1. INTRODUCTION

1.1. Definitions of mycotoxins

The name "mycotoxin" is derived from the combination of the Greek word for fungus '*mykes*' and the Latin word '*toxicum*' meaning poison (Turner, Subrahmanyam, & Piletsky, 2009). A toxin is substance that is naturally produced by a plant species, an animal or by microorganisms, that is harmful to another organism. The term 'mycotoxin' is usually reserved for the relatively small molecules (molecular weight $\leq$ 700); and highly toxic chemical products formed as secondary metabolites by a few fungi that readily colonize crops in the field or after harvest. Mycotoxins are produced by saprophytic moulds that grow during storage of food or feed or a variety of endophytic moulds during plant growth.

Generally, crops that are being stored for more than a few days become a potential target for the growth of mould and mycotoxin contamination. Factors contributing to their presence or production in food and feed include temperature, moisture content and insect activity, though the interrelations between all these factors are not yet well understood and toxin production cannot reasonably be predicted (Coulumbe, 1993). Moulds grow over a temperature range of 10-40°C, at pH range of 4-8 and above 12-13% moisture content. Therefore, mycotoxins can occur in temperate and tropical regions of the world, depending on the types of fungus. They are usually genotypically specific, but can be produced by one or more fungal species for example OTA, which is produced by *Aspergillus ochraceus*, mainly in tropical regions and also by *Penicillium verrucosum*, a common storage fungus in temperate areas (Kabak, 2009; Thrane, 1989), and in some cases one species can form more than one mycotoxin (Table 1.1).

The orderly classification of mycotoxins is quite complicated, because of their different chemical structures, biosynthetic origins and their production of a large number of fungal species. A first approach is to classify them according to their differences in fungal origin, chemical composition and biological activity. In addition, the classification can be done according to how frequently they occur and in what amounts. This task is more complicated because mycotoxin contamination of food and feed relies on the environmental and climatic conditions, harvesting techniques, storage conditions and other factors.

The usual classification schemes reflect the expertise of the investigator doing the classification. For clinicians the classification is according to their effects on the organ: hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, etc. For cell biologists the classification is according to the generic groups such as teratogens, mutagens, carcinogens and allergens. Organic chemists classified mycotoxins according to their chemical structures e.g. lactones, coumarines, etc. Biochemists classified mycotoxins according to their biosynthetic origins e.g. polyketides, amino acid-derived, etc. Lastly, the physician will classify mycotoxins according to the illnesses caused by the mycotoxin e.g. St. Anthony's Fire, yellow rice disease, stachybotrytoxicosis, etc. The major groups of fungi and mycotoxins produced are presented in Table 1.1.

Table 1.1
Classification of mycotoxin producing fungi

Major classes of mycotoxin-producing fungi	Fungi species	Mycotoxins
Aspergillus	***A. flavus***	**Aflatoxin**
	A. parasiticus	
	A. nomius	
	A. flavus	**Ochratoxin**
	A. ochraceus	
	A. carbonarius	
	A. niger	
	A. clavatus	**Patulin**
	A. terreus	
	A. flavus	**Cyclopiazonic acid**
	A.versicolor	
Penicillium	***P. verrucosum***	**Ochratoxin**
	P. virridicatum	
	P. citrinum	**Citrin**
	P. verrucosum	
	P. roqueforti	**Roquefortine**
	P. cyclopium	**Cyclopiazonic acid**
	P. camemberti	
	P. expansum	**Patulin**
	P. claviforme	
	P. roquefortii	
Fusarium	***F. moniliforme***	**Fumonisin**
	F. proliferatum,	
	F. graminearum,	**Type A Trichothecenes**
	F. culmorum	
	F. crookwellense	
	F. sporotrichioides	
	F. poa	**Type B Trichothecenes**
	F. acuminatum	
	F. sambucinum	
	F. sporotrichioides	
	F. graminearum	**Zearalenone**
	F. culmorum	
	F. sporotrichioides	

1.2. Diversity and impact of mycotoxins

Mycotoxins are potentially harmful to man and domestic animals. The general interest on mycotoxins, began in 1960 with the outbreak of the Turkey X disease in the U.K, which was later shown to have been caused by secondary metabolites from *Aspergillus flavus* (aflatoxins), which appeared in farm animals in England (Whitlow & Hagler Jr, 2002; Yiannikouris & Jouany, 2002). This event highlighted the risk that other mysterious metabolites from moulds might also be deadly. Consequently, it was found that aflatoxins are hepatocarcinogens in animals and humans, and this has catalyzed research on mycotoxins.

Thousands of mycotoxins exist, but only a few pose a threat to food safety. Mycotoxins, when present in high concentrations in food and feed can impose a health risk to animals and humans and create economic losses. However, assessment of the adverse health effects is complicated by many factors including the intake levels, toxin species, age, duration of exposure, mechanisms of action and metabolism. In addition, there is a lack of research on the availability of good methods, sensitivity differences by animal species, errors in sampling and analysis and the co-existence of a variety of mycotoxins and their interactions (Whitlow & Hagler Jr, 2002).

The major mycotoxin-producing fungal genera, in terms of research in the U.S., are *Aspergillus, Fusarium and Penicillium* (Whitlow & Hagler Jr, 2002) whereas, the most prominent mycotoxins, with the most severe effects in humans and animals, are aflatoxins, deoxynivalenol, zearalenone, ochratoxin A, fumonisin, and Patulin (Bennett & Klich, 2003). Exposure to these compounds can cause adverse health effects such as kidney and liver damage (deterioration), mutagenic and teratogenic effects, birth defects, and cancers (specially liver cancer) that result in symptoms ranging from skin irritation to immune suppression, neurotoxicity, and death (WHO, 2002). The most common food commodities

that are affected are cereals, nuts, dried fruits, coffee, cocoa, spices, oil seeds, dried peas, beans and fruits, particularly apples.

The toxic syndromes resulting from mycotoxin intake are known as mycotoxicoses (Richard *et al.*, 2003). This syndrome affects human and animal health through the consumption, dermal contact or inhalation of foodstuffs and feedstuffs prepared from these commodities. In addition, they can enter the human food chain through animal products such as meat, milk, eggs and cheese resulting from livestock eating contaminated feed.

The known chemical and biological properties of the mycotoxins are varied and their toxic effects are extremely variable. The various effects from mycotoxin exposure are carcinogenicity, genotoxicity, teratogenicity, nephrotoxicity, hepatotoxicity and immunotoxicity (FAO, 2001). A summary of the some common mycotoxins, with a description of their health effects and the affected commodities is presented in Table 1.2.

Mycotoxins exhibit a wide range of non-specific actions with reference to the usual levels of exposure, such as immunosuppression and can therefore increase the risk of exposure to other diseases. Currently, there is no conclusive evidence that the normal daily exposure to some mycotoxins listed in Table 1.1 would lead to health disorders or these can occur only at high levels of exposure (e.g. for Patulin), but the maximum tolerable daily intake and residual levels in foods have been established for safety and health reason (Cigić & Prosen, 2009).

Table 1.2
Some common mycotoxins with their possible health effects and the affected commodities

Mycotoxins	Commodities	Possible Health Effects
Aflatoxins	Cereals (corn, wheat, barley, maize, oats and rye), nuts (hazel nuts, peanuts and pistachios), dried fruits (figs) and spices (Diener *et al.*, 1987)	Carcinogenicity, mutagenicty, teratogenicity and immunosuppression (Aycicek, Aksoy, & Saygi, 2005)
Ochratoxins	Cereals (maize, oats, barley and wheat), green coffees, dried fruits, spices coffee, beer and wine (Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, & Hedili, 2009)	Nephrotoxic, hepatotoxic, immunosuppressive, teratogenic and have carcinogenic effects on animals and humans (IARC, 1993)
Trichothecenes	Cereal commodities (maize, oats, barley and wheat) (WHO, 2001; Zöllner & Mayer-Helm, 2006)	Vomiting and loss of appetite; while high concentrations of type-B trichothecenes can cause chronic intoxication, leading to extensive haemorrhage, and subsequent haematological toxicities (Berthiller, Schuhmacher, Buttinger, & Krska, 2005).

1.3. Current situation of mycotoxins

On a worldwide basis, 99 countries have mycotoxin regulations for food and/or feed in 2003 (FAO, 2004). The number of countries regulating mycotoxins is significantly increasing over the years. Compared to the situation in 1995 and 2003, more mycotoxins have been regulated in more commodities, whereas the tolerance limits generally remain the same or have been reduced. This reflects a public concern on the potential adverse effects that mycotoxins can have on the health of humans and animals. Regulations become more diverse and detailed with new requirements on the official procedure for sampling and analytical methodology. For several mycotoxins, specific regulations do exist for the

aflatoxins (B1, B2, G1, G2, M1 and M2); the trichothecenes (deoxynivalenol, T-2 toxin and HT-2 toxin); the fumonisins (B1, B2 and B3); ochratoxin A and zearalenone.

For the three main toxin/matrix combinations, aflatoxins in nuts (221 regulations) ochratoxin A in cereals and cereal products (37 regulations) and patulin in fruits and vegetables (161 regulations) there are regulated for their control (FAO, 2004; van Egmond & Jonker, 2004).

1.4. Mycotoxins selected for this study

1.4.1. Aflatoxins

Aflatoxins are secondary metabolites of certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* and the less common *A. nomius* (Battilani, Barbano, & Logrieco, 2008). Hence, their name is derived from the "a" from *Aspergillus* and the "fla" from flavus. The B and G classes depicting the blue and green fluorescence emitted by their metabolites under ultraviolet (UV) light, and the sub-type 1 and 2 refers to the major and minor compounds respectively. *A. flavus* produces the type B aflatoxin only, while the other two fungal species produce both types of aflatoxins, B and G (Aycicek *et al.*, 2005; D'Mello & Macdonald, 1997). Aflatoxins have been found to be a contaminant in a wide variety of important foodstuff such as cereals (corn, wheat, barley, maize, oats and rye), nuts (hazel nuts, peanuts and pistachios), dried fruits (figs) and spices (Diener *et al.*, 1987). Among the 18 different types of identified aflatoxins, the most common naturally occurring aflatoxins in foodstuff are aflatoxin B1, B2, G1 and G2 (Battilani *et al.*, 2008). When animals consume aflatoxin-contaminated feeds, they biologically metabolize aflatoxin B1 and B2 into the hydroxylated form called aflatoxin M1 and M2, respectively. The International Agency for Research on Cancer (IARC) has classified aflatoxin B1, the most toxic, as a group 1 carcinogen, which primarily affects the liver (IARC, 1993). Exposures to aflatoxins

may cause carcinogenicity, mutagenicty, teratogenicity and immunosuppression (Aycicek *et al.*, 2005).

As a result of the toxicity of the aflatoxins, many countries have enacted regulations and also established regulatory limits for their control in foods of plant origin that are intended for human and animal consumption (FAO, 2004). Currently, aflatoxins are regulated in 93 countries and the average range for these regulatory limits for aflatoxin B1 and total aflatoxins are from 1-20 µg/kg and from 0-35 µg/kg, respectively (FAO, 2004). However, in the European Union regulations, the maximum allowed levels for aflatoxin B1 and total aflatoxins are 2 and 4 µg/kg respectively (European Commission, 2006b).

Physical and chemical properties of the aflatoxins

Aflatoxins can be categorized into two groups according to the chemical structure, namely, difurocoumarocyclopentenone series and ifurocoumarolactone. Aflatoxin B1 contains dihydrobisfuran and a coumarin nucleus, fused to form cyclopentanone, while in aflatoxin G1, the six-membered lactone is substituted with the cyclopentanone of aflatoxin B1. Aflatoxins B2 and G2 are produced from the hydrogenation of aflatoxins B1 and G1 respectively. The chemical structures of the aflatoxins B1, B2, G1, G2, M1 and M2 are shown in Figure 1.1.

Aflatoxins occur as colorless to pale-yellow crystals at room temperature (IARC 1976, 1993). Aflatoxins are slightly soluble in water, insoluble in non-polar solvents and freely soluble in moderately polar solvents such as chloroform, methanol, dimethyl sulfoxide and acetonitrile. Aflatoxin solutions prepared in chloroform or benzene can be stored for years if kept cold and in the dark. The aflatoxins fluoresce strongly under UV radiation (ca. 365 nm). Some important chemical and physical properties of the aflatoxins are presented in the Table 1.3.

Table 1.3
Physical and chemical properties of aflatoxins

Aflatoxin	Molecular Formula	Weight (g/mol)	Melting Point (°C)
B1	$C_{17}H_{12}O_6$	312	268-269
B2	$C_{17}H_{14}O_6$	314	286-289
G1	$C_{17}H_{12}O_7$	328	244-246
G2	$C_{17}H_{14}O_7$	330	237-240
M1	$C_{17}H_{12}O_7$	328	299
M2	$C_{17}H_{14}O_7$	330	293

Crystalline aflatoxins, in the absence of moisture, are extremely stable, if kept away from light and UV radiation. However, destruction can occur in aflatoxins by opening of the lactone ring followed by decarboxylation at elevated temperatures whether in oilseed meals or in aqueous solution at pH 7.

The lactone ring makes them undergo alkaline hydrolysis such as sodium hypochlorite, chlorine, potassium permanganate, sodium perborate, hydrogen peroxide and ozone. These oxidizing agents can react with aflatoxins and the aflatoxin molecule loses their fluorescence properties. However, the mechanisms of these reactions are not completely understood (Correia, Hotza, & Segadães, 2004), and in addition, the reaction products remain unidentified in most cases.

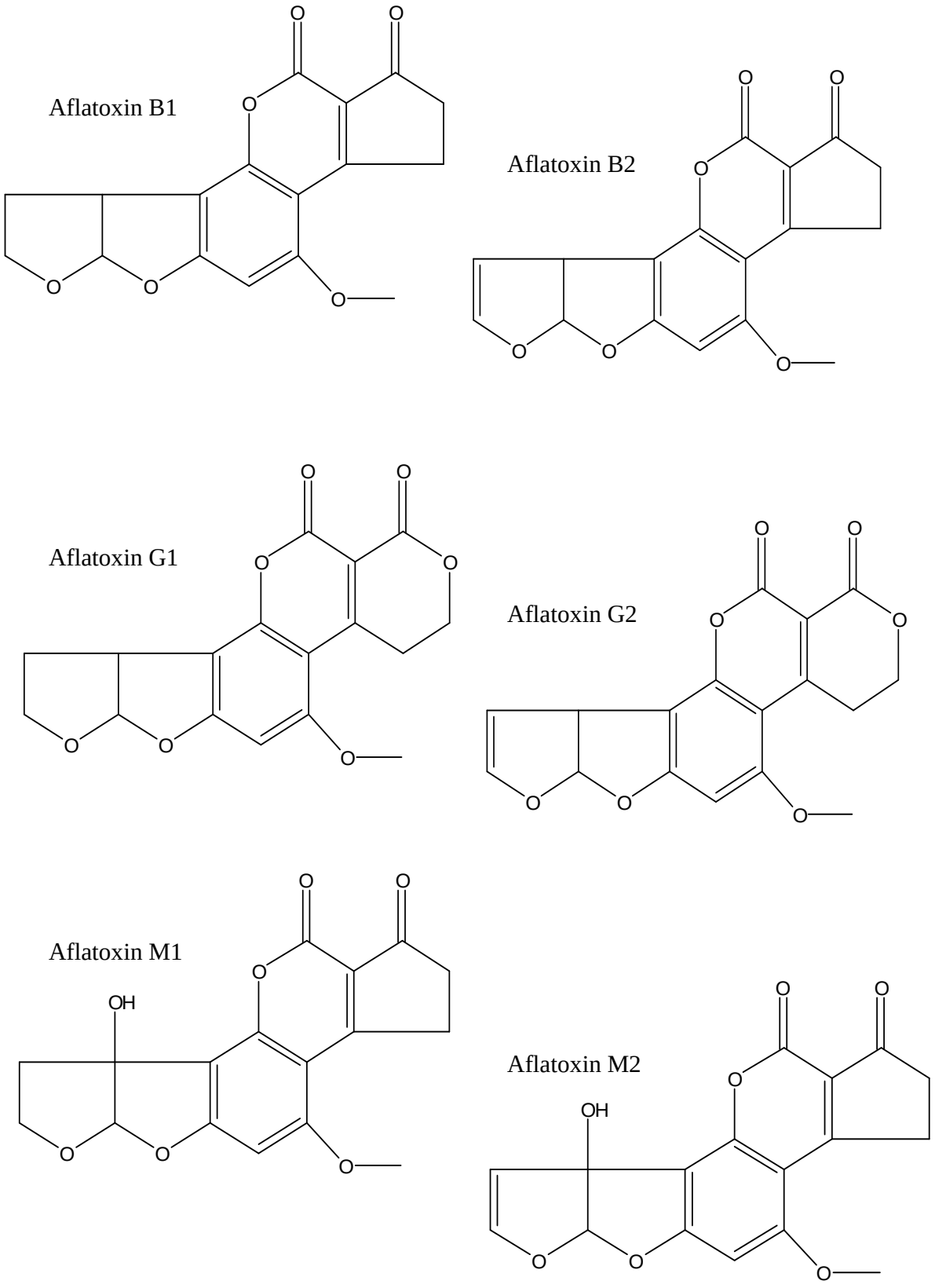


Figure 1.1: *Chemical structures of aflatoxins B1, B2, G1, G2, M1 and M2*

1.4.2. Ochratoxin

Ochratoxins are secondary metabolites produced from moulds particularly *Aspergillus* and *Penicillium* (Atkins & Norman, 1998; Ghali *et al.*, 2009). Among the family of ochratoxins, which consists of three members, A, B, and C, the most toxic and commonly detected type in foodstuff is ochratoxin A (OTA) (Atkins & Norman, 1998; Peraica, Radić, Lucić, & Pavlović, 1999).

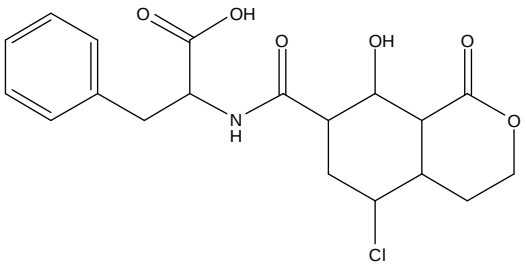
The primary source of ochratoxin contamination in food and feedstuff is cereal commodities (maize, oats, barley and wheat) in addition to groundnuts, dried fruits and coffee beans, which have been infected by the *A. ochraceus*, *A. niger*, *A. carbonarius*, *A. flavus*, *A. auricomus*, *A. glaucus*, *A. melleus*, *A. alliaceus*, *P. verrucosum* and *P. virridicatum* (Bennett & Klich, 2003).

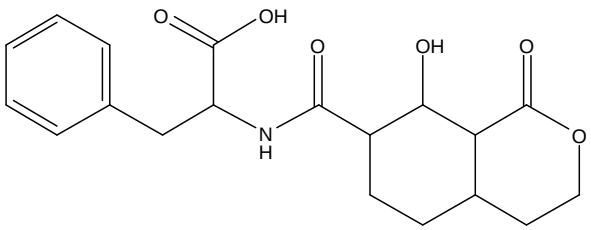
The International Agency for Research on Cancer (IARC) has classified OTA as a group 2B carcinogen, as it has been shown to be nephrotoxic, hepatotoxic, immunosuppressive, teratogenic and possess carcinogenic effects on animals and humans (IARC, 1993). Because of its toxicity and frequent occurrence, several countries have established legal regulations or recommendations for OTA levels in one or more commodities to control mycotoxin contamination of various food products (van Egmond & Jonker, 2004). Currently, OTA is regulated in cereal and cereal products in 37 countries and the average range for these regulatory limits for OTA is from 3-50 µg/kg. A total of 37 countries have set the maximum limits for OTA in the cereal and cereal products as 5 µg/kg (van Egmond & Jonker, 2004). The European Commission (EC) has published Commission Regulation No. 1881/2006 setting the maximum limits for OTA in unprocessed cereals as 5 µg/kg and, in the case of unprocessed cereal products the level is 3 µg/kg (European Commission, 2006b).

Physical and chemical properties of the ochratoxins

Ochratoxins are considered as a derivative of isocoumarin. Ochratoxins A, B, and C differ slightly from each other in their chemical structures, and this affects their respective toxic properties. The chemical structures of the ochratoxin A, B and C are shown in Figure 1.2.

Ochratoxin A





Ochratoxin B

Ochratoxin C

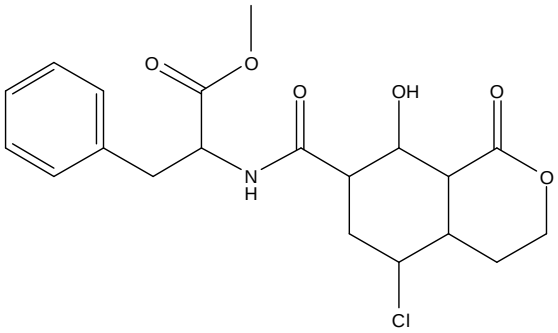


Figure 1.2: *Chemical structures of ochratoxin A, B and C.*

OTA is unstable when exposed to light, especially in very humid conditions; however, it is stable in the dark in ethanol solutions (Akron 2010). OTA is also fairly stable to heat; in cereal products, up to 35% of the toxin survives autoclaving for up to 3 hours (IARC, 1976).

