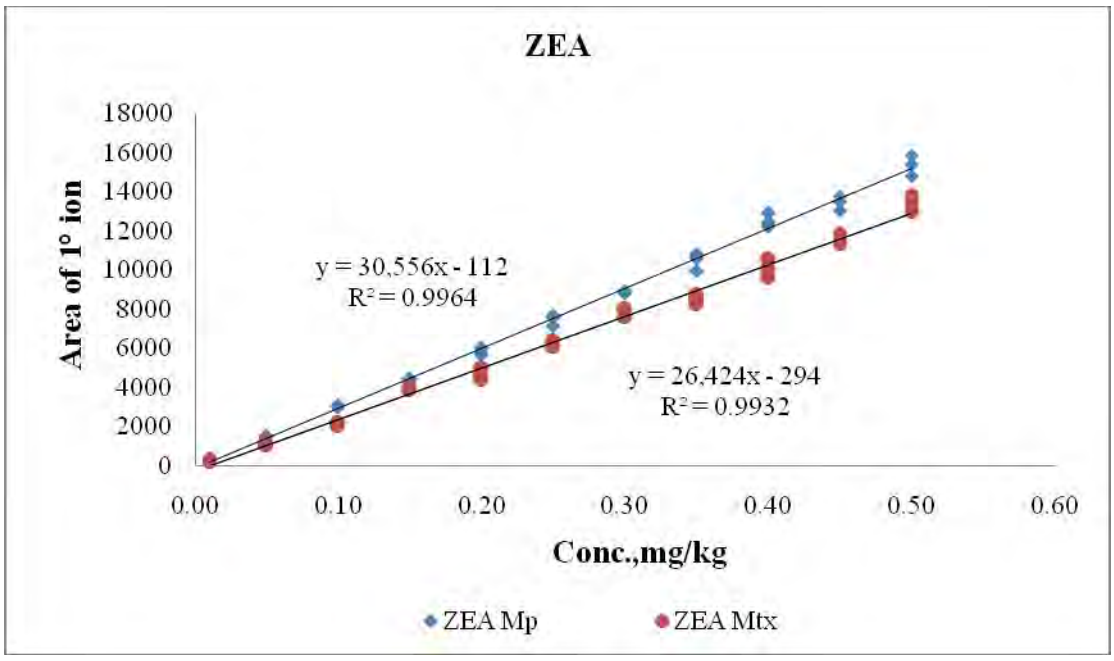

Figure B-14 The standard calibration curve of zearalenone (ZEA)

APPENDIX C

Table C-1 The result of *t*-test for paired two samples for means of AF-B1 for investigation of matrix effect.

AF-B1	*Mp*	*Mtx*
Mean	73920	130391.0606
Variance	2151920520	6782782642
Observations	33	33
Pearson Correlation	0.998033802	
Hypothesized Mean Difference	0	
df	32	
t Cal	-8.967043007	
P(T<=t) one-tail	1.52193E-10	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	3.04385E-10	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-2 The result of *t*-test for paired two samples for means of AF-B2 for investigation of matrix effect.

AF-B2	*Mp*	*Mtx*
Mean	33822.60606	42076.30303
Variance	442269602.6	825720188
Observations	33	33
Pearson Correlation	0.993394671	
Hypothesized Mean Difference	0	
df	32	
t Cal	-5.777348203	
P(T<=t) one-tail	1.03496E-06	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	2.06992E-06	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-3 The result of *t*-test for paired two samples for means of AF-G1 for investigation of matrix effect.

AF-G1	*Mp*	*Mtx*
Mean	64883.30303	93787.33333
Variance	1651604729	3841575825
Observations	33	33
Pearson Correlation	0.997540762	
Hypothesized Mean Difference	0	
df	32	
t Cal	-7.676837393	
P(T<=t) one-tail	4.73392E-09	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	9.46784E-09	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-4 The result of *t*-test for paired two samples for means of AF-G2 for investigation of matrix effect.

AF-G2	*Mp*	*Mtx*
Mean	44869.18182	64015.06061
Variance	769550082.8	1748381512
Observations	33	33
Pearson Correlation	0.997817238	
Hypothesized Mean Difference	0	
df	32	
t Cal	-7.71732407	
P(T<=t) one-tail	4.23666E-09	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	8.47332E-09	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-5 The result of *t*-test for paired two samples for means of CIT for investigation of matrix effect.