OTA is slightly soluble in water, moderately soluble in chloroform and soluble in acetonitrile, methanol and ethyl acetate. OTA solution prepared in polar organic solvents such as ethanol or methanol can be stored for more than a year without any loss if kept cold and in the dark. The OTA exhibits green and blue fluorescence under UV radiation. Some important chemical and physical properties of the OTA are presented in the Table 1.4. Crystalline OTA is colorless, in the absence of moisture, are extremely stable up to 3 hours of autoclaving, if kept away from light and UV radiation.

Table 1.4
Physical and chemical properties of OTA

Property	**Information**
Molecular weight	403.8
Density	1.366 g/mL
Melting point	90°C in benzene and 171°C in xylene
Log K_{ow}	4.74
Water solubility	1.31 mg/L at 25°C
Vapor pressure	7.56×10^{-15} mm Hg at 25°C
Dissociation constant (p*K*a)	4.2−4.4 and 7.0−7.3

1.4.3. Trichothecenes

Trichothecenes belong to a group of mycotoxins, which are produced by a number of fungal genera, including *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichothecium*, *Trichoderma*, *Cephalosporium* and others (Bennett & Klich, 2003; Cole & Cox, 1981).

Hence, the name “trichothecenes” is derived from the trichothecen as the first member of the family identified (Bennett & Klich, 2003). A total of 180 different structures have been discovered and they fall into four distinct groups namely from A through D (Murphy, Hendrich, Landgren, & Bryant, 2006). The primary source of trichothecene

contamination in food and feedstuff is cereal commodities (maize, oats, barley and wheat) which have been infected by the *Fusarium* fungi. While type-A and type-B trichothecenes are commonly circulated by means of these crops, type-C and type-D trichothecenes, although more toxic, rarely occur in food and feed. *Fusarium* species such as *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. moniliforme*, *F. proliferatum*, *F. poae*, *F. sporotrichioides* and *F. verticillioides* is the major genus concerned in producing the type-A and type-B trichothecenes (Yiannikouris & Jouany, 2002).

Type-A and type-B trichothecenes exhibit acute toxicity, and when consumed, can result in vomiting and loss of appetite; while high concentrations of type-B trichothecenes can cause chronic intoxication, leading to extensive haemorrhage, and subsequent haematological toxicities. Type-A and Type-B poisoning may also inhibit both in vitro and in vivo protein synthesis and mitochondrial function, as well as manifest symptoms of immunosuppression at low concentrations. In particular, DAS exposure causes suppression in the macrophage phagocytic function. Agricultural produce from Europe are known to be free from type-A- trichothecenes, and contain low concentrations of DON, one of the most common mycotoxin pollutants. This is unlike crops from elsewhere in which the presence of DON, as well as other *Fusarium* mycotoxins, especially type-B trichothecenes, is common.

As a result of their toxicity and frequent occurrence, several countries have established legal regulations or recommendations for DON, HT-2 and T-2 toxins. The Food and Drug Administration (FDA) in the USA recommends maximum contamination levels of 1000 µg/kg for cereal products meant for human consumption, while the EU countries have set standards of between 100 and 1000µg of DON per kilogram for food, and between 400 and 5000 µg/kg for feedstuff (Zöllner & Mayer-Helm, 2006).

Physical and chemical properties of the trichothecenes

Trichothecenes are low-molecular-weight (MW 250–550) compounds characterized by specific structural features and all of them share a common tetracyclic, sesquiterpenoid 12, 13-epoxytrichothec-9-ene ring system, but are different in the side chain substitutions. Type-A trichothecenes have a saturated carbon at C-8, characterized by an oxygen functional group and they include T-2 (T-2) and HT-2 toxins (HT-2), neosolaniol (NEO) and diacetoxyscirpenol (DAS). Type-B trichothecenes are functionalized by a carbonyl group at the C-8 position and include deoxynivalenol (DON), nivalenol (NIV), fusarenon X (FUSX), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) and their derivatives. Type-C trichothecenes have a unique second epoxy group while the type-D trichothecenes are distinguished by a macrocyclic structure. The chemical structures of the trichothecenes investigated are shown in Fig. 1.3.

Trichothecenes are insoluble in water and freely soluble in moderately polar solvents such as ethyl acetate, dimethyl sulfoxide, chloroform, methanol, ethanol, and propylene glycol. Trichothecenes are extremely stable compounds in the presence of light, UV radiation and air. However, destruction occurs for trichothecenes by heating up to 482^{o}C for 10 minutes or 260^{o}C for 30 minutes as well as by treating with oxidizing agents, such as 3% to 5% solution of sodium hypochlorite. Some important chemical and physical properties of the trichothecenes are presented in the Table 1.5.

Table 1.5
Physical and chemical properties of trichothecenes

Trichothecenes	Molecular Formula	Weight (g/mol)	Melting Point (°C)
Nivalenol (NIV)	$C_{15}H_{20}O_7$	312	482.54
Deoxynivalenol (DON)	$C_{15}H_{20}O_6$	296	425.96
Fusarenon-X (FUS X)	$C_{17}H_{22}O_8$	354	486.59
3-Acetyldeoxynivalenol (3-ADON)	$C_{17}H_{22}O_7$	338	430.01
15-Acetyldeoxynivalenol (15-ADON)	$C_{18}H_{26}O_6$	338	406.18
Diacetoxyscirpenol (DAS)	$C_{22}H_{28}O_6$	366	365.84
HT-2 toxin (HT-2)	$C_{22}H_{32}O_8$	424	441.23
T-2 toxin (T-2)	$C_{24}H_{34}O_9$	466	445.28

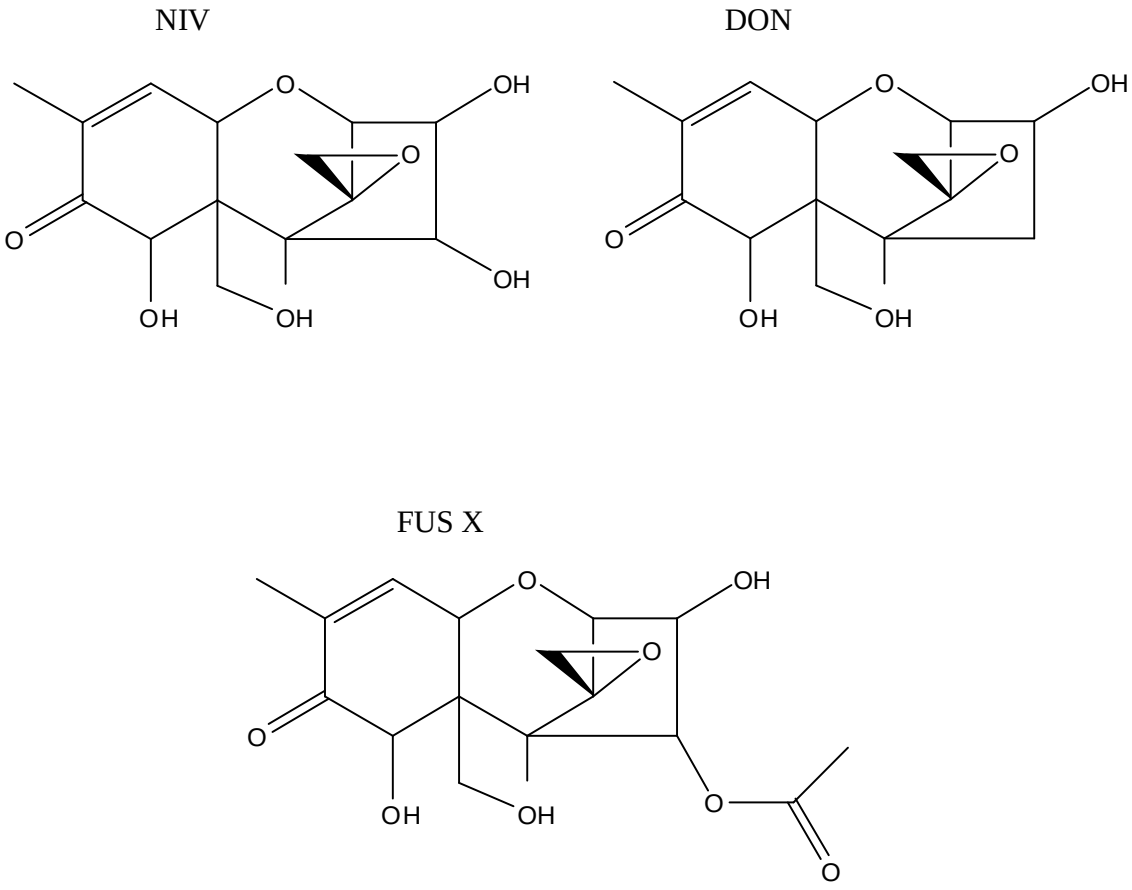


Figure 1.3: *Chemical structures of NIV, DON and FUS X*

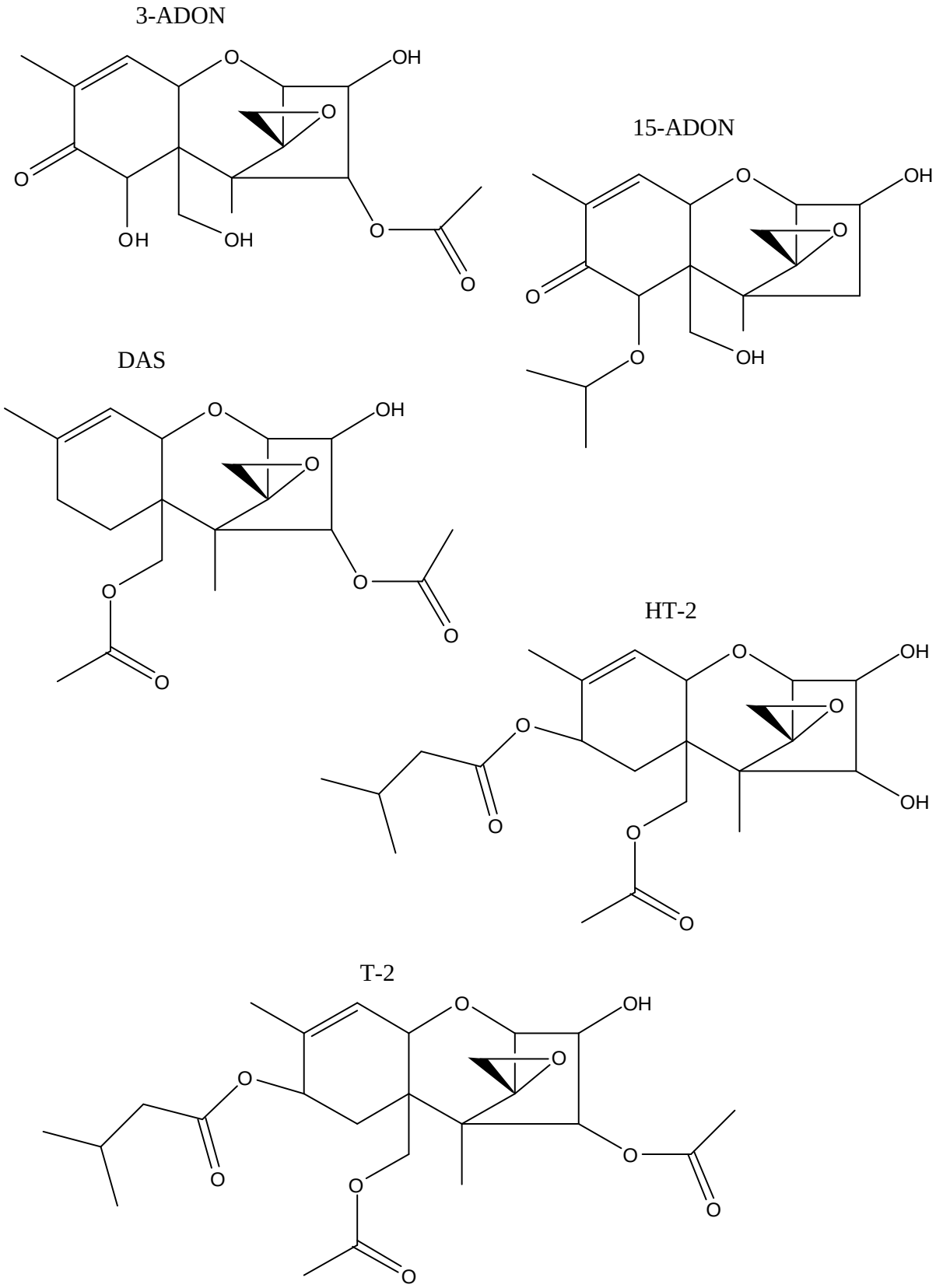


Figure 1.4: *Chemical structures of 3-ADON, 15-ADON, DAS, HT-2 and T-2*

1.5. Scope and Objective of Study

The overall objective of this thesis is to develop and validate analytical techniques for the determination of naturally occurring toxins known as mycotoxins. The main purpose of this research is the development and validation of an efficient analytical methodology for the implementation of worldwide regulations for mycotoxins in food and feed. It includes the need for method development and subsequently the validation of these analytical techniques. The structure of this work is divided into four main parts, from which independent conclusions are drawn.

The first part is the development of a method for the quantification of aflatoxin B1, B2, G1 and G2 in cereals using liquid chromatography coupled with electrospray ionization quadrupole time of flight mass spectrometry (LC-ESI-QTOF-MS/MS). To attain this goal, the chromatographic conditions and the ESI parameters are all taken into consideration and optimized.

Secondly, the implementation and validation of the optimized LC-ESI-QTOF-MS/MS method and the development of a new method based on Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) technique for the determination of eight(8) type-A and type-B trichothecenes in cereal samples are carried out.

The third part is focused on optimizing the sample pretreatment conditions of the developed QuEChERS method and the optimization of the chromatographic conditions of a high performance liquid chromatography with fluorescent detection (HPLC-FLD) with postcolumn photochemical reactor for enhanced detection (PHRED) method, for quantification of four(4) aflatoxins B1, B2, G1 and G2 in food. The developed QuEChERS-HPLC method was then validated and compared with the fluorometric determination method. The methods were then used for the analysis of the selected

aflatoxins in a total of 669 domestic and imported food samples in Jordan using a QuEChERS-HPLC method.

The final component of this study, is the implementation and validation of the optimized QuEChERS-HPLC method for the determination of ochratoxin A in cereal samples.

2. REVIEW OF ANALYTICAL METHODS FOR MYCOTOXINS ANALYSIS IN FOOD

2.1. Introduction

The importance of the determination of mycotoxins in food and animal feeds cannot be overemphasized, because of their toxic effects on humans and animals at very low concentration. For that reason, it is necessary to have sensitive, reliable and an accurate method for the determination of mycotoxins in different food commodities (Krska, 1998). The analytical determination of mycotoxins is complicated by three main factors: - the complexity of the sample matrix, the very low levels of mycotoxins in the food and the differences in physicochemical properties (Rahmani, Jinap, & Soleimany, 2009). Therefore, the analysis of mycotoxins in food and feed is composed of a multistep process which includes sampling, sample preparation, extraction from the matrix, clean up (Köppen *et al.*, 2010; Krska, 1998) and measurements.

To attain this goal, several methods have been developed for the analysis of mycotoxins including: - thin-layer chromatography (TLC) high performance liquid chromatography (HPLC) with ultraviolet-visible detection (UVD) and fluorescence detection (FLD), gas chromatography with electron capture detector (GC-ECD), enzyme linked immunosorbent assay (ELISA), capillary electrophoresis (CE), liquid chromatography coupled with mass spectrometry (LC-MS) and gas chromatography coupled with mass spectrometry (GC-MS).

2.2. Sampling strategy

The first step is to obtain a representative sample and it includes an adequate sampling, which means that within a sample lot, every single item should have an equal probability of being randomly selected. This process is tedious since several small samples are taken from the lot and are composed into one larger "lot sample." The lot sample is ground in a mill to reduce the particle size, and a subsample is removed for extraction and analysis. Except in case of liquids samples such as milk and blended food, such as peanut butter, the lack of homogeneity in the agricultural commodity or food susceptible to the growth of toxigenic moulds is common and the mycotoxin concentration of a bulk lot is usually estimated by measuring the mycotoxin in a sample taken from the lot.

Then, the decision will be made to classify the lot as acceptable or unacceptable based upon a comparison of the measured sample concentration with a legal limit. If the sample concentration does not accurately reflect the bulk concentration, then it may be misclassified and result in undesirable consequences, both economic and health. Due to the heterogeneous distribution nature of mycotoxins in food commodities (Zheng, Richard, & Binder, 2006), which may contain a large number of interfering compounds, the sampling plans should be designed to minimize the misclassification and to reduce the undesirable consequences associated with regulatory decisions concerning the fate of bulk lots.

As a consequence of the importance that sampling plays in the precision of the determination of the levels of mycotoxins, methods of sampling for mycotoxins in agricultural commodities are carefully defined in the Commission Regulation (EC) No 401/2006 of 23 February 2006 (European Commission, 2006a).

2.3. Sample Preparation

Sample preparation steps, which are necessary for the successful determination of mycotoxins, is a tedious, time consuming and most important part of mycotoxins analysis because they are more prone to error and must be carefully and diligently monitored. Furthermore, sample preparation relies largely on physiochemical properties of the commodities that are contaminated with mycotoxins as well as on the mycotoxin. Commodities with high fat and pigment content require more treatment. On other hand, mycotoxins consist of a large number of secondary metabolites that have different physiochemical properties. Due to this variety, no general procedures can be applied for the isolation and purification of all of the different mycotoxins. In all other cases, the traditional strategies for the determination of a mycotoxin in food commodities include the first step extraction from ground solid matrices with a solvent or mixture of organic solvents such as chloroform, ethyl acetate, methanol, acetone and acetonitrile, followed by reconcentration of the analyte and purification of the sample.

Different extraction methods, which have been developed for the removal of mycotoxins from food commodities and are dependent on the structure and nature of the mycotoxins such as liquid-liquid extraction (LLE), solid-phase extraction (SPE), Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) and Immunoaffinity columns (IAC). Polar mycotoxins (e.g. fumonins) are best extracted in the presence of water, while hydrophobic toxins require the use of organic solvents (Shephard, 1998; Turner *et al.*, 2009).

2.3.1. Liquid-liquid extraction (LLE)

Liquid-liquid extraction (LLE) is an extraction method in which the sample is distributed between two immiscible solvents with the analyte having different solubility's in the

solvents. It is used for the extraction of mycotoxins and is based on the different solubility of mycotoxins in aqueous phase and in the water insoluble organic phase. A non-polar solvents, such as n-hexane, cyclohexane and toluene, are used for the extraction of non-polar contaminants (Turner *et al.*, 2009). The extraction method was found to be effective for several toxins, but works better for small-scale sample preparation. It is time consuming, makes use of toxic solvents, and solely depends on the nature of sample matrix to be analyzed and the analyte compounds to be determined. Its disadvantages lie in the formation of emulsion (Dean, 1998), loss of sample by adsorption onto the glassware (Turner *et al.*, 2009), and possible contamination by the solvent. Therefore, it is important to use analytical grade reagent with highest purity.

Water has been used as a polar solvent to dissolve mycotoxins and extract them from the ground sample. The use of relatively polar solvents has been found to be more effective in the LLE extraction of mycotoxins. Acetonitrile and methanol are considerably the most common solvents used for extracting the most important mycotoxins.

Chlorinated solvents, such as methylene chloride, have been used to extract aflatoxins from corn. Methanol has been used to extract OTA from rice (Juan, Gonzalez, Soriano, Molto, & Manes, 2005), acetone and acetonitrile was effectively used for the extraction of fumonisin in rice (Hinojo *et al.*, 2006). Ethyl acetate followed by diethyl ether was used for the extraction of mycotoxins (cyclopiaozonic acid) in corn and rice (Hayashi & Yoshizawa, 2005), while the mixture of acetonitrile, water and methanol (25:25:50, v/v/v) was used for the extraction of fumonisin in corn (Zinedine *et al.*, 2006). The use of mixture of inorganic salt (0.5% KCl) in 70% methanol was also reported to be effective for the extraction of aflatoxin B1 in rice, but was followed by an ELISA method (Reddy, Reddy, & Muralidharan, 2009). The extraction method used for mycotoxins gave good recoveries and detection limits but is disadvantageous in the use of toxic solvents. The

extraction method has also been widely used for the extraction of mycotoxins from liquid samples (Rahmani *et al.*, 2009).

2.3.2. Solid-phase extraction (SPE)

Solid-phase extraction (SPE), also called Liquid-solid extraction (LSE), is a technique which involves the selective adsorption of analyte onto the surface of a solid phase, when the sample is brought in contact with it (Dean, 1998). The principle is based on the variation of chromatographic techniques, where the stationary/solid phase is packed with small disposable cartridges containing silica gel, or a bonded phase. The SPE cartridges, which can be used either on or off line have high adsorption capacity for small molecules and contain different types of bonding phases (Turner *et al.*, 2009). The phases are generally divided into three; normal, reversed and ionic exchange. Normal phase contains polar functional group and includes unmodified silica, aminopropyl and florosil, while the reversed phase contains non-polar functional group and include octadecylsilane (C_{18} silica), styrene-divinylbenzene phenyl, the ion exchange materials have either cationic or anionic functional group and include benzene sulphonic acid (Dean, 1998) Generally, silica gel based cartridges are the most commonly used.

The SPE cartridges were designed in relation to the number and nature of the sample to be analyzed. The most commonly used is the syringe cartridge, which is made of polypropylene containing the sorbent materials, and has a wide open entrance for sample introduction and a narrow exit.

The use of different SPE procedures has been investigated for the extraction of type B trichothecenes in wheat followed by GC-ECD analysis (Valle-Algarra *et al.*, 2005). MycoSep 225 column was used with acetonitrile–water (84:16, v/v) as the extraction solvent, florisil cartridge in which its procedure was slightly modified by substituting light

petroleum with hexane. A column containing mixtures of alumina-charcoal-celite 545 (5:7:3, w/w/w), alumina-charcoal (100:15, w/w), alumina-charcoal-C_{18} silica at 3 different ration (75:3:40, 75:1:40 (containing 5mL of sample extract) and 75:1:40 (with containing 5mL of sample extract), w/w/w) and alumina-charcoal-silica (90:1.5:5, w/w/w) were investigated. The elutes in the columns were concentrated to dryness by a gentle stream of nitrogen prior to chromatographic analysis. The best recoveries were obtained with MycoSep 225, alumina-charcoal-C_{18} silica and alumina-charcoal-silica cartridges, with alumina-charcoal-C_{18} giving the best result in terms of better reliability, low cost and shorter analysis time.

In most cases the extract from the SPE are cleaned-up prior to instrumental analysis, in order to remove any co-extracted material which often cause interference in the determination of target analytes (Rahmani *et al.*, 2009). The combination of polar and non polar sorbent materials for SPE was found to be an effective clean-up technique, which helped in the complete removal of possible interfering co-extracts (Stecher *et al.*, 2007).

The efficiency of SPE procedure was also investigated for the extraction of type B-trichothecenes in cereals (Krska, 1998), with MycoSep 225 consisting of packing materials that are made up of different adsorbents like charcoal, celite and ion-exchange resins. The use of MycoSep was found to be less time consuming, requires no rinsing step and gave good recovery. The mean recoveries range from 95 to 103% and provides a quick reliable and rugged method. SPE sample preparation method provides better advantages over LLE method, which include the use of less quantity of toxic solvents, less analytical time and giving a better recoveries and detection limits.

Aflatoxins B1, G1, B2 and G2 were extracted from medicinal herbs (Rasmussen, Storm, Rasmussen, Smedsgaard, & Nielsen, 2010) with a mixture of methanol and water (80:20,v/v) followed by SPE clean-up by a polymeric sorbent. The eluted extract was

analyzed with liquid chromatography coupled with electrospray ionization-mass spectrometry (LC-ESI-MS) operating in the positive ion mode. The injection involved the use of reverse-phase C18 short column with an isocratic mobile phase composed of methanol-water (70:30, v/v). The average recoveries of the investigated aflatoxins range from 77-110% with RSD between 5.0 – 7.1%. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 10 ng and 25 ng respectively. The linearity ranged from 10 to 600 ng with correlation coefficients greater than 0.999 (Ventura *et al.*, 2004).

A SPE clean–up method for the determination of aflatoxins B1, B2, G1 and G2 in groundnut cake was developed. The method involves the clean-up of an acetone and water (85:15, v/v) extract on a bonded-phase cartridge and was quantified by HPLC-FLD following a post-column derivatization with iodine. The average recoveries range from 82.1 to 88.0% with LOD between 1.6 and 3.2 µg/kg. The developed method showed no significant different with the AOAC official method at 5% confidence level, but the developed extraction method extracted more aflatoxins B1. The developed extraction method, with acetone was also compared with slurry extraction method and the two methods showed similar precision, but the slurry method was found to extract more aflatoxins B1 and B2 (Roch, Blunden, Coker, & Nawaz, 1995).

2.3.3. Immunoaffinity column (IAC)

An Immunoaffinity column (IAC) is the method of extraction of contaminants, which is based on the specific biological interactions between antigen and antibodies. The inherent specificity of antibodies is used to bind the target analytes (Maragos, 2004). The IAC contains anti-mycotoxin antibody that is coated on the surface of a solid spherical beads like agar-rose gel contained in a phosphate buffer and is packed in a small plastic cartridge column. The diluted mycotoxins is extracted by applying the sample to an IAC containing

specific antibodies to a particular mycotoxins, by passing through the antibody, the mycotoxin is bonded to it, this is followed by passing water through the column to remove impurities. The bonded mycotoxin is then removed from the antibody by passing solvent such as methanol through the column, and can be detected with a fluorometer or instrumental techniques. Most mycotoxins have low molecular weight (Maragos, 2004; Zheng *et al.*, 2006), and thus the immunoassay procedures for their extraction is limited.

It is disadvantageous, because IACs have limited loading capacities and the sample clean-up procedure is complicated. Mycotoxins (T-2 and HT-2) were extracted in by LLE method using methanol/ water (90:10, v/v) and the extract was cleaned-up by IAC. The antibody used (monoclonal) showed 100% cross-reactivity with both mycotoxins and the IAC clean-up was found to be very effective up to a concentration of 1.4 µg/kg of both analytes. The recoveries ranged from 70% to 100% and RSD was found to be lower than 8%, while the LOD was between 3–5 µg/kg (Visconti, Lattanzio, Pascale, & Haidukowski, 2005).

The IAC method was used as the sample preparation procedure for the quantification of zearalone mycotoxins in corn. The method involved the extraction of corn sample in acetonitrile/water (90:10, v/v), followed by dilution of the extract with water (1:10, v/v) and then applied to Zearala Test IAC. The column was washed with water to remove the impurity and the analyte was eluted with methanol and quantified. The recoveries of the analyte with the Zearala Test column were found to be higher than 95% and the column could effectively hold a maximum of 4 mg of mycotoxin. The RSD and LOD were 6% and 3 µg/kg respectively. The method was compared with the AOAC official method 985.18 and a correlation coefficient of 0.87 was observed (Visconti & Pascale, 1998).

A mycotoxin (DON) in wheat was also quantified using immunoaffinity chromatography. The wheat samples were extracted, filtered twice and transferred into the IAC. Impurities were washed with water while the bonded mycotoxin was removed with methanol. The specificity of the IAC was determined and was found to be greater than 80% in DON and between 40 to 50% in 3-ADON while 3-ADON, NIV, T-2 and FUS X were not recognized by the antibody. The average recoveries for all analytes determined were 90% with an average RSD of 8.3%. The LOD was 0.1 mg/kg, linearity between 0.1 to 1.0 mg/kg with correlation coefficient of 0.999, while the IAC capacity was determined to be 3.3 mg (Cahill *et al.*, 1999). This method was found to be simple, accurate, and rapid and it offers high selectivity, in the extraction and subsequent determination of mycotoxins, but the limitation lies in a single use of the column.

A method for the determination of aflatoxins B1, B2, G1 and G2 in food matrices was developed. The method involved the IAC clean-up step after extraction with methanol and subsequent determination with TLC. The investigated aflatoxins were quantified by densitometry. The LOD ranged from 0.1 to 0.2 µg/kg and LOQ between 0.2 and 0.3 µg/kg for all investigated aflatoxins. The average recoveries ranged from 76 – 87% with RSD between 1.4 and 8.9% (Stroka, Otterdijk, & Anklam, 2000). The LOQ of the developed method was found to be significantly lower than the current regulatory limits for aflatoxins control outside and within the European community. The recoveries and precision of the developed showed that the method is likely to give satisfactory performance if tested in a future collaborative trial.

2.3.4. Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) is an extraction technique, which makes use of supercritical fluids. A supercritical fluid is a substance, which does not condense or

evaporate above its critical temperature and pressure, but its properties changes from gas-like to liquid-like as the pressure changes. It involves extraction of samples by a flowing stream of supercritical fluid at a particular temperature and pressure over a period of optimized time (Clifford, 1998). Supercritical fluids such as CO_2, which is non-toxic and non-inflammable, environmentally friendly and chemically inert (Ambrosino *et al.*, 2004; Krska, 1998), is commonly used, because they generally have low viscosities between 10–3–10–4 g/cm/s, with high diffusivities between 10–3–10–4 cm^2/s and moderate densities of around 0.3 – 0.8 g/cm. This enables rapid and efficient extractions due to the combination of two properties; high gas-like solvating power and liquid-like mass transfer properties (Josephs, Krska, Grasserbauer, & Broekaert, 1998). It has been shown to be an effective method for the extraction of non-polar mycotoxins from many different sample matrices.

The efficiency of SFE depends strongly on the analyte-matrix interaction (Krska, 1998), and therefore the need for the optimization of SFE parameters, to determine the optimum conditions for effective extraction of analytes. The solubility's of the analytes are controlled by their polarities and temperature and supercritical fluid density which is pressure dependent (Josephs *et al.*, 1998). The optimization of parameters can be performed at temperatures ranging from 20 to 40^oC, and therefore can be used for temperature sensitive analytes (Ambrosino *et al.*, 2004). The selectivity of the method can be achieved by varying the temperature and pressure during extraction. The efficiency of SFE is also enhanced by addition of polar modifiers (e.g. methanol) which increases the densities and polarities, and therefore enhances the solvating power of the supercritical fluid, and thus can be used for the extraction of moderately polar analytes (Josephs *et al.*, 1998).

The SFE method with CO_2 as the supercritical fluid has widely been used in the extraction of mycotoxins from foods such as cereal. The dual properties of supercritical

CO_2 provide ideal condition for extracting analytes with a high degree of recoveries in a short period. Trichothecene mycotoxins was determined in wheat by the use of supercritical fluid extraction, and it gave a good recoveries, 90.1±10.6% for spiked sample and 53.0±3.2% for naturally contaminated samples, with RSD of 9.6%. The LOD was found to be 1.6 mg/kg and LOQ of 5.35 mg/kg (Josephs *et al.*, 1998).

The method was also used effectively to determine beauvericin mycotoxins in maize. The method involved the optimization of extraction parameters such as temperature, pressure and time. The best extraction conditions were determined to be at a temperature of 60^{o}C, pressure of 3200 psi, static extraction time of 30 min and uses of methanol as modifier. The extraction recovery of 36% was achieved without the use of modifier which was found to increase to 76.9% in the presence of modifier with an RSD of between 3–5% (Ambrosino *et al.*, 2004).

The optimum condition for the extraction of B-trichothecenes in cereals was also investigated and was found at a pressure of 38 bar and extraction temperature of 40^{o}C, with a dynamic extraction time of 15 min and a fluid flow rate of 2.0 mL/min in the presence of methanol as modifier, which was preceded by a static extraction time of 30 min (Krska, 1998).

The SFE technique was also found to be effective in the extraction of macrocyclic lactone mycotoxins in maize flour following optimization of necessary parameters. The best recoveries were achieved at temperature of 80^{o}C and fluid flow ratio of 1 mL/min with static extraction time of 10 min and 30 min of dynamic extraction time, using 9.1% of methanol as modifier. The flow rate of 0.05 mL/min of the modifier was observed to increase the recovery from 84-88% to 90-92%, and the flow rate of 0.1 mL/min of modifier was found to be enough for quantitative extraction (97–100%) of the mycotoxins (Zougagh & Ríos, 2008). The SFE extraction method is less expensive, fast, the supercritical fluids

can easily be removed and are readily available, and the SFE can be automated which prevents contamination and helps in the optimization of parameters for effective extraction

The SFE method was also developed for the extraction of aflatoxins B1, B2, G1 and G2 from spiked corn sample using modified supercritical carbon dioxide. The extraction method developed involve holding the corn in static extraction at 65^{o}C and 51.7 MPa for 15 min followed by a dynamic extraction with 20 mL of liquid carbon dioxide. Methanol was added as modifier to the SFE extraction cell containing the corn layered between Hydromatrix, and it helps to prevent the clogging of the frits and reduced the effect of moisture on the extraction. The extract was collected in chloroform and was further cleaned up with a Florosil Sep-Pak. The cleaned up extract was analyzed with HPLC with fluorescence detector after post-column derivatization with iodine. The average recoveries of the aflatoxins B1, B2, G1 and G2 over a linear range of 3 to 11 µg/kg were found to be 77.3, 82.9, 75.4 and 80.3% with RSD of 3.7, 3.8, 7.77 and 10.57% respectively. Dynamic extraction with up to 60 mL of liquid CO_2 was found to improve the recoveries of aflatoxins G1 and G2, but decreased that of aflatoxins B1 and B2. Therefore reducing the dynamic extraction volume, depressurization of the SFE extraction cell and reading the modifier helped to improve the recoveries and reproducibility of all investigated aflatoxins (Holcomb, Thompson Jr, Cooper, & Hopper, 1996).

2.3.5. Accelerated Solvent Extraction (ASE)

Accelerated Solvent Extraction (ASE) also known as pressurized liquid extraction (PLE) is a technique which makes use of organic solvents at high pressure and elevated temperature at or above the boiling point, in order to achieve efficient and almost 100% extraction of analytes from solid and semi-solid samples with lower solvent volumes and shorter extraction time (Juan *et al.*, 2005; Rahmani *et al.*, 2009; Sheibani & Ghaziaskar, 2009). The

technique involves placing a solid sample in a cell and sealed it after it has been filled with extraction solvent and used to statically extract the sample under elevated temperature of between 50–200^{o}C and pressure of 500–3000 psi for short period of time (Rahmani *et al.*, 2009; Richter, Jones, Ezzell, & Porter, 1996). The high temperature and pressure cause the solvent to be in the liquid state, helps the solvent to penetrate into the pore of the sample and encountered the analyte.

The effect of experimentally controllable ASE parameters, such as solvent composition, temperature, pressure, static time, number of extraction cycle and sample cell size are very important (Juan *et al.*, 2005; Rahmani *et al.*, 2009; Urraca, Marazuela, & Moreno-Bondi, 2004), and need optimization for an effective and efficient extraction. The use of high temperature helps to increase the solubilzing capacity of solvents and leads to faster diffusion rate; it also helps in the disruption of the strong solute-matrix interaction that is caused by van der Waals forces and hydrogen bonding. Extraction at elevated temperature increases the viscosity of the solvent, thereby allowing better penetration of the solvent into the pore of the sample. The use of high pressure forces the solvent into inner pore of the matrices and facilitate the extraction of analyte that is trapped in sample matrix pores (Richter *et al.*, 1996).

The ASE method has been used for the extraction of aflatoxins B1 and B2 from contaminated pistachio samples following the optimization of the required parameters. The pressure variation of 10 to 100 bar was observed to have no significant effect on the extraction of aflatoxins. A mixture of methanol and water (80:10, v/v) used as extraction solvent and carried out at a flow rate of 0.5 mL/min and at a temperature higher than 80^{o}C was found to be the optimized conditions which resulted in efficient extraction (Sheibani & Ghaziaskar, 2009).

A method for the determination of zearalenone in cereal flour was also developed by the application of ASE using a mixture of methanol and acetonitrile (50:50, v/v) as the extraction solvent, and the technique was found to be very effective in the removal of the analyte from the sample (Pérez-Torrado, Blesa, Moltó, & Font, 2010).

An ASE method for the analysis of OTA in bread was described, and parameters affecting the extraction were investigated thoroughly. The optimized conditions were found at 80^{o}C, at the pressure of 2000 psi with a 5 min cycle using methanol as the extraction solvents. The total extraction time was observed to be 12 min with extraction yield of more than 90%. A relationship between the pressure and extraction recovery was established and was shown that increase in pressure above 2000 psi decrease extraction recoveries at 80^{o}C (Osnaya, Castillo, Cortés, & Vinuesa, 2006).

The ASE procedure for wheat sample was also optimized for the extraction of zearalenone and the optimum extraction conditions were obtained at 50^{o}C, a pressure of 1500 psi, a static extraction time of 5 min in the presence of methanol/acetonitrile (50:50, v/v) as the extraction solvent in an 11 mL extraction cell and a 60% flush volume (Urraca *et al.*, 2004).

The ASE method was found to have no significant difference when compared to standard EPA extraction methodology in terms of recovery and precision (Richter *et al.*, 1996). The ASE method uses less solvent compared to other traditional extraction methods and is more effective than SFE, which in some cases lacked the required strength for the extraction of polar analytes (Recovery $\leq$20) from complex matrices. The use of higher temperature leads to the breakage of intermolecular interaction between the analyte and the sample matrices. Its limitation lies in the loss of volatile analytes when used for the extraction of solid samples.

2.3.6. Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS)

A Quick, Easy, Cheap, Effective, Rugged and Safe **(**QuEChERS) method which was developed by Anastassaides and his co-workers (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003), was initially developed for the extraction of pesticides from fruits and vegetables (Aguilera-Luiz, Plaza-Bolaños, Romero-González, Vidal, & Frenich, 2011), and has widely been employed for analysis of mycotoxins in many food matrices. It involves the partitioning of the sample matrix by the use of mixture of solvents, induced by addition of inorganic salts. The analytes are extracted by the organic solvent, while the more polar impurities are held-up in the aqueous layer. The residual sugars, fatty acids and other H-bonding matrix co-extractable, are removed from the organic solution by the addition of primary and secondary amine (PSA), followed by dispersive solid phase extraction (dSPE) (Zachariasova *et al.*, 2010), as a clean-up step.

Samples are homogenized at a low temperature and the solvent is added, followed by the spinning of the mixture for about 1 min in a centrifuge. An inorganic salt such as anhydrous magnesium sulphate ($MgSO_4$) (to absorb water and contaminants present in the sample) and the PSA are added to the supernatant, shake and re-centrifuged, this is followed by instrumental analysis. The selection of the extraction solvent is a very important factor in QuEChERS method development (Sospedra, Blesa, Soriano, & Mañes, 2010). The sample extraction procedure can be performed with mixture of several solvents depending on the type of mycotoxins to be extracted. Mixtures of several solvents have been employed, such as dichloromethane, ethyl acetate, acetonitrile, methanol methanol/acetonitrile, methanol/acetonitrile/water and methanol/water in different ratio (Desmarchelier *et al.*, 2010; Romero-González, Garrido Frenich, Martínez Vidal, Prestes, & Grio, 2011).

The extraction of five mycotoxins; three type A- trichothecenes such as DAS, T-2 and HT-2, and two type B-trichothecenes such as DON and NIV, from wheat flour, was optimized using a modified QuEChERS method (Sospedra *et al.*, 2010). The use of different extraction solvents were investigated for effective and better recoveries. Methanol was found to be effectively extracting all the five mycotoxins under investigation, with recoveries of more than 50%, while acetonitrile showed good recoveries for the type-A trichothecenes. The mixture of the two solvents (methanol/acetonitrile) in two different ratios by volume (75:25, 85:15 and 95:5, v/v) gave better recoveries which ranged from 86 to 108%. The solvent mixture with the 85:15, v/v combination gave the highest recoveries with RSD of between 5.6 and 9.0%, which confirmed the good repeatability and reproducibility of the extraction method. The extraction LOD values ranges from 1–30 µg/kg and the LOQ of between 4 to 100 µg/kg, with linearity of 100 to 500 µg/kg and correlation coefficients greater than 0.99.

The extraction method was also developed for simultaneous determination of pesticides and biopesticides such as pyrethrins, rotenone, azadirachtin, and mycotoxins, such as aflatoxins B1, B2, G1, G2, OTA, T-2 and HT-2, from wheat flour. The method allows the extraction of large number (80) of compounds in one-step. The method developed involved single extraction with acidified acetonitrile, which is followed by portioning with salts (anhydrous $MgSO_4$ and anhydrous sodium acetate (Na_2SO_4)), without any clean-up step. The recoveries were in the range of 70 and 120% with intra-day RSD lower than 20% and inter-day RSD lower than 24%. LOQ was found to be lower than 10µg/kg. The QuEChERS method developed was compared with other traditional extraction techniques and was found to provide better sensitivity and selectivity. The good recoveries obtained also indicated the reliability of the extraction method (Romero-González *et al.*, 2011).

The analysis of 17 mycotoxins from cereal-based commodities such as corn, wheat and rice, was carried out using QuEChERS technique. The extraction solvent contained a mixture of water and acidified acetonitrile (50:50, v/v) and the inorganic salts made up of mixture of $MgSO_4$: NaCl (4:1, w/w). The resulting supernatant was cleaned-up using n-hexane under agitation, then centrifuged followed by evaporation to dryness under a stream of nitrogen. The extract was then reconstituted in methanol and water, re-centrifuged to give a clear supernatant for subsequent chromatographic analysis with no clean-up step (Desmarchelier *et al.*, 2010). The technique was compared with ASE and was observed to give similar performance in term of linearity ($r^2 > 0.98$), precision (RSD< 20%), but the QuEChERS method was found to be easier, less solvent use and also allowed high sample throughput.