CIT	*Mp*	*Mtx*
Mean	105203.2121	214074.3939
Variance	4566543831	18037605932
Observations	33	33
Pearson Correlation	0.993191861	
Hypothesized Mean Difference	0	
df	32	
t Cal	-9.245226358	
P(T<=t) one-tail	7.45849E-11	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	1.4917E-10	
t Critical two-tail	2.036933334	
|tCal|>|t Crit| : **Significant difference**		

Table C-6 The result of *t*-test for paired two samples for means of DON for investigation of matrix effect.

DON	*Mp*	*Mtx*
Mean	3879.969697	4129.333333
Variance	5844309.905	6940754.354
Observations	33	33
Pearson Correlation	0.998360026	
Hypothesized Mean Difference	0	
df	32	
t Cal	-5.493647903	
P(T<=t) one-tail	2.3616E-06	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	4.72319E-06	
t Critical two-tail	2.036933334	
|tCal|>|t Crit| : **Significant difference**		

Table C-7 The result of *t*-test for paired two samples for means of FUM-B1 for investigation of matrix effect.

FUM-B1	*Mp*	*Mtx*
Mean	10224.72727	13682.24242
Variance	40712466.83	90815082.88
Observations	33	33
Pearson Correlation	0.994074359	
Hypothesized Mean Difference	0	
df	32	
t Cal	-6.089856046	
P(T<=t) one-tail	4.18528E-07	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	8.37055E-07	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-8 The result of *t*-test for paired two samples for means of FUM-B2 for investigation of matrix effect.

FUM-B2	*Mp*	*Mtx*
Mean	18351.69697	32903.60606
Variance	130757120.6	404404462.6
Observations	33	33
Pearson Correlation	0.995381544	
Hypothesized Mean Difference	0	
df	32	
t Cal	-9.503163914	
P(T<=t) one-tail	3.88496E-11	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	7.76991E-11	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-9 The result of *t*-test for paired two samples for means of HT-2 for investigation of matrix effect.

HT-2	*Mp*	*Mtx*
Mean	4378.454545	6376.363636
Variance	7843099.756	17487076.86
Observations	33	33
Pearson Correlation	0.993241083	
Hypothesized Mean Difference	0	
df	32	
t Cal	-7.984808598	
P(T<=t) one-tail	2.04512E-09	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	4.09025E-09	
t Critical two-tail	2.036933334	
|tCal|>|t Crit| : **Significant difference**		

Table C-10 The result of *t*-test for paired two samples for means of OTA for investigation of matrix effect.

OTA	*Mp*	*Mtx*
Mean	69862.36364	102714.7273
Variance	2016488068	4056837653
Observations	33	33
Pearson Correlation	0.997062952	
Hypothesized Mean Difference	0	
df	32	
t Cal	-9.814008031	
P(T<=t) one-tail	1.79084E-11	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	3.58169E-11	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-11 The result of *t*-test for paired two samples for means of OTB for investigation of matrix effect.

OTB	*Mp*	*Mtx*
Mean	39889.51515	191196.1515
Variance	698965808.9	14596459597
Observations	33	33
Pearson Correlation	0.991037803	
Hypothesized Mean Difference	0	
df	32	
t Cal	-9.180230559	
P(T<=t) one-tail	8.80291E-11	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	1.76058E-10	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-12 The result of *t*-test for paired two samples for means of NIV for investigation of matrix effect.

NIV	*Mp*	*Mtx*
Mean	2096.121212	1703.242424
Variance	1675801.922	1260740.064
Observations	33	33
Pearson Correlation	0.996806423	
Hypothesized Mean Difference	0	
df	32	
t Cal	11.4629142	
P(T<=t) one-tail	3.62782E-13	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	7.25564E-13	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-13 The result of *t*-test for paired two samples for means of T-2 for investigation of matrix effect.

T-2	*Mp*	*Mtx*
Mean	25788.69697	38547.72727
Variance	268693500.3	589126417.5
Observations	33	33
Pearson Correlation	0.997728222	
Hypothesized Mean Difference	0	
df	32	
t Cal	-9.168835357	
P(T<=t) one-tail	9.06296E-11	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	1.81259E-10	
t Critical two-tail	2.036933334	
|t Cal|>|t Crit| : **Significant difference**		

Table C-14 The result of *t*-test for paired two samples for means of ZEA for investigation of matrix effect.

ZEA	*Mp*	*Mtx*
Mean	7554.545455	6335.030303
Variance	23727544.82	17800356.84
Observations	33	33
Pearson Correlation	0.994921564	
Hypothesized Mean Difference	0	
df	32	
t Cal	8.798999631	
P(T<=t) one-tail	2.35302E-10	
t Critical one-tail	1.693888703	
P(T<=t) two-tail	4.70604E-10	
t Critical two-tail	2.036933334	
|tCal|>|t Crit| : **Significant difference**		

VITA

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