A QuEChERS-based extraction procedure was developed for the determination of 10 mycotoxins such as beauvericin, enniatin A, A1, B1, citinin, OTA, aflatoxins B1, B2, G1 and G2 in egg sample at a trace level. The extraction step, chromatographic and detection conditions were optimized in order to increase efficiency and sample throughput and sensitivity. Matrix-matched calibration was used for quantification. Blank samples were fortified at 10, 25, 50 and 100 µg/kg, and recoveries ranged from 70% to 110%, except for OTA and aflatoxin G1 at 10 µg/kg, and aflatoxin G2 at 50 µg/kg. The RSDs were lower than 25% in all the cases. LOD ranged from 0.5 µg/kg (for aflatoxins B1, B2 and G1) to 5 µg/kg (for enniatin A, citrinin and OTA) and LOQ ranged from 1 µg/kg (for aflatoxins B1, B2 and G1) to 10 µg/kg (for enniatin A, citrinin and OTA). Seven samples were analyzed using the optimized conditions and aflatoxins B1, B2, G1, G2, and beauvericin were detected at trace levels (Frenich, Romero-González, Gómez-Pérez, & Vidal, 2011).

Rasmussen *et al.* (2010) described a method for the determination of 27 mycotoxins of different types in maize, using QuEChERS procedure. The method involve the development of a simple pH-buffering, which ensures that the pH in the sample was effectively maintained at a stable pH value (pH<4). The extracted analytes were analyzed qualitatively and quantitatively with LC-MS without any clean-up. Quantification was performed by using matrix-matched calibration standard which yielded recoveries ranging from 37 to 201% with most of the investigated mycotoxins in the range of 60 and 115% (RSD = 5-27%). The LODs ranged from 1 to 739 µg/kg (Rasmussen *et al.*, 2010).

The QuEChERS extraction technique has been found to offer significant advantages over the traditional sample extraction technique, because extraction can be achieved in short period of time and the use of toxic solvent is also minimized, it also eliminates clean-up and additional sample processing, thereby reducing errors. It gave high analyte recoveries for a wide range of mycotoxins and also allowed high repeatability and reproducibility with ease of use.

2.4. Qualitative Analysis of Mycotoxins

2.4.1. Enzyme Linked Immuno-Sorbent Assay (ELISA)

The Enzyme linked immuno-sorbent assay **(**ELISA) method is a qualitative technique that is based on the principle of a sandwich, where an antigen and the corresponding antibody specific to the analyte are immobilized on a microtiter plate in a solid phase. The assay involves immobilizing an analyte or the antibody on the bottom of a commercially available 96-well microwell assay plate, or on the walls of a plastic tube, using an enzyme system as the reporter signal. The nature of binding of the antibody and antigen can either be measured directly or indirectly (Stanker & Beier, 1996). The direct measurement involves the modification of the antibody molecule to contain a reporter system, while the

indirect measurement involves the use of a second antibody, which carries the reporter system and binds specifically to the original antibody. The structural and chemical interactions that occur within the antibody-combining site, controls the ability of the antibody molecule to specifically bind an antigen molecule. The antigen-antibody interaction is reversible and does not result to the formation of covalent bond (Stanker & Beier, 1996). It is a convenient screening tool that can be used to analyze a large number of samples simultaneously and does not require sophisticated instrumentation (Zhang, Wang, & Fang, 2011), but the procedure allows for qualitative and semi-qualitative analysis (Nilufer & Boyacioglu, 2002; Rahmani *et al.*, 2009).

The ELISA protocol that may be applied to different antigen-antibody interaction can either be competitive direct (cdELISA) or competitive indirect (ciELISA) (Christensen, Yu, & Chu, 2000; Yu, Chi, Liu, & Su, 2005). The cdELISA involves immobilizing the analyte specific antibody in the wells of a microassay plate, followed by addition of the unknown sample and a fixed amount of enzyme-labeled analyte to the antibody-coated tube. The sample is incubated for about 5 to 60 min, and the labeled and the unlabeled analytes are left to compete for binding sites on the antibody. The unbinding material is then washed away while the amount of labeled analyte bonded to the immobilized antibody is quantified using an enzyme substrate that forms a colored product. The ciELISA involves coupling the analyte to a carrier protein and then immobilized on the bottom of a microassay plate well, followed by addition of the unknown sample to the microassay well and a fixed amount of analyte-specific antibody. This is followed by the incubation of the microassay plate, which allows the partition of the antibody between the unbound analyte present in the unknown sample and the analyte immobilized on the bottom of the microassay. The unreacted materials are then washed and quantification of the amount of the analyte-specific antibody bounded to the bottom of the microassay well is achieved by

addition of a second antibody that specifically binds the first antibody and incubated again. This is followed by washing away of the unreacted materials and addition of appropriate substrate (Stanker & Beier, 1996). The sensitivity of this technique is dependent on the affinity of the specific antibodies and the sensitivity of the detection system used (Quan, Zhang, Wang, Lee, & Kennedy, 2006).

The amount of color developed measured in term of optical density, in both cdELISA and ciELISA is inversely proportional to the amount of analyte present in the sample. The cdELISA assay in which mycotoxin-protein conjugates are coated onto microassay plate as solid phase are widely employed for qualitative analysis of mycotoxins in food compared to the cdELISA which is time-consuming (Yu *et al.*, 2005). The two types of assay can be used based on polyclonal antibody (Christensen *et al.*, 2000; Quan *et al.*, 2006; Yu *et al.*, 2005), or monoclonal antibody (Maragos, 2004), and has been used for mycotoxins analysis because of their adaptability, simplicity, selectivity, and low cost (Quan *et al.*, 2006).

The cdELISA based on monoclonal antibody was used for the analysis of fumonisin B1 mycotoxins. The monoclonal antibody was prepared against hydrolysis product of the fumonisin B1 (HFB1), that cross reacts with hydrolysis product of fumonisin B2, B3 and B4. There was no reaction between the antibody and the intact fumonisin. The antibody was applied to the extracts of grinded corn and the performance of the cdELISA was found to be affected by the amount of solvent and the matrix components. The antibody used (P2F11-3-H7) was found to be specific to HFB1 and reactive with the hydrolysis products of fumonisin B2, B3 and B4. The lowest IC50 was found in HFB1 with value of 36 µg/L followed by HFB3, HFB2 and HFB4 with values of 174 µg/L, 331 µg/L and 1700 µg/L respectively. The used of cdELISA for the analysis of corn gave the recovery value of 79% with LOQ of 10 µg/kg HFB with IC50 of 41 µg/kg. The linearity range from 50 to 500

µg/kg and reproducibility of less than 20 µg/kg ad coefficient of variation of 8.1% (Maragos, 2004).

An ELISA method based on polyclonal antibody coupled to an enhanced chemiluminescent (ECL) detection was developed for the analysis of fumonisin B1 in food sample (Quan *et al.*, 2006). The assay involves the optimization of concentrations of the antibody, enzyme conjugate and competition time. The optimized condition gave a linear working range of 0.14–0.9 µg/L, IC50 value of 0.32 µg/L and LOD of 0.09 µg/L. The effect of amount of antibody per well, competition time and the amount of antigen was investigated. The result showed that, 0.5 µg/well of antibody, with 60 min competition time and 0.5% BSA-PBST as a fumonisin B1-HRB diluent buffer produced the best RLUmax/IC50. The investigation of the effect of solvent mixture (methanol and acetonitrile) and their concentrations showed that less than 10% and 5% methanol and acetonitrile respectively did not have any effect on the sensitivity of the assay, while the solvent concentration of more than 20% methanol and 10% acetonitrile reduced the detection sensitivity. Methanol was then chosen for the extraction of fumonisin B1 mycotoxins after it was reduced to 10%, and the results showed that the assay developed for the antibody is specific to fumonisin B1.

Two ciELISA methods were investigated for the determination of aflatoxins B1. It involves the coating of the antigen (Aflatoxin BrBSA) to the solid phase made of polystyrene microtiter plates. One of the procedures involves the conjugation of the specific antibody with peroxidase, and there was no conjugation in the other procedure but a second antibody labeled with alkaline phosphate was employed. The study showed that the affinity constant of the complex solid and solution, and the amount of antigen in the solid are the main factors which affect the dynamic range of the method (Pesavento, Domagala, Baldini, & Cucca, 1997). Mycotoxins were analyzed in sesame butter using ELISA and compared

with two other analytical methods; HPLC and fluorometry. The coefficient of variation was found to be in the range of 27.16 – 39.94% and recovery ranging between 145.6 t0 161.4%.

The recovery was observed to be inversely proportional to the coefficient of variation and directly proportional to the spiking levels. The comparison showed that the ELISA method is only applicable for screening purposes (Nilufer & Boyacioglu, 2002).

A polyclonal antibody was used in the analysis of fumonisin B4, by assaying with a competitive direct ELISA. The result showed that the concentrations of fumonisin B1, B2, B3 and B4 in the fumonisin B1-HRP- based cdELISA that caused 50% inhibition of binding of enzyme marker (IC50) were 9.0, 2.1, 9.0 and 6.5 µg/L, with relative cross-reactivities of 58.5, 309.5, 58.5 and 100% toward fumonisin B1, B2, B3 and B4 respectively. The IC50 values in the fumonisin B3-HRP-based were 7.1, 1.9, 7.6 and 5.3 µg/L with relative cross-reactivities of 74, 280, 70 and 100% towards fumonisin B1, B2, B3 and B4 respectively. The recovery obtained when the fumonisin B3-HRP-based cdELISA was employed using a corn cultured materials was 65% in the range of 100-1000 mg/L, and the LOD obtained assaying a clean corn matrix was between 10-100 mg/kg (Christensen *et al.,* 2000).

An OTA was assayed with cdELISA and ciELISA using polyclonal antibodies that were generated from rabbits after the animals were separately immunized with OTA-γ-globulin and OTA-keyhole limpet henocyanin (KLH), and were used for the analysis of soybeans. The investigation showed that the antibody titers in the serum of rabbits immunized with OTA-γ-globulin were considerably higher than those in rabbits immunized with OTA-KLH, and therefore the higher antibodies serum were used for further characterization. In the cdELISA assay, the concentrations of OTA, ochratoxin B and ochratoxin C, causing 50% inhibition (IC50) of binding of OTA-horseradish peroxidase to the antibodies were found to be 0.90, 110 and o.54 µg/L respectively. The recovery was

found to be 85.9% when 10 to 250 µg/kg of standard OTA was spiked to soybean samples and extracted with 50% aqueous methanol (Yu *et al.*, 2005).

The ELISA method was also used for the analysis of aflatoxins in two varieties of hulled rice, which was artificially contaminated at different concentrations and processed using dehulling and polishing methods to produce four fractions (hull, bran, polished broken grains, and polished whole kernels). The fractions were then analyzed for total aflatoxins including B1, B2, G1 and G2. The processing was shown to have an average removal of 96% and 97% of the initial aflatoxins present in the polished broken grains and polished whole kernel respectively. The processing was found to effectively remove aflatoxins from the rice and reduced initial contamination by more than 90% and was considered to be a good step in improving the safety of the final fraction (Castells, Ramos, Sanchis, & Marin, 2007).

An ELISA method followed by HPLC for the screening of aflatoxins was designed by Li *et al.* (2009). The method involved the use of three-class specific monoclonal antibodies against aflatoxins, and aflatoxins G2 was used as competitor in the ELISA system. The ELISA method showed high cross-reactivity (65%) to aflatoxin G2, antibody 10C9 had the most similar sensitivity for five aflatoxins (B1, B2, G1, G2 and M1), with I50 values were in a range of 2.1–3.2 µg/L. After careful optimization, antibody 10C9 was selected to develop an ELISA for determination of aflatoxin B1, B2, G1 and G2 in peanut samples. The average recovery of the method ranged from 87.5 to 102% with RSD between 6.8 and 11.2% (Li *et al.*, 2009). The results indicate that the ELISA developed can accurately determine total aflatoxins in samples of peanuts after the simple and rapid extraction procedure.

2.4.2. Fluorescent Polarization Immunoassay (FPIA)

Fluorescent polarization Immunoassay (FPIA) is a rapid qualitative technique which is based on measuring the competitive interactions between mycotoxins and a mycotoxins-fluorescent labeled tracer for a mycotoxin specific antibody . The fluorescence polarization measures the orientation of the fluorescence emission from horizontal and vertical directions. This is related to the measurement of rate of rotation of the fluorescent molecules in solution, rather than the fluorescence intensity. The rate of rotation is in turn directly related to the size of the fluorescent molecules in solution. The rate of rotation is in a direct proportion, while polarization has an indirect proportion, to the size of the molecules, with small molecules having higher rates and give low fluorescent polarization than that of larger molecules (Chun, Choi, Chang, Choi, & Eremin, 2009; Maragos, 2004; Nasir & Jolley, 2002; Zheng *et al.*, 2006). Therefore, it allows for the determination and detection of small molecular weight analytes in solution.

The FPIA method was developed for the screening test for zearalenone in cereals and their products, based on the change in fluorescent polarization of flourscent-labelled small antigen bounded to a specific antibody. The synthesized fluorescent labeled zearalenone tracer containing three linkers of different carbon lengths (2, 3 and 6-carbon bridge), ethylene diamine, 1,2-diaminopropane and hexamethylenediamine (HMDF) were assayed and their binding response with zearalenone-specific antibody was evaluated. The zearalenone-HMDF conjugate which contain 6-carbon bridge was found to the most sensitive FPIA for the detection range of 150 -1000 µg/kg with a detection limit of 137 µg/kg and was completed in less than 2 min. The average recovery of the spiked corn sample was found to be 106.4% with RSD of 12.5%. The result was compared to ELISA and HPLC and the correlation coefficients between FPIA and ELISA and between FPIA and HPLC was found to be 0.76 and 0.72 respectively, implying that the zearalenone-

HMDF tracer is suitable for the FPIA screening of mycotoxins in grains without the need for a time-consuming and complicated clean-up (Chun *et al.*, 2009).

The FPIA method was used for the analysis of a series of naturally contaminated grain (corn, sorghum, peanut butter, and peanut paste) and spiked popcorn samples. The analysis of spiked popcorn samples containing a mixture of aflatoxins (B1, B2, G1 and G2) gave a good correlation value of 0.99. The comparison of the assay with an HPLC reference method gave a good correlation coefficient ($r^2 = 0.97$) for naturally contaminated samples. Although the assay was found to consistently underestimate the aflatoxin content, this could be as a result of low cross-reactivity of the antibody used towards aflatoxins B2, G1and G2. This showed that considering the portability and simplicity of the FPIA technique, the assay can be used for screening of total aflatoxins in grains (Nasir & Jolley, 2002).

An FPIA method for rapid screening of DON in wheat and maize samples were developed, based on the competition between DON and a novel DON-fluorescence tracer (DON-FL2) for a DON-specific monoclonal antibody solution. The assay was compared with HPLC method, and the FPIA methods agreed well with linear regression of 0.936 for wheat and did not agree well for maize with linear regression of 0.849. The recoveries from the two samples using the aqueous extraction and testing by FP, was excellent for maize with recovery of 94.6% and was poor for wheat with recovery value of 71.2%. The study also investigated the impact of the incubation time on the calibration curve and was found that the assay was most sensitive with a short incubation time (15 s), but an identical calibration curve was observed at an incubation time ranging from 1 to 12 min with equilibrium reached at 1 min and thus was used for the rest of the assay (Maragos & Platiner, 2002)

The FPIA method is a homogenous assay conducted in the solution phase. It does not require the separation of the free and bound tracer, and an enzyme reaction, which distinguishes it from the ELISA method, which is a heterogeneous assay that requires the separation of the free and bound tracer and enzyme reaction. Thus, there is minimal effect of coloured and cloudy solutions (Maragos, 2009; Nasir & Jolley, 2002; Zheng *et al.*, 2006). Therefore FPIA does not require any washing or clean-up step, although matrix effect does exist and it makes an extensive validation study a necessity for application of the FPIA method in different commodities (Zheng *et al.*, 2006). The matrix effect can be controlled by dilution, matrix matched calibration curve and data normalization; with dilution providing better selectivity and does not adversely affect the operating range of the assay for a simple matrix (Maragos, 2009).

2.5. Quantitative Analysis of Mycotoxins

2.5.1. Gas Chromatography (GC)

Gas chromatography (GC) analysis is a separation technique that is based on the partition of analytes between a liquid stationary phase and a gas mobile phase. GC has been regularly used widely for the qualitative and quantitative analysis of food samples. It has been used widely to identify and quantify the presence of mycotoxins in food (Turner *et al.*, 2009). It is mostly used for the analysis of trichothecenes (Valle-Algarra *et al.*, 2005), because they do not absorb intensively in the ultraviolet-visible range, are non-fluorescent compounds and vary considerably in polarity (Koch, 2004; Lattanzio, Pascale, & Visconti, 2009).

GC is suited for the analysis of thermally stable, non-polar, semi-polar, volatile and semi-volatile compounds (Köppen *et al.*, 2010), such as pesticides, oils and steroid. Most mycotoxins are non-volatile; hence, derivatization is always used to increase the volatility

of mycotoxins and improve their responses to GC detection system (Koch, 2004; Köppen *et al.*, 2010; Turner *et al.*, 2009). This derivatization involves the reaction of the hydroxyl groups of the mycotoxins with; silylating agent using trimethylsilyl (TMS) to form TMS ester, fluorinating agent using pentfluoropropyl (PFP), heptafluorobutyl (HFB) or trifluoroacyl (TFA), and acetylation with acetic anhydride to form their respective esters (Koch, 2004; Langseth & Rundberget, 1998). The choice of derivatization agent depends on the nature of the mycotoxins to be analyzed and the type of detection system employed (Langseth & Rundberget, 1998). Fluorinating agents provide better sensitivity and selectivity for type A and B trichothecenes (Radova, Holadova, & Hajslova, 1998; Valle-Algarra *et al.*, 2005).

Type-B trichothecenes (DON, NIV, 3-ADON and 15-ADON) have been analyzed in wheat samples using GC-ECD techniques that are preceded with the SPE procedure for clean-up. The study involved comparative studies of two derivatization agents (pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA) and two chromatographic columns (HP-1701 and HP-5) (Valle-Algarra *et al.*, 2005). The LOD for NIV and 3- and 15-ADON were found to be similar irrespective of the derivatization used, while for DON using HFBA derivatization gave higher chromatographic signal than PFPA, thereby providing higher sensitivity. The linearity was also the same for all analytes for both derivatization agents and ranges from 0.006 to 0.625 mg/L, with PFPA providing better correlation coefficients (0.9900-0.9993) and was used for further studies. The HP-1701 column gave good and well-separated peaks for 3-ADON and 15-ADON and all analyzed trichothecenes were well separated with a better limit of detection.

A method for the multi-determination of trichothecenes (DON, NIV, DAS, FUS X, T-2, and HT-2) in wheat was developed using two clean-up procedures followed by derivatization with trifluoroacetic acid anhydride (TFAA) and were separated with high

resolution GC-ECD (Radova *et al.*, 1998). The ECD response was found to be linear over the range of 20-300 pg/ injection with correlation coefficients of 0.99. The detection sensitivity of the ECD for the derivatized trichothecenes was found to be dependent on the number and position of the hydroxyl functional groups available for acetylation. The detector response increases with the number of hydroxyl groups, thus DON with three hydroxyl groups in its molecule gave higher relative response. It was observed that determining trichothecenes below a spiking level of 2 mg/kg was not reliable and that good recoveries (86 – 97%) could only be achieved when this spiking level is exceeded.

A powerful and practical GC–MS system was developed for the simultaneous detection and confirmation of trichothecenes and zearalenone with TMS used as the derivatization agent (Tanaka, Yoneda, Inoue, Sugiura, & Ueno, 2000). Recoveries of eight mycotoxins from wheat was found to range from 83 – 94% and in corn from 81 – 93% with RSD values 0f 3.9 – 5.5 and 3.9 – 5.7 respectively. The LODs in all mycotoxins in cereals range from 5 – 10 µg/kg. Schollenberger *et al.* (1998) also determined eight trichothecenes in wheat with TFAA used for derivatization. The LODs were between 20 – 120 pg, which corresponds to 2 – 12 mg/kg of sample, while LOQs were between 60 – 380 pg, corresponding to 6 – 38 mg/kg of sample. The recoveries ranged from 73 to 91% with RSDs between 4.0 and 11.7% .The same recoveries were obtained when rice and whole bread samples were spiked with 100 µg/kg of the mycotoxins. It was also observed that trichothecenes were destroyed during derivatization, which was related to the destruction of active centers in the glass wall (Schollenberger *et al.*, 1998).

A comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC x GC-TOF-MS) used for the analysis of trichothecenes A and B in wheat was described (Jelen & Wasowicz, 2008). The trichothecenes were analyzed and quantified following their derivatization as TFAA. It was observed that the type-A trichothecenes

provided a better detector responses when derivatized with TFAA, while type-B trichothecenes provided higher sensitivity when analyzed using TMS derivatives. The TMS was also found to produce less interference than TFA. The LOD for analyzed mycotoxins was between 0.01 and 0.02 mg/kg and linearity between 25 – 2500 pg with correlations coefficients of 0.999 and RSD less than 9%.

Although, some analytical problems were found in certain cases to be associated with the GC methods, such as changes induced in the mass spectra of unknown analytes due to derivatization, over-estimation due to matrix effect and poor repeatability, it has some advantages that include simultaneous detection of two or more mycotoxins and at relatively low LODs. Little work has been done on the use of GC for quantitative analysis of aflatoxins.

2.5.2. Liquid Chromatography (LC)

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid and a solid stationary phase. It is also based on the partition between the liquid mobile phase and the stationary phase. It is the most popular method for the separation, detection and quantification of mycotoxins in food. LC analytical methods for the analysis, purification and separation of mycotoxins in foods can either be normal-phase liquid chromatography (NPLC) or reversed-phase liquid chromatography (RPLC), coupled to different detector systems. HPLC is a modern analytical technique for the analysis of mycotoxins using various adsorbents depending on the physical and chemical structure of the mycotoxins (Rahmani *et al.*, 2009; Turner *et al.*, 2009). HPLC is quantitative technique that is suited for online clean-up of sample extracts and has been combined with different detectors, which provided different sensitivities and selectivities.

The detectors that have been described include UVD, DAD, FLD and MS, but the most common are the MS and FLD. FLD relies on the presence of flourophore on the mycotoxins molecules. MS is regarded as the industry standard detection method, which allows better accuracy, eliminates the need for sample derivatization and allows for selective and sensitive detection of toxins, but its high cost, complex laboratory requirements and limitations in the type of solvents used for extraction and for the mobile phase have limited its use (Rahmani *et al.*, 2009; Turner *et al.*, 2009). For these reasons FLD became the detector of choice and also because of its high sensitivity (Rahmani *et al.*, 2009), but its limitation lies in the use of derivatization for mycotoxins fluorescence activity enhancement.

A LC-MS-MS method was used for the analysis of a trichothecene (DON) in wheat. The study also involved the comparison of the LC-MS-MS method using different quantification methods (internal standard and standard additions) and with HPLC-DAD. All methods were found to have similar sensitivity, except for the HPLC-DAD. The linearity of LC-MS-MS were between 50 to 1,500 µg/L, with correlations greater than 0.99, LODs and LOQs were 0.2 µg/kg and 0.5 µg/kg correspondingly while the recovery was 98% for both quantification method adopted. The RSD was 4.7% using internal standard and 1.8% with standard addition method. The recovery of the HPLC-DAD was higher with 91.6%, with LOD and LOQ of 63 µg/kg and 195.7 µg/kg and RSD of 5.7%. Due to its high LOQ the LC-DAD was found to be suitable for the measurement of highly contaminated samples (Neuhof, Ganzauer, Koch, & Nehls, 2009).

In another study, OTA was determined in rice sample using HPLC-FLD with methyl ester derivatization (Juan *et al.*, 2005). The HPLC analysis was preceded by an ASE using methanol as the extracting solvent after the optimization of the extraction step. The method detection and quantification limits were 0.01 and 0.03 µg/kg respectively, with a

mean recovery of 94% and RSD of 2.5% of fortified rice sample at a concentration level of 5 µg/kg of OTA. HPLC-FLD method was also used for the simultaneous determination of type-A trichothecenes (T-2 and HT-2) in cereal samples (wheat, maize and barley), after the extraction of the mycotoxins from the sample with methanol/water (90:10. v/v) followed by IAC clean-up containing monoclonal anti T-2 antibodies (Visconti *et al.*, 2005). The toxins were quantified using a reverse-phase HPLC-FLD. The recoveries from spiked samples ranged from 70 to 100%, with RSD lower than 8%, while the LODs were 5µg/kg for T-2 and 3µg/kg for HT-2. The method described in this study allows the determination of the two mycotoxins at levels that can occur in naturally contaminated samples, because it combined the selectivity of the IAC clean-up with the selectivity of the HPLC-FLD.

A method for the determination of 27 mycotoxins and other secondary metabolites in maize was described using LC-MS-MS after the extraction of the mycotoxins using QuEChERS method with no clean-up step (Rasmussen *et al.*, 2010). The method was validated for the determination of eight analytes quantitatively and 19 quantitatively. The average recoveries ranged between 60 to 105% with RSD of 5–27 %. The LOD ranged from 1 to 739 µg/kg. The same extraction method was also used for the simultaneous determination of type-A and -B trichothecenes in wheat flour, followed by LC-MS analysis (Sospedra *et al.*, 2010). The LOD and the LOQ of the investigated mycotoxins ranged from 1 to 30 µg/kg and 4 to 100 µg/kg. The recoveries were determined using the method of standard addition and compared with an external standardization method and were in the range of 86.2 to 108.5%. The RSD ranged from 3.4 to 6.7% and its low value (<10) confirmed the reproducibility and repeatability of the described method.

An ultrasensitive and selective method was developed for the determination of various regulated mycotoxins (aflatoxins G1, G2, B1, B2, M1 and OTA) in baby food and

milk by Beltran et al, (Beltrán *et al.*, 2011), using ultra high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS). The sample was pre extracted with SPE with IAC after sample extraction with mixture of acetonitrile and water (80:20, v/v). The method validation using samples spiked at 0.025 and 0.100 µg/kg gave satisfactory recoveries between 80 and 110% with RSD lower than 15%. The LOQ ranged from 18 – 25 ng/kg for cereals infant formula, 6 – 18 ng/kg for milk for infants with cereals, 5 – 15 ng/kg in raw milk and 3 – 5 ng/kg in milk for babies. While the LOD were between 5 and 9 ng/kg in cereals infant formula, 2 and 5 ng/kg both in milk for infants with cereals and in raw milk, and 1 to 2 ng/kg in milk for babies. The sensitivity of the method was ascertained because no relevant interferences were observed in the blank at the analytes retention times. Due to the absence of matrix effects, quantification was done using external standard calibration.

2.5.3. Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a separation technique, which consists of a stationary phase that is immobilized on a glass or plastic plate, and placed in an organic solvent. The sample, either liquid or solid dissolved in a volatile solvent is deposited as a tiny spot on the stationary phase. TLC has two treatment methods for the chemical confirmation of mycotoxins; these include the impregnation of the TLC plates with acidic-organic solvent and the exposure of the TLC plate with the developed chromatogram to vapor of pyridine or acetic anhydride or dipped into aluminium chloride reagent. This 2 step treatment results in the conversion of the mycotoxins into fluorescent compounds and observation of the TLC plate under ultraviolet (UV) light of 365 nm (Rahmani *et al.*, 2009). TLC is a powerful and popular chromatographic method used for the qualitative and semi-quantitative analysis of mycotoxins at levels as low as 1 µg/kg, because it enables the

screening of large number of samples with higher sample throughput, rapid analysis, low operating cost and ease of identification of target analytes by comparison of spot color, retention factor (Rf) values and using ultraviolet-visible (UV-Vis) spectral analysis (Köppen *et al.*, 2010; Lin, Zhang, Wang, Wang, & Chen, 1998; Turner *et al.*, 2009).

Although, like other chromatographic techniques, TLC requires the sample preparation and clean-up step (Lin *et al.*, 1998; Turner *et al.*, 2009), due to the complex nature of the sample matrices, and the extraction procedure and clean-up is dependent on the physicochemical properties and types of the mycotoxins under investigation. Several TLC methods have been developed for the analysis of mycotoxins. Its sensitivity and accuracy is found to be less than that of HPLC-FLD, but the results obtained with high performance Thin layer chromatography (HPTLC) were similar (Jaimez *et al.*, 2000; Stroka *et al.*, 2000). In another study, a one-dimensional TLC method for the analysis of aflatoxins (B1, B2, G1 and G2) in various food matrices (paprika powder, peanut butter and pistachios) following IAC sample clean-up step after extraction with methanol was carried out (Stroka *et al.*, 2000). The method LODs and LOQs calculated at the 95% confidence interval of the calibration curve ranged from 0.1 to 0.6 µg/kg and 0.4 to 1.7 µg/kg respectively for all aflatoxins. The recoveries were between 76 to 87% with RSD values between 1.3 and 8.9%. The LOQ of the method was found to be significantly lower than the EC regulatory limit and the recovery showed that the method is likely to give satisfactory performance when validated.

In another study a bi-directional HPTLC was employed for the analysis of aflatoxins in maize using a silica gel and phenyl non-polar bonded –silica gel for the separation of the mycotoxins after extraction with various combinations of aqueous methanol, acetone and their mixtures (1:1). The mean recoveries of the method was found in the range of 92 to 99% with RSD values between 1.7 and 10.8% and linearity ranged

from 3.4 to 90.1µg/kg (Bradburn, Coker, Jewers, & Tomlins, 1990). A method was also developed for the qualitative recovery of aflatoxins B1 and B2 in pistachio and peanut samples using a pressurized fluid extraction using mixture of methanol and water (80:20, v/v) as the extraction solvent, followed by TLC analysis (Sheibani & Ghaziaskar, 2009). The method performance was compared to the AOAC method and it shows a higher mean recovery (100%) and RSD of 13.5%, which was found to be 20% higher than the AOAC method.

3. DETERMINATION OF AFLATOXINS IN CEREALS USING LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION QUADRUPOLE TIME OF FLIGHT MASS SPECTROMETRY (LC-ESI-QTOF-MS/MS)

3.1. Introduction and scope of the work

Aflatoxins are secondary metabolites of certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* (Battilani *et al.*, 2008). Aflatoxins have been found to contaminate a wide variety of important foodstuff such as cereals (corn, wheat, barley, maize, oats and rye), nuts (hazel nuts, peanuts, pistachios), dried fruits (figs) and spices (Ardic, Karakaya, Atasever, & Durmaz, 2008; Bacaloni *et al.*, 2008; Battilani *et al.*, 2008). Among the 18 different types of identified aflatoxins, the most common naturally occurring aflatoxins in foodstuff are aflatoxin B1, B2, G1 and G2 (Battilani *et al.*, 2008). In fact, the International Agency for Research on Cancer (IARC) has classified aflatoxin B1 as the most toxic group 1 carcinogen which primarily affects the liver (IARC, 1993). In the European Union regulations, the maximum allowed levels for aflatoxin B1 and total aflatoxins are 2 and 4 µg/kg respectively (European Commission, 2006b).

Therefore, several methods have been developed for the analysis of aflatoxins including: - thin-layer chromatography (TLC) (Richard, 2007) high performance liquid chromatography (HPLC) fitted with ultraviolet detection (UV) and fluorescence detection (FLD) (Herzallah, 2009), enzyme linked immunosorbent assay (ELISA) (Var, Kabak, & Gök, 2007), capillary electrophoresis (CE) (Maragos, 2006), liquid chromatography coupled with mass spectrometry (LC/MS) (Nonaka, Saito, Hanioka, Narimatsu, & Kataoka,

2009) and gas chromatography coupled with mass spectrometry (GC/MS) (Peter M, 1993). However, liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) is considered as the method of choice for the accurate measurement of mycotoxins in food (Cavaliere *et al.*, 2007; Silva *et al.*, 2009). The hybrid tandem mass spectrometer with quadrupole time of flight (QTOF-MS/MS) provides a high sample throughput, with high resolution and good specificity due to the mass accuracy. In addition, it can provide the structural information when employed in the MS/MS mode, which allows the acquisition of the full range mass spectral data instead of just a single ion.

The aim of this study is to develop an LC-ESI-CID-MS/MS analysis using a hybrid QTOF-instrument for the rapid determination of aflatoxin B1, B2, G1 and G2 in cereals. In this study, the use of CID-MS/MS in the quantification of aflatoxins in food is discussed. Furthermore, the sample preparation, chromatographic conditions, and the electrospray ionization (ESI) interface conditions are optimized in order to maximize the sensitivity. The instrument optimization procedure was carried out by varying one parameter while holding the other parameters constant until the best sensitivity was achieved. This simple approach is based on the monitoring of the molecular ion of the standard aflatoxin B1 and G1 at 50 µg/kg and the final selection is based on this parameter, which gave the highest intensity as well as a clean spectral background. For the quantification studies a minimum number of fragment ions are necessary, while for structural studies the full extent of the fragmentation pattern has to be maximized (Ardrey, 2003).

3.2. Experimental

3.2.1. Reagents and materials

Certified mixed standard solutions of aflatoxin B1, B2, G1 and G2 were obtained from Sigma-Aldrich (Darmstadt, Germany). Subsequently, daily working standard solutions

were prepared by diluting the stock solutions in the mobile phase (water containing 2 mM ammonium formate and 1% formic acid: methanol containing 1% formic acid, 40%: 60%). Ammonium formate was purchased from Agilent Technologies (USA). Formic acid, HPLC-grade, was supplied by Sigma-Aldrich (Darmstadt, Germany). Sodium chloride was purchased from Merck (Germany). Water was purified by reverse osmosis with an electrodeionization (EDI) system (Maxima Ultra Pure Water, England). A 0.2 μm disposable membrane filter was purchased from Cronus Filter (UK).

3.2.2. Sample preparation

The aflatoxins B1, B2, G1 and G2 were extracted from the samples using a solid-liquid extraction procedure. 0.5 g of the ground sample and 0.2 g of sodium chloride were weighed into a 250 mL conical flask and 40 mL of methanol/water (80:20, v/v) was added. The mixture was stirred for three minutes at a high speed and filtered through a Whatman No. 1 filter paper. The filtrate was rinsed twice with 5 mL methanol. After that, the extracts were dried with anhydrous sodium sulfate (Fisher Scientific, UK) and evaporated until dryness using a rotary evaporator (TOKYO PIKAKIKAI, Japan) at 45°C under vacuum. Finally, the residue was reconstituted with 0.5 mL methanol and diluted 10 times with the mobile phase (water containing 2 mM ammonium formate and 1% formic acid: methanol containing 1% formic acid, 40%: 60%, v/v) and passed through a 0.2 μm disposable membrane filter prior to the LC-ESI-CID-MS/MS analysis.

3.2.3. Analytical procedure

Quantification of the aflatoxin B1, B2, G1 and G2 were performed using an Agilent 6530 Q-TOF-MS/MS spectrometer coupled with an Agilent 1200 Series HPLC system. The HPLC system consists of a vacuum degasser (G1379B) with a thermostatted autosampler

(G1330B) and binary pump (G1312B), and a thermostatted column compartment (G1316B). The instrument was also equipped with an electrospray ionization (ESI) source and the proprietary Agilent jet stream dual nebulizer.

3.2.4. Instrumental conditions

The injection volume was 40 μL for the samples and standards. In the HPLC analysis, a mobile phase of 1% formic acid and 2 mM ammonium formate in water (A) and 1% formic acid in methanol (B), was employed in the isocratic mode with 40% solvent A: 60% solvent B, for 4 minutes. The separation was performed with the ZORBAX Eclipse XBD-C_{18}, 2.1 mm × 100 mm, 1.8 μm (P.N. 928700-902) column at a flow rate of 0.3 mL/min. The mass spectrometer was operated in the positive electrospray ionization mode with the Agilent jet stream technology. The optimum ESI operational conditions are as follows: capillary voltage and fragmentor voltage were 3500 V and 200 V, respectively. The drying gas and sheath gas temperatures were set at 150°C and 350°C, respectively, while the drying gas and sheath gas flows were set at 5 L/min and 12 L/min, respectively. Finally, the nebulizer pressure was set at 25 psi and the nozzle voltage was set at 0 V.

3.2.5. Food samples

In the month of December 2009, 28 samples of cereals (barley, wheat, soybeans and corn) were randomly obtained from groceries and stores in Kuala Lumpur, Malaysia. 1-2 kg of each type of cereal sample was obtained. The samples were stored in a dark place at room temperature (25–30°C). The samples were ground and mixed at room temperature for 10 min till a fine and homogeneous powdered material was obtained. The powdered samples were then stored in plastic bags at 4°C in a refrigerator prior to analysis.

3.3. Results and discussion

3.3.1. Optimization of the liquid chromatography (LC) conditions

The liquid chromatography conditions such as the mobile-phase composition, flow rate, and buffer composition can greatly affect the operation and the sensitivity of the ESI interface, specifically the ionization efficiency.

3.3.1.1. Effects of mobile phase composition

The mobile phase in the ESI interface is a prerequisite for generating the small aerosol droplets, which leads to an increase in the solvent evaporation. Hence, a volatile solvent is preferred and large amounts of water together with solvents of high surface tension and high viscosity should be avoided (Ardrey, 2003). Methanol and acetonitrile were chosen for the optimization of the most suitable mobile phase composition in the aflatoxins analysis. Methanol was more suitable than acetonitrile, since it resulted in well-defined chromatographic peaks of higher intensity (Figure 3.1). This could be due to the fact that methanol is a proton donor providing hydrogen for the protonation of the aflatoxins. Previous studies have shown that using methanol leads to higher signal enhancement in the positive ESI mode compared to acetonitrile. The addition of 2 mM ammonium formate to the mobile phase decreased the droplet size and thus enhances the ionization efficiency (Figure 3.1).

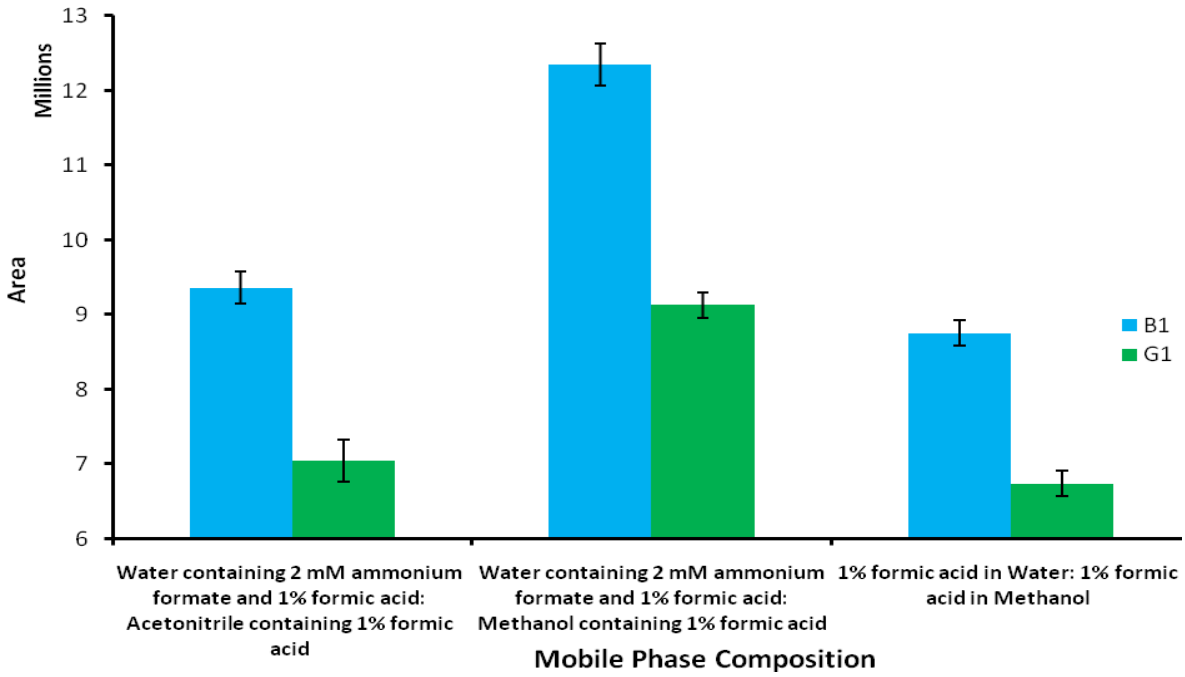


Figure 3.1: *Effects of mobile phase composition on the chromatographic peak areas of aflatoxins B1 and G1*

On the other hand, increasing the percentage of the organic solvent will increase the ionization of the polar aflatoxins, which leads to enhanced sensitivity (Figure 3.2). Frenich *et al.* (2009) reported that increasing the percentage of water decreases the ionization signal (Frenich, Vidal, Romero-González, & Aguilera-Luiz, 2009).

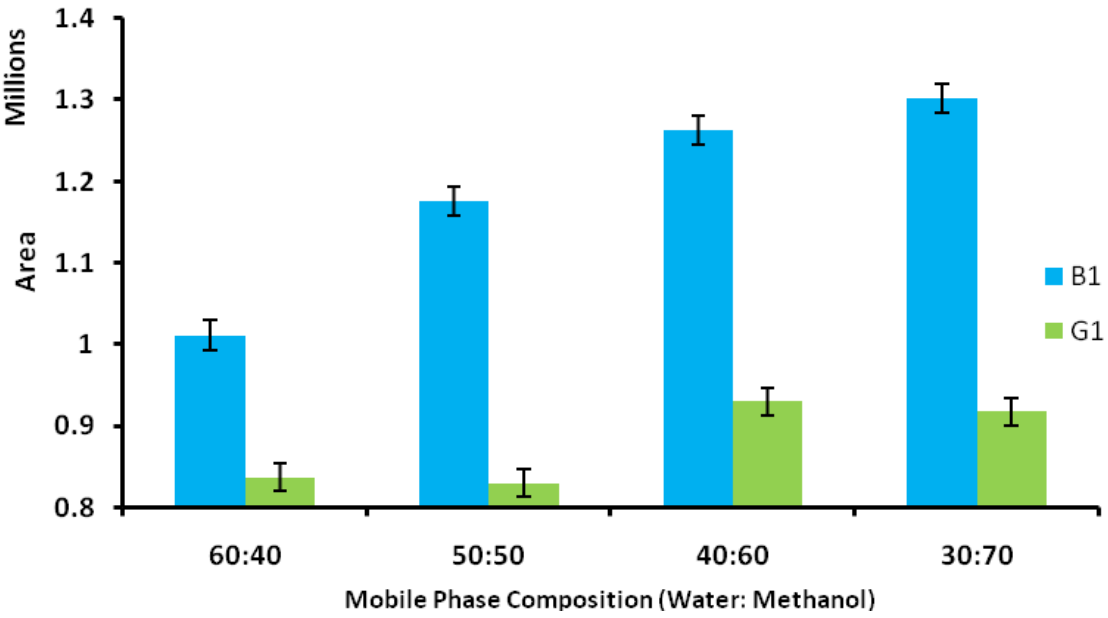


Figure 3.2: *Effects of percentage of the organic solvent in the mobile phase on the chromatographic peak areas of aflatoxins B1 and G1*

A high percentage of the aqueous solvent (80:20, v/v) leads to a broadened chromatographic peak and a longer retention time. In contrast, a high percentage of the organic solvent (20:80, v/v) leads to a sharp chromatographic peak with a shorter retention time. However, the aflatoxin chromatographic peaks were not completely resolved with this mobile phase composition. Therefore, the mobile phase composition of water/methanol (40:60, v/v) was selected in this study (Figure 3.3). As an alternative, the extracted ion chromatogram (EIC) mode can be applied to differentiate between overlapping chromatographic peaks obtained when a high percentage of the organic solvent (20: 80, v/v) was employed.

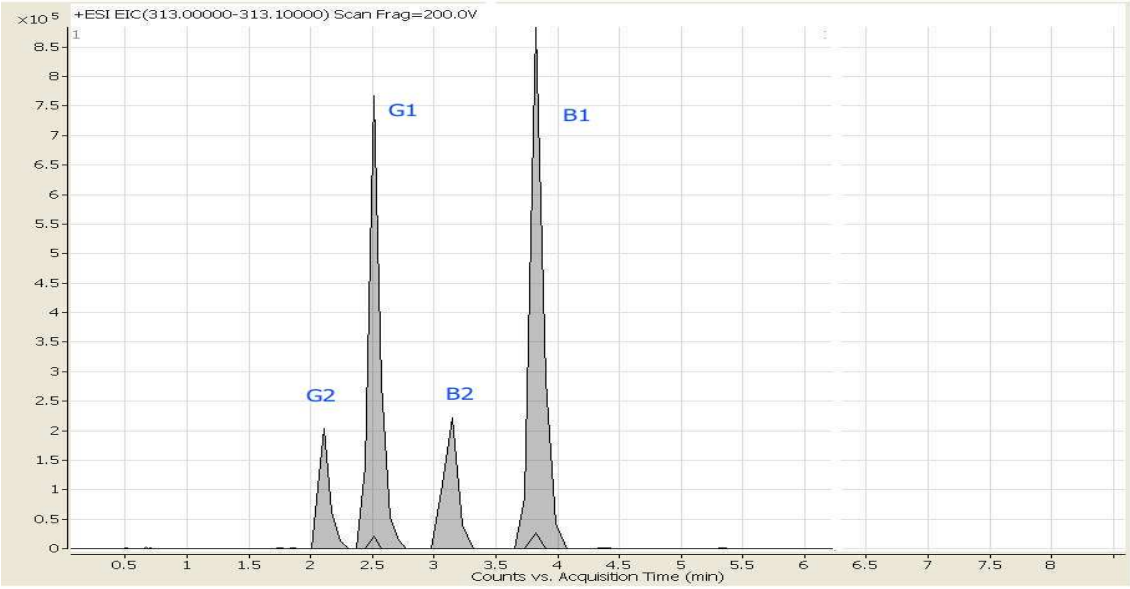


Figure 3.3: *LC–QTOF Chromatogram of aflatoxin standard solutions containing 100 µg/L of aflatoxin B1 and G1 and 30 µg/L of aflatoxin B2 and G2*

3.3.1.2. Effects of mobile phase flow rate

The flow rate of the mobile phase in the ESI interface plays an important role in the performance of a mass spectrometer. Increasing the flow rate tends to increase the droplet size which subsequently decreases the yield of the gas-phase ions from the charged droplets. The flow rate was examined in the range of 0.15 to 0.30 mL/min. It was found that the ionization efficiency increased as the flow rate was increased from 0.15 to 0.30 mL/min for both aflatoxin B1 and G1 (Figure 3.4). This indicates that the vaporization of a constant fraction of the analyte droplet ions is independent of the flow rate and the ESI interface becomes mass sensitive rather than concentration sensitive. Therefore, the application of a flow rate of 0.30 mL/min, which would also decrease the run time as well as enhance the sensitivity was employed in this study.

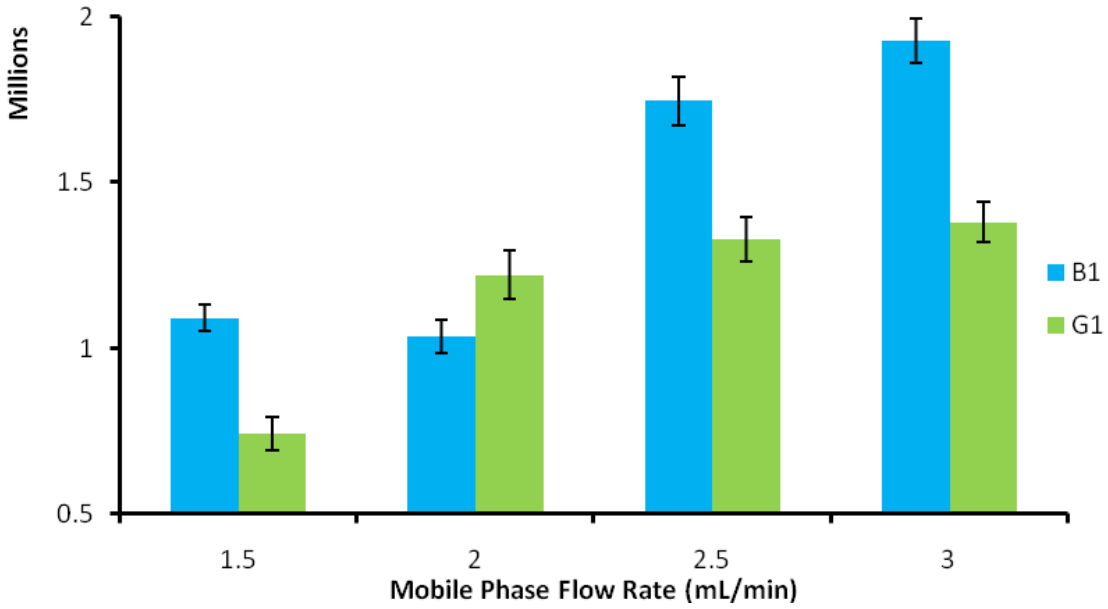


Figure 3.4: *Effects of mobile phase flow rate on the chromatographic peak areas of aflatoxins B1 and G1*

3.3.2. Optimization of the ESI parameters

3.3.2.1. Effects of capillary voltage

The capillary voltage is that applied to the inlet of the capillary. Besides its effects on the transmission efficiency of the ions through the capillary sampling orifice, the capillary voltage affects the ionization efficiency by producing the desired charged aerosol droplets. To obtain the optimum voltage, a voltage range from 2000 to 5500 V was selected at increments of 500 V. The greatest peak area for aflatoxin B1 (3.55E+06) and G1 (2.73E+06) were obtained when the voltage was set at 3500 V, while at 2000 and 5500 V, the ionization process gave the lowest peak areas (2.93E+06 and 2.78E+06; 2.08E+06 and 2.14E+06) for aflatoxin B1 and G1, respectively (Figure 3.5).

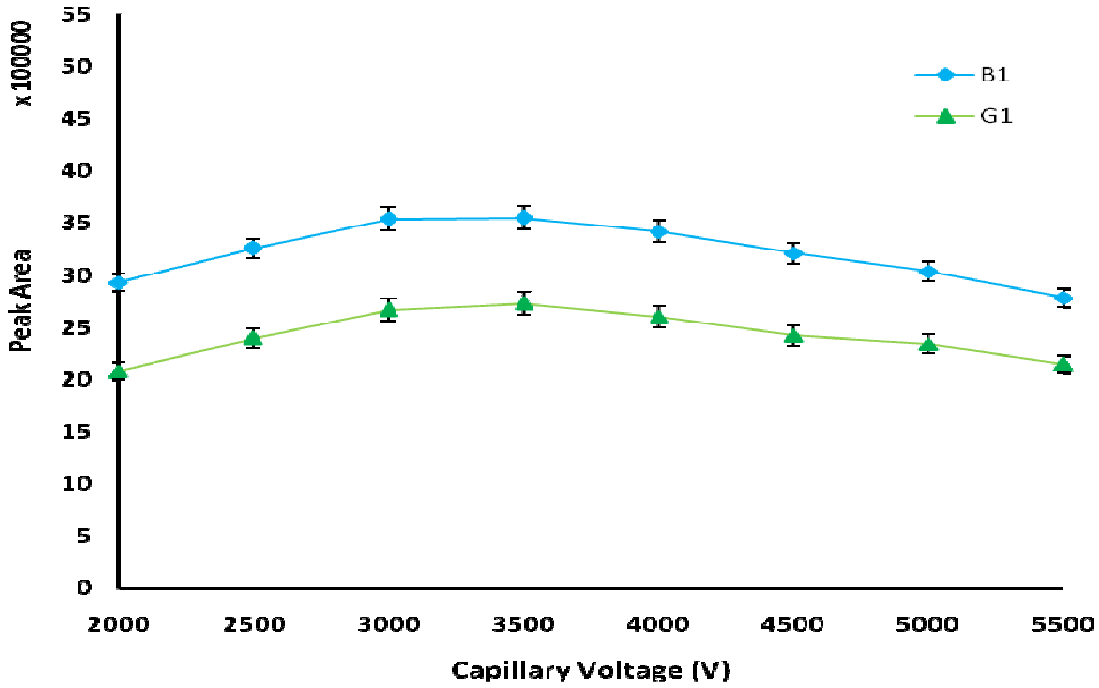


Figure 3.5: *Effects of the capillary voltage on the chromatographic peak areas for both aflatoxins B1 and G1*

Increasing the capillary voltage will accelerate the analyte movement through the capillary and may cause an increase in the transmission efficiency. In addition, increasing the capillary voltage may cause a large number of charged analyte droplets to be formed, which further enhances the sensitivity. However, instead of droplet formation, the use of a high capillary voltage would result in electrical discharge of the droplets. Hence, the optimum capillary voltage of 3500 V is employed.

3.3.2.2. Effects of fragmentor voltage

The fragmentor voltage (cone voltage or orifice voltage) is the difference between the voltage applied to the cone and that applied to the skimmer. Increasing the potential difference between the two would increase the kinetic energy and the velocity of the charged analyte droplets with more ions being transferred towards the skimmer resulting in a significant enhancement of the transmission efficiency and sensitivity. Optimization of

the fragmentor voltage was carried out by modifying the voltage from 50 to 300 V at increments of 25 V (Figure 3.6).

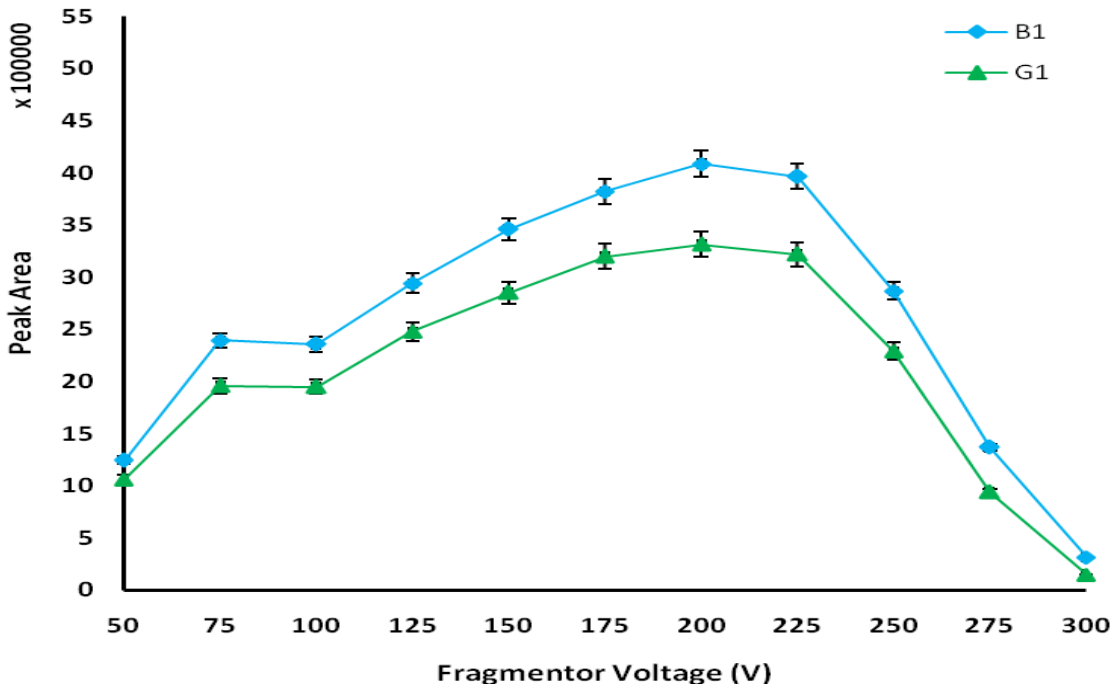


Figure 3.6: *Effects of the fragmentor voltage on the chromatographic peak areas for both aflatoxins B1 and G1*

The peak areas for aflatoxin B1 and G1 gradually increased from 1.25E+06 and 1.06E+06 to 4.09E+06 and 3.32E+06, respectively, as the fragmentor voltage increased from 50 to 200 V. However, by applying voltages higher than 200 V resulted in a sudden reduction in the peak areas for both aflatoxin B1 and G1. A high fragmentor voltage increases the internal energy of the molecular ion and the collisions between the analyte molecular ions and the solvent vapor and residual drying gas (N_2) leading to more fragmentation and a significant drop in peak areas. Hence, the optimum fragmentor voltage of 200 V is employed.

3.3.2.3. Effects of drying gas

The flow and temperature of the drying gas (N_2) are important factors for desolvation and reduction of the solvent droplet size (Ardrey, 2003). The angle between the sample spray and the MS inlet was set at 90° to discard neutral solvent clusters and to attract the charged droplet ions. The flow of the drying gas is usually set between 5 to 7 L/min with a maximum of flow of 11 L/min (Figure 3.7). The drying gas temperature, on the other hand, is usually set at temperatures between 250 and 350°C (Figure 3.8).

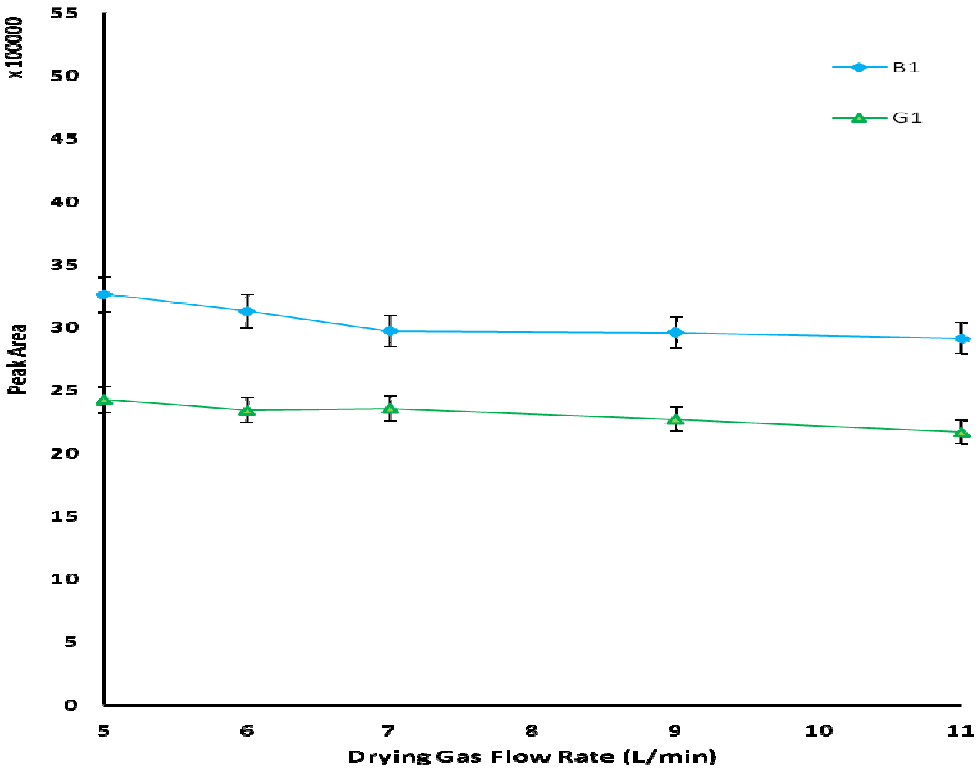


Figure 3.7: *Effects of the flow of the drying gas on the chromatographic peak areas for both aflatoxins B1 and G1*

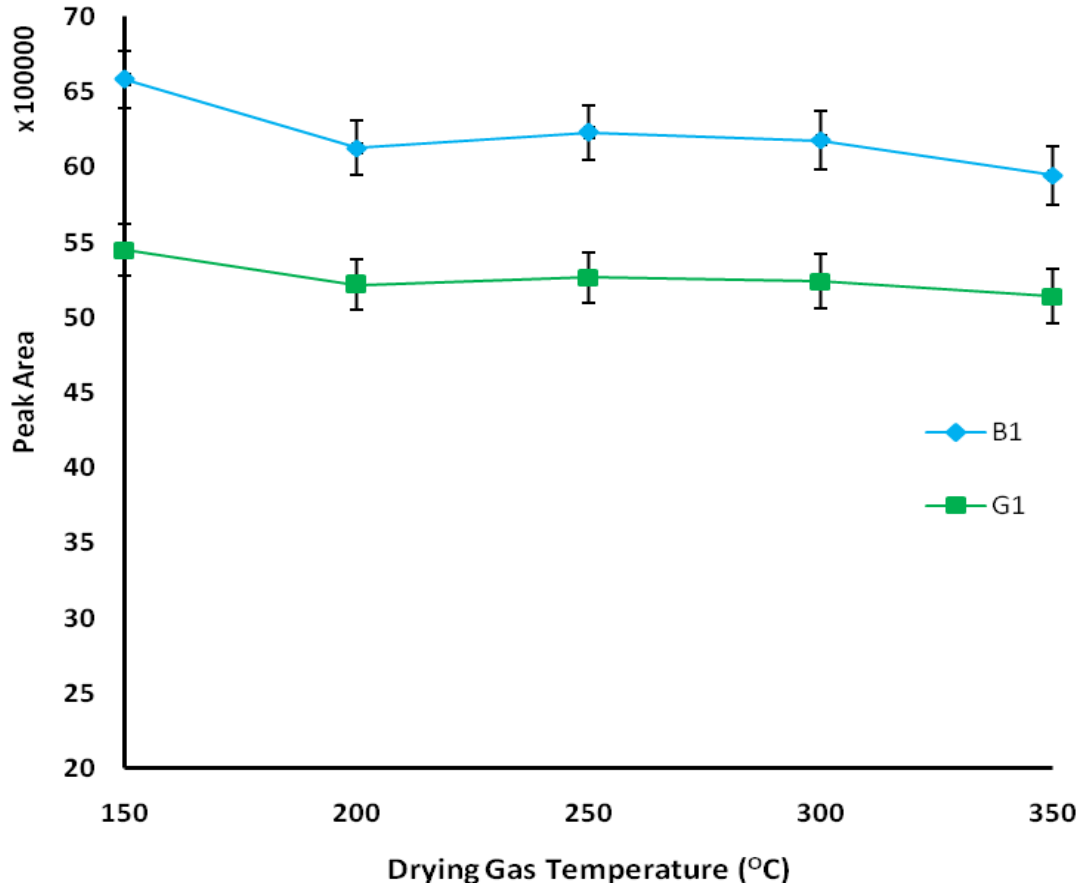


Figure 3.8: *Effects of the drying gas temperature on the chromatographic peak areas for both aflatoxins B1 and G1*

A higher temperature is recommended when the mobile phase contains a higher percentage of water and is flowing at a faster rate. However, during gradient elution, a high temperature of the drying gas may lead to compound decomposition. Hence, an optimum value for the flow and temperature has to be carefully selected to obtain the highest possible sensitivity. The optimization procedure for the drying gas parameters was carried out by modifying the drying gas temperature between 150 and 350°C until the maximum sensitivity was obtained. At a fixed temperature setting, the drying gas flow was varied from 5 to 11 L/min. The peak areas for both aflatoxin B1 and G1 decreased from 6.58E+06 and 5.45E+06 to 5.95E+06 and 5.14E+06 respectively as the drying gas temperature increased from 150 to 350°C. In contrast, when the drying gas flow was increased from 5 to

11 L/min, the peak areas for aflatoxin B1 decreased from 3.26E+06 to 2.91E+06, while that for aflatoxin G1 decreased from 2.43E+06 to 2.17E+06. Hence, the optimum drying gas temperature and flow are set at 5 L/min and 150°C, respectively.

3.3.2.4. Effects of sheath gas

The Agilent Jet Stream is a patented technology that uses a drying gas (N_2) as a sheath to collimate the nebulizer spray and to increase the solvent evaporation of the analyte droplet ions. Temperature settings of the sheath gas were varied between 200°C and 400°C, at 50°C increments (Figure 3.9) while the sheath gas flow was increased from 8 to 12 L/min, at 2 L/min increments (Figure 3.10).

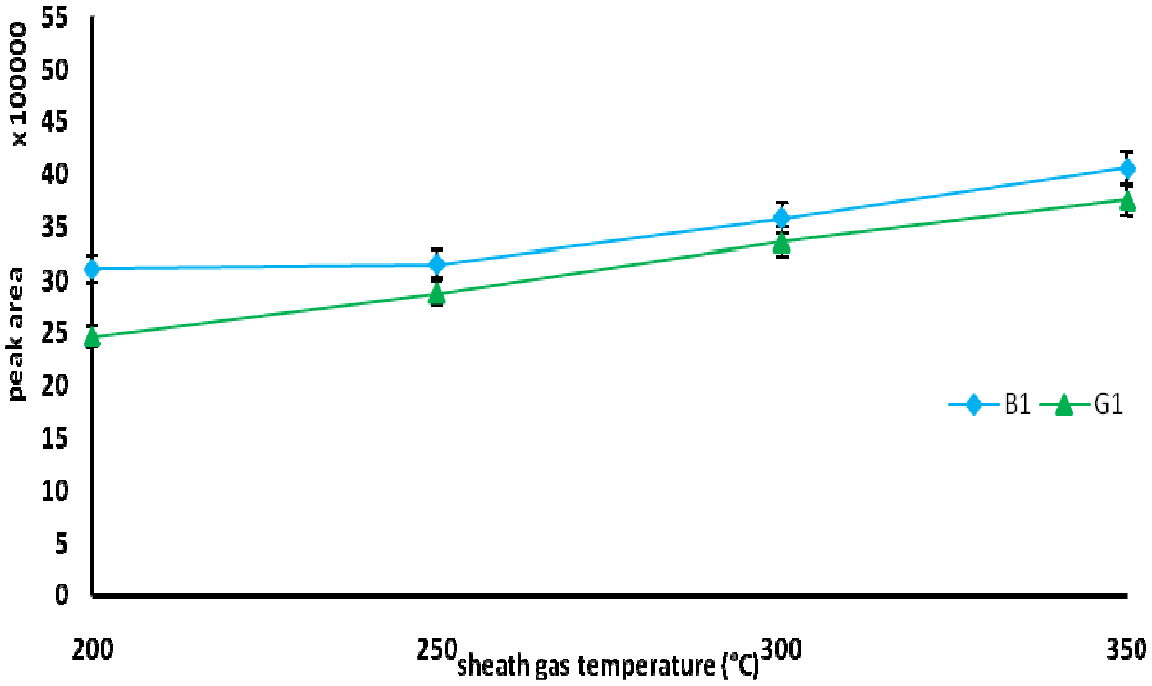


Figure 3.9: *Effects of the sheath gas temperature on the chromatographic peak areas for both aflatoxins B1 and G1*

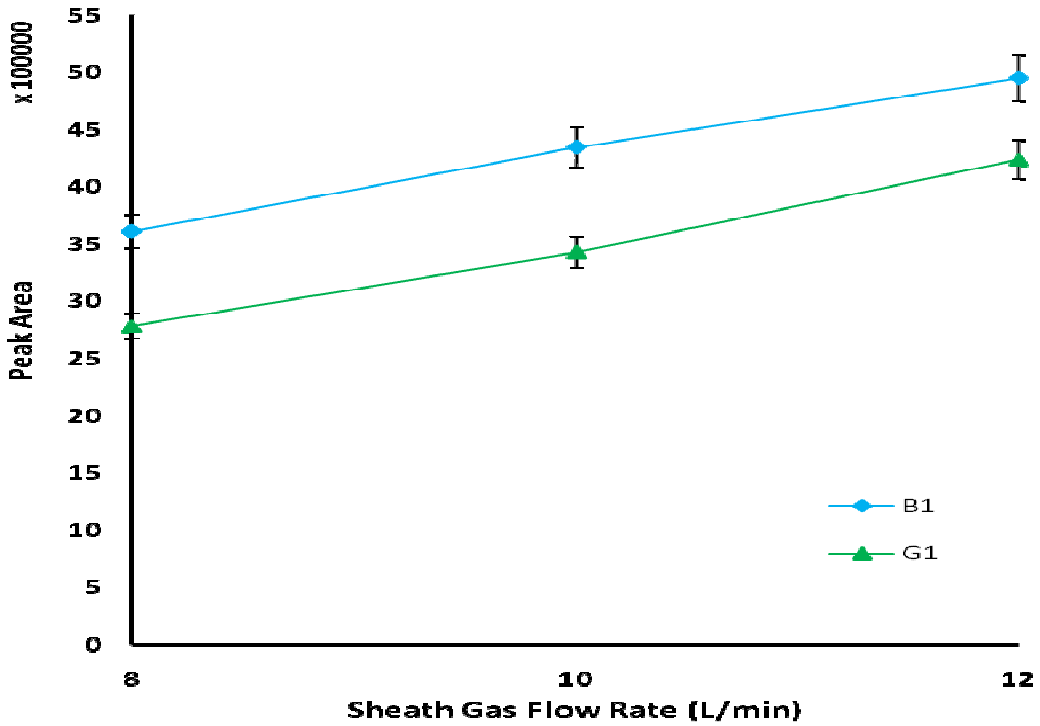


Figure 3.10: *Effects of the sheath gas flow on the chromatographic peak areas for both aflatoxins B1 and G1*

Increasing the sheath gas flow from 8 to 12 L/min caused an increase in the ion density in the front of the MS inlet leading to an increase in the peak areas of both aflatoxin B1 and G1 from 3.61E+06 to 4.95E+06 and from 2.78E+06 to 4.24E+06, respectively (Figure 310). The highest peak areas of both aflatoxin B1 and G1 were obtained when the sheath gas temperature was fixed at 400°C indicating that the desolvation and containment function of the sheath gas (Figure 3.9). The peak area for aflatoxin B1 increased from 3.11E+06 to 4.42E+06, while that for aflatoxin G1 increased from 2.47E+06 to 4.05E+06 as the sheath gas temperature in creased from 200 to 400°C. Hence, the sheath gas flow and the temperature were set at 12 L/min and 400°C, respectively, to obtain the maximum sensitivity. The results from these experiments show that as the sheath gas flow and temperatures were increased the sensitivity was enhanced , as opposed to the effects from the drying gas flow and temperature, which showed a decrease in sensitivity when these parameters were increased. This is because the laminar flow of the sheath gas leaves the

analyte molecular ion intact while the drying gas approaching from the orthogonal direction leads to analyte decomposition. In addition, the drying of the analyte molecular ion might have been accomplished by the sheath gas before reaching the drying gas zone, which may cause further fragmentation of the analyte molecular ion.

3.3.2.5. Effects of nebulizer pressure and nozzle voltage

The role of the nebulizer gas pressure is to break up the mobile phase stream into an aerosol mist of different droplet sizes as it moves towards the MS inlet. In practice, as the nebulizer pressure increases the solvent droplet size decreases leading to an increase in the sensitivity. The peak areas of aflatoxin B1 and G1 were monitored as the nebulizer pressure was increased from 20 to 60 psi (Figure 3.11); this resulted in a dramatic decrease in the sensitivity.

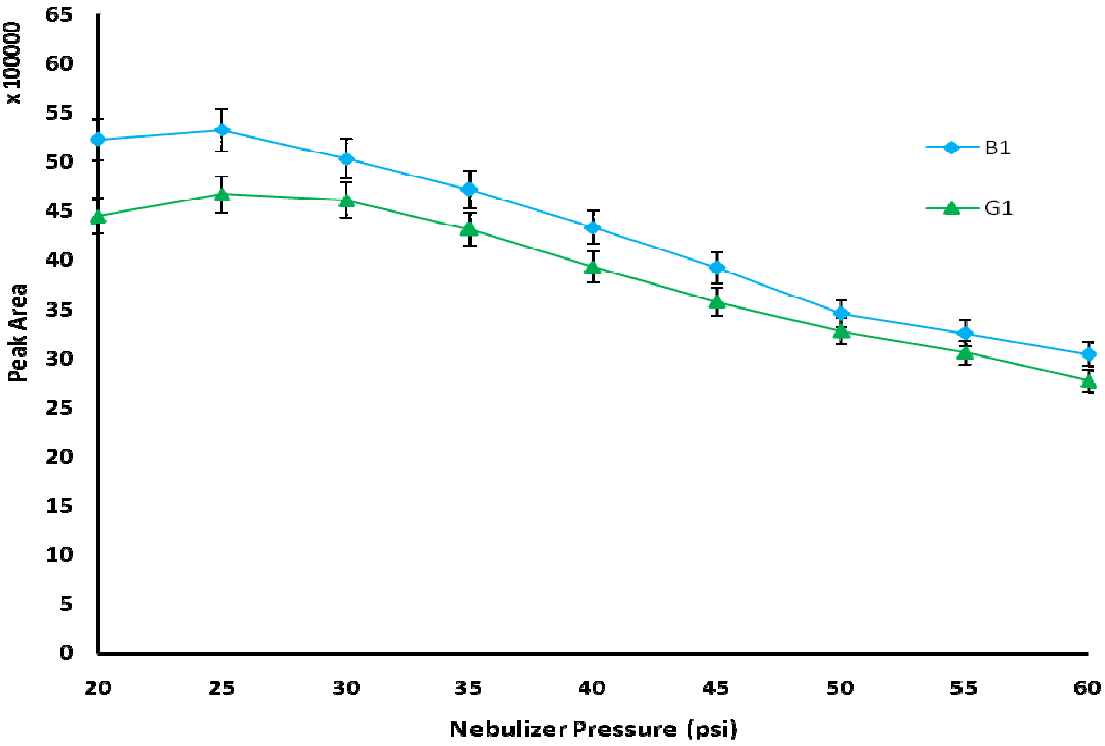


Figure 3.11: *Effects of the nebulizer pressure on the chromatographic peak areas for both aflatoxins B1 and G1*

As predicted, an increase in the nebulizer pressure from 25 to 60 psi resulted in a decline in the peak areas and sensitivities for both types of aflatoxins B1 and G1 from 7.94E+05 to 5.19E+05 and from 6.46E+05 to 4.58E+05 respectively (Figure 3.11) . This might be due to the uneven dissipation of the analyte droplet ions to smaller secondary droplets upon an increase of the nebulizer pressure. The nozzle voltage, one of the Agilent Jet Stream parameters, which can affect the sensitivity, was studied between 0 and 2000 V (Figure 3.12).

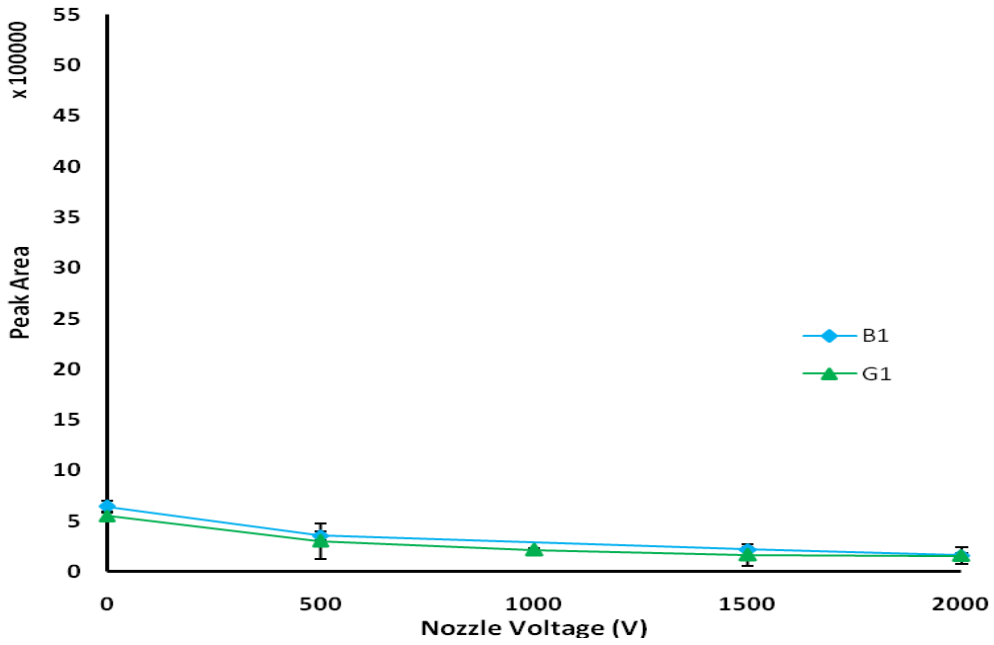


Figure 3.12: *Effects of the nozzle voltage on the chromatographic peak areas for both aflatoxins B1 and G1*

The results from these experiments show that at higher nozzle voltages the sensitivity for both aflatoxin B1 and G1 decreased from 6.42E+05 and 5.5E+05 to 1.63E+05 and 1.59E+05 respectively as the nozzle voltage increased from 0 to 2000 V (Figure 3.12). Hence, the nebulizer pressure and the nozzle voltage were set at 25 to 60 psi and 0 V, respectively.

3.3.3. Accurate mass measurements

In order to achieve accurate mass information of the molecular ion, the effect of the accuracy of the mass measurements of the selected aflatoxins was evaluated at different concentration levels in the range of 3-50 µg/L. In this way, the accurate mass information for the molecular ion was obtained. The LC-ESI-QTOF-MS/MS accurate mass spectrum of the four(4) aflatoxins B1, B2, G1 and G2 are shown in Figure 3.13 to Figure 3.16.

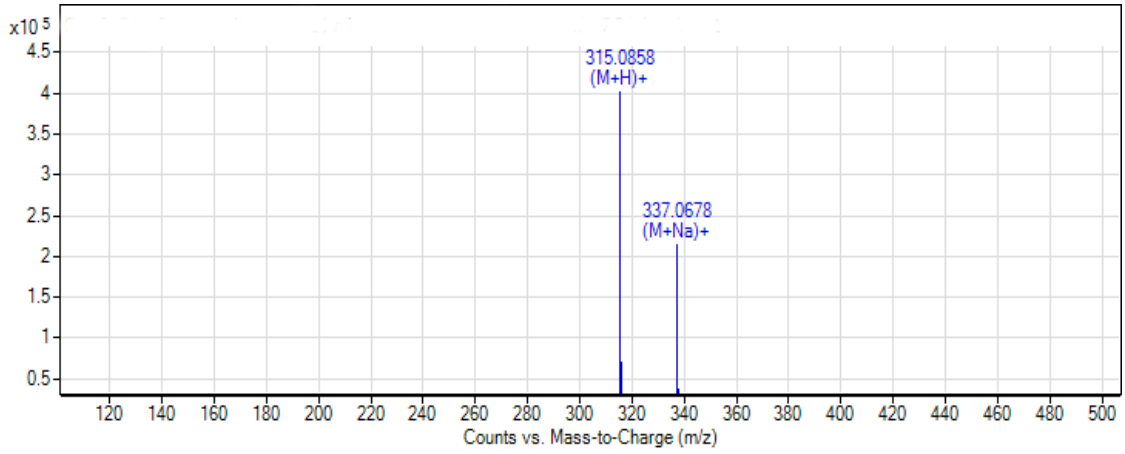


Figure 3.13: *Full scan ESI (+) production mass spectra of aflatoxin B1*

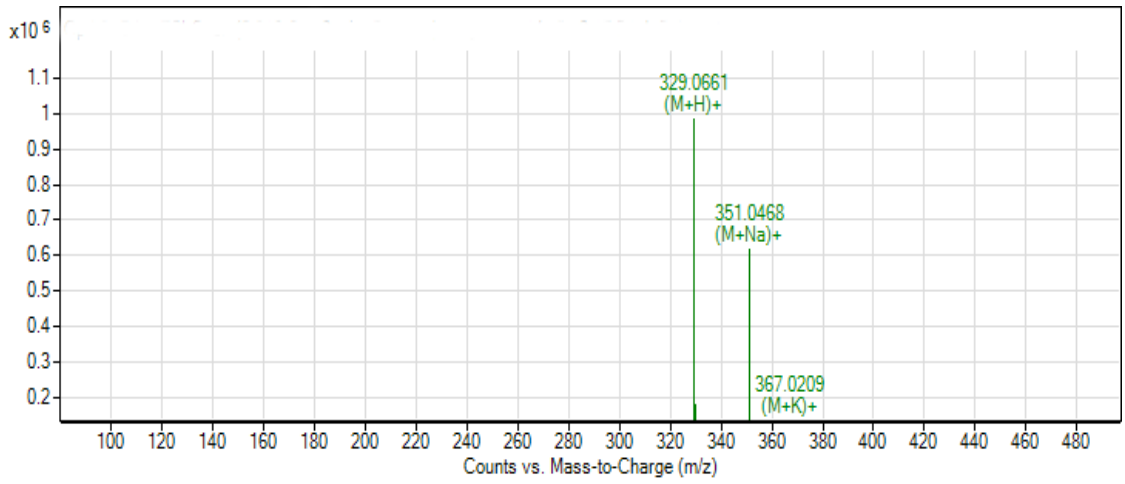


Figure 3.14: *Full scan ESI (+) production mass spectra of aflatoxin B2*

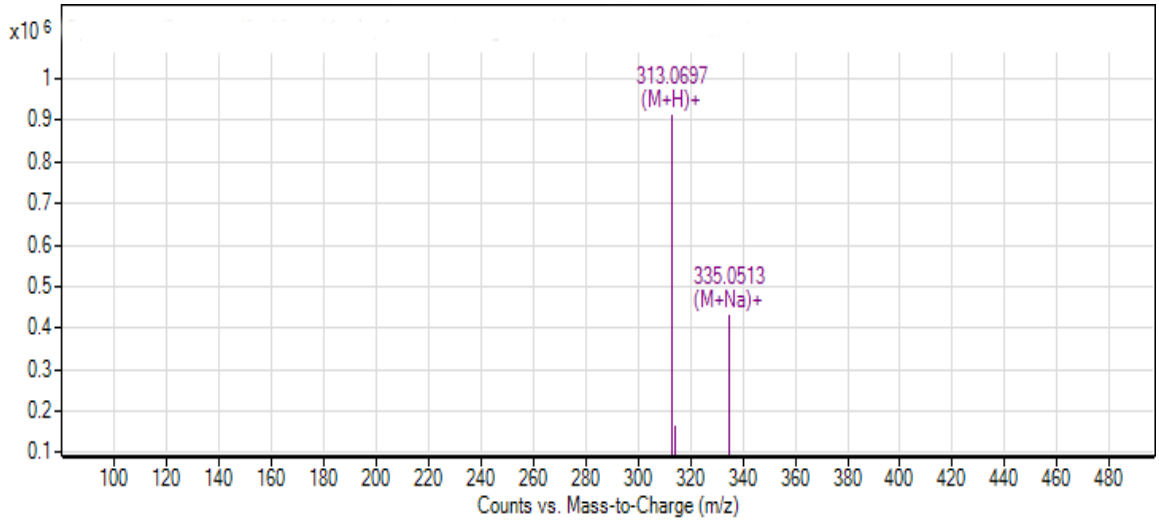


Figure 3.15: *Full scan ESI (+) production mass spectra of aflatoxin G1*

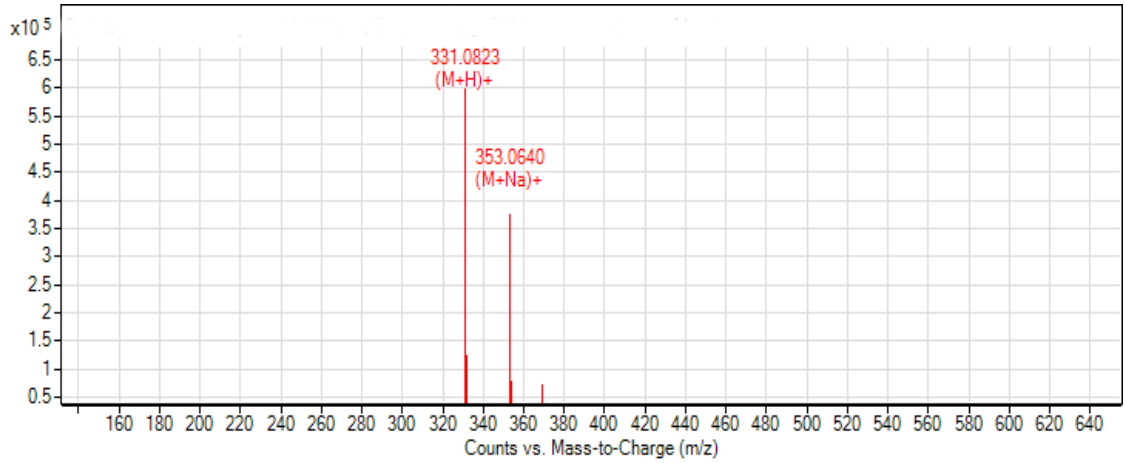


Figure3.16: *Full scan ESI (+) production mass spectra of aflatoxin G2*

The results for the determination of the mass accuracies of the molecule ion are as summarized in Table 3.1.

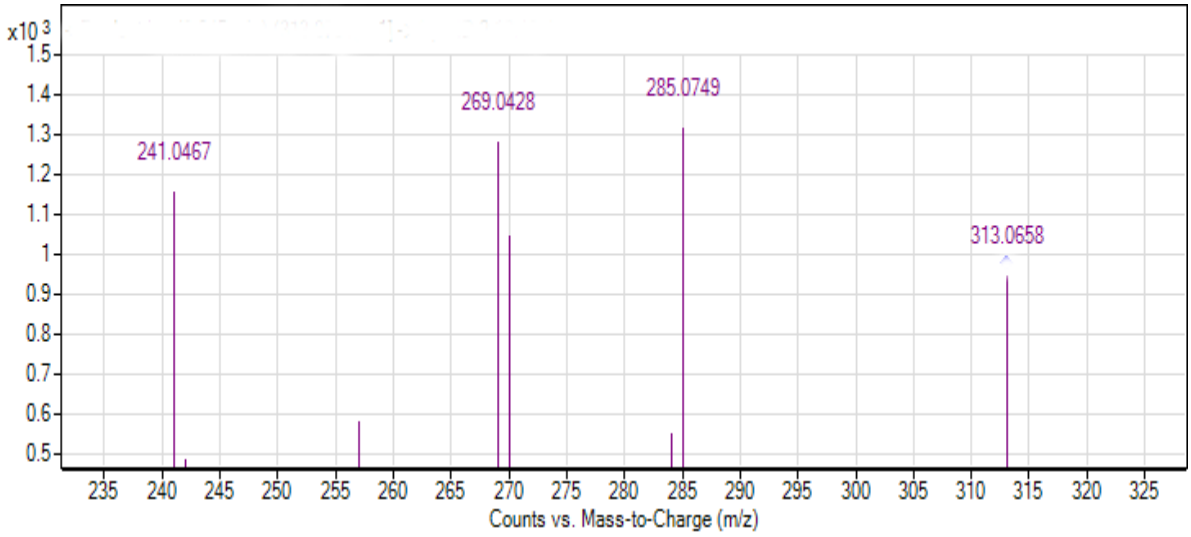
Table 3.1
Accurate mass information of the measured aflatoxins

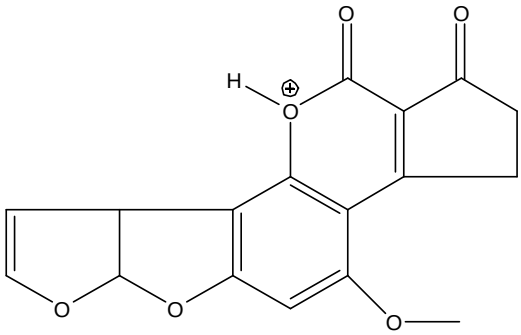
Aflatoxin	**Molecular Formula**	**Observed Mass** (m/z)	**Exact Mass** (m/z)	**Difference** (ppm)
B1	$C_{17}H_{12}O_6$	312.0634	312.0634	0.04
B2	$C_{17}H_{14}O_6$	314.0794	314.0790	1.06
G1	$C_{17}H_{12}O_7$	328.0583	328.0583	0.07
G2	$C_{17}H_{14}O_7$	330.0742	330.0740	0.80

It can be observed from Table 3.1 that, there were no significant differences in the mass accuracy between the observed mass and the exact mass, since the differences were between 0.04 ppm of aflatoxin B1 and 1.06 ppm of aflatoxin B2. Therefore, it can be deduced that the accurate mass measurements have capability for unmistakable confirmation of these analytes at different concentration levels.

3.3.4. Selection of product ions

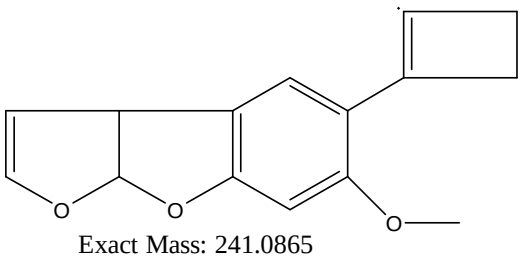
The selection of product ions was carried out by varying the collision energy between 5 and 45 V, at increments of 5 V. Figure 3.17 and Figure 3.20 show the chemical structures of aflatoxins B1, B2, G1, and G2, the corresponding molecular weights, and the main MS/MS fragmentations, with arrows indicating the formation of the product ions used in the quantification against the optimal collision energy. Two product ions were selected, the more intense as the quantifier ion and the other as the qualifier ion. Furthermore, the selection of the product ions was carried out for confirmation of the identity of the analyte.



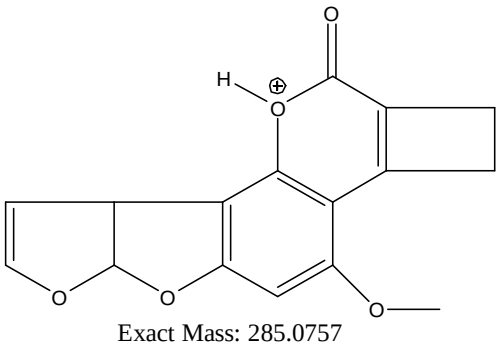


Chemical Formula: $C_{17}H_{13}O_6^+$
Exact Mass: 313.0707

Collision Energy 30 V

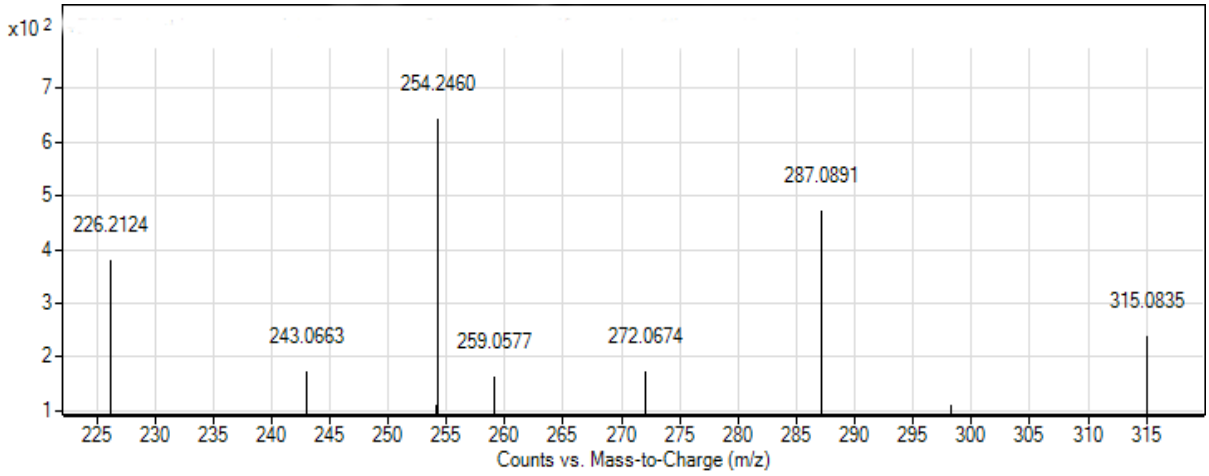


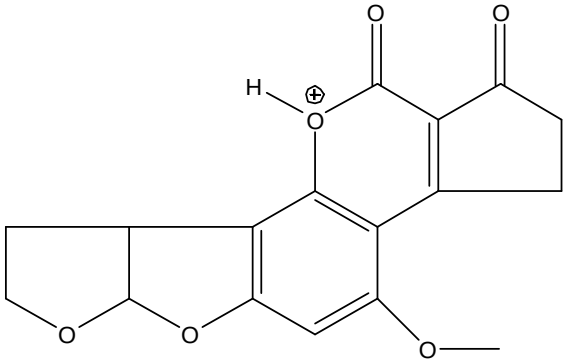
Exact Mass: 241.0865



Exact Mass: 285.0757

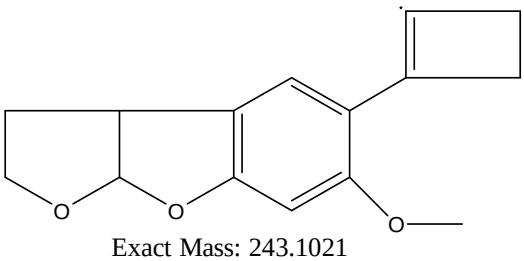
Figure 3.17: *The mass spectra and the proposed fragmentation scheme of aflatoxin B1*



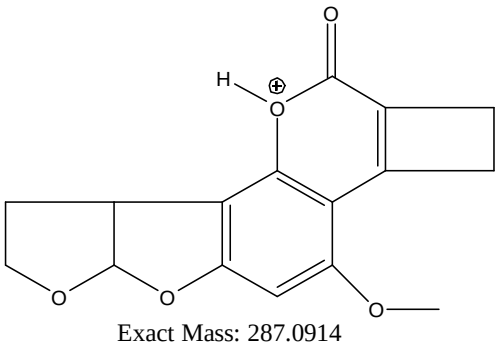


Chemical Formula: $C_{17}H_{15}O_6^+$
Exact Mass: 315.0863

Collision Energy 25 V

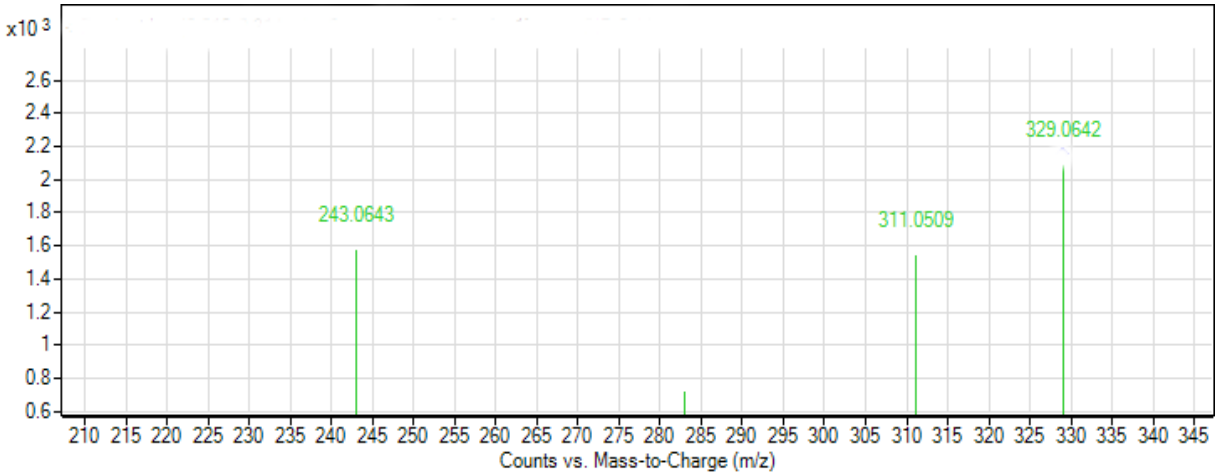


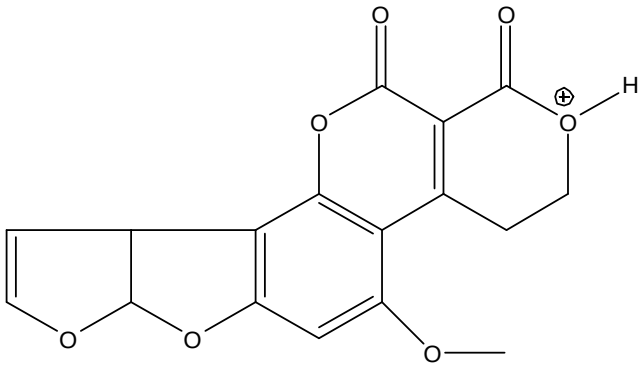
Exact Mass: 243.1021



Exact Mass: 287.0914

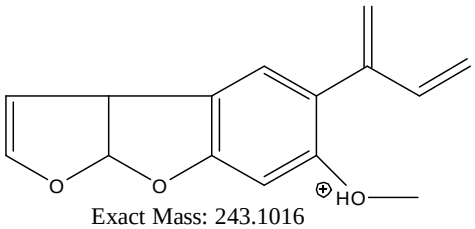
Figure 3.18: *The mass spectra and the proposed fragmentation scheme of aflatoxin B2*



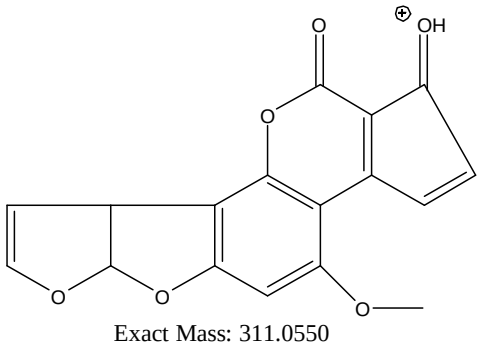


Chemical Formula: $C_{17}H_{13}O_7^+$
Exact Mass: 329.0656

Collision Energy 25 V

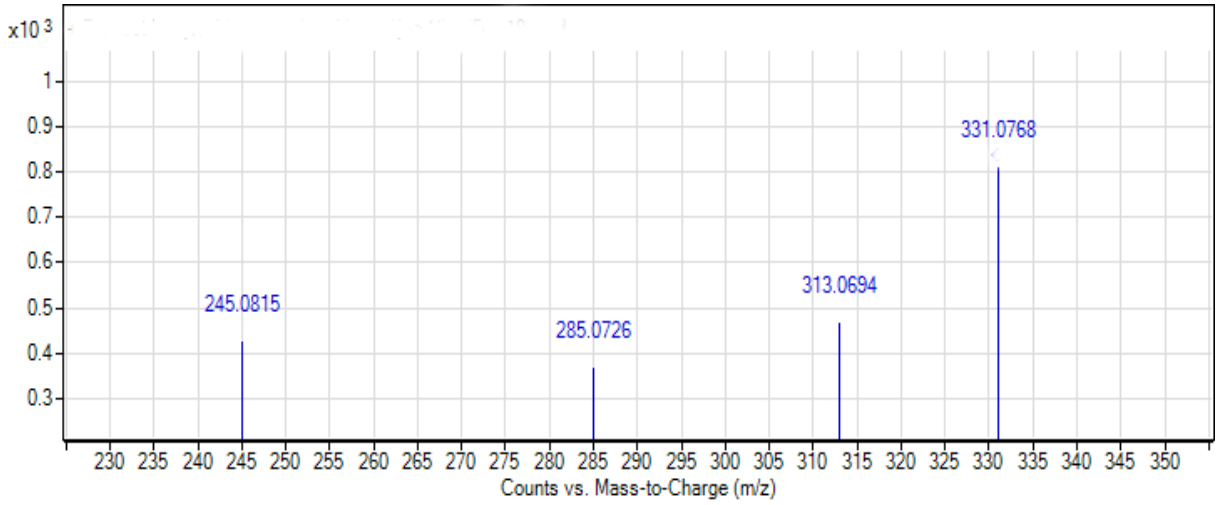


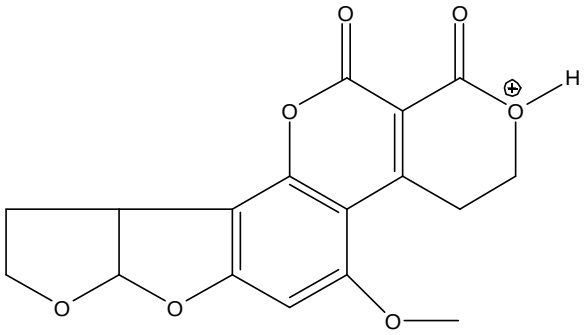
Exact Mass: 243.1016



Exact Mass: 311.0550

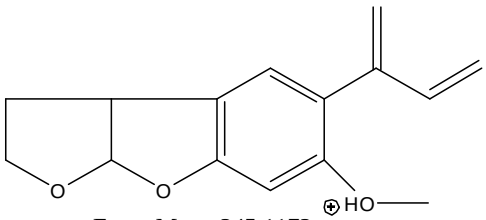
Figure 3.19: *The mass spectra and the proposed fragmentation scheme of aflatoxin G1*



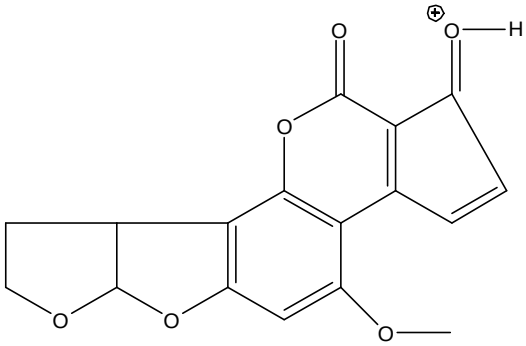


Chemical Formula: $C_{17}H_{15}O_7^+$
Exact Mass: 331.0812

Collision Energy 25 V



Exact Mass: 245.1172



Exact Mass: 313.0707

Figure 3.20: *The mass spectra and the proposed fragmentation scheme of aflatoxin G2*

3.3.5. Aflatoxin database table

An aflatoxin database table was created by injecting the four(4) aflatoxins in the LC-ESI-QTOF-MS/MS, working in the TOF-MS mode, at a concentration of 50 µg/L for aflatoxin B1 and G1 and from 15 µg/L for aflatoxin B2 and G2. This table can be continuously updated to extend the aflatoxin database table to include new mycotoxins.

Retention times add an extra degree of confidence in the identification of the unknown compound having the same empirical formula and the same exact accurate mass. The repeatability and reproducibility of the retention times as measured by their relative standard deviation (RSD) ranged from 0.8% to 1.2% and 2.6% to 5.1%, respectively.

By using the database table, the automatic screening method was carried out by investigating the retention time, the observed mass and the exact mass of each compound that were collected on a Microsoft Excel sheet. In this way, the analytes were confirmed by their retention time, the accurate mass measurements of the TOF analyzer and the product ions (Table 3.2).

Table 3.2
Aflatoxin database table

Molecular Formula	**Aflatoxin**	**Retention Time** (min)	**Observed Mass** (m/z)	**Calculated Mass** (m/z)	**Difference** (ppm)	**Product Ions** (m/z)
$C_{17}H_{12}O_6$	B1	3.82	312.0634	312.0634	0.04	285.0757 241.0863
$C_{17}H_{14}O_6$	B2	3.15	314.0794	314.0790	1.06	287.0914 243.1021
$C_{17}H_{12}O_7$	G1	2.51	328.0583	328.0583	0.07	311.0550 243.1016
$C_{17}H_{14}O_7$	G2	2.11	330.0742	330.0740	0.80	313.0707 245.1172

3.3.6. Sample pretreatment optimization

Other solvents which have been used previously were tested with respect to the extraction efficiency: chloroform (Liu, Gao, & Yu, 2006), acetonitrile: water (84:16, v/v) (Elbert, Czapiewski, Bujara, Kunze, & Giger, 2008), acetonitrile: water: acetic acid (79:20: 1, v/v) (Sulyok, Berthiller, Krska, & Schuhmacher, 2006), ethyl acetate: formic acid (Monbaliu *et al.*, 2009), methanol: water: formic acid (79:20:1, v/v), and methanol: water containing 0.2 g of sodium chloride (80:20, v/v) (Countryman, Huq, & Mathews, 2009).

The results indicate that a high extraction efficiency and good recovery of aflatoxins was obtained when a mixture of methanol: water (80:20, v/v) containing 0.2 g of sodium chloride was used. The dilution of the sample extracts is usually performed to reduce the matrix effects on the suppression or enhancement of ionization (Sulyok *et al.*, 2006). In this study, blank wheat extracts were fortified with 10 µg/kg of aflatoxin B1, G1 and 3 µg/kgof aflatoxin B2, G2. Then, the fortified extracts were diluted in the ratio of 1:1, 1:5 and 1:10

with the mobile phase together with an undiluted fortified extract. These extracts were then injected into the LC-MS instrument, analyzed, and their respective recoveries were compared (Figure 3.21).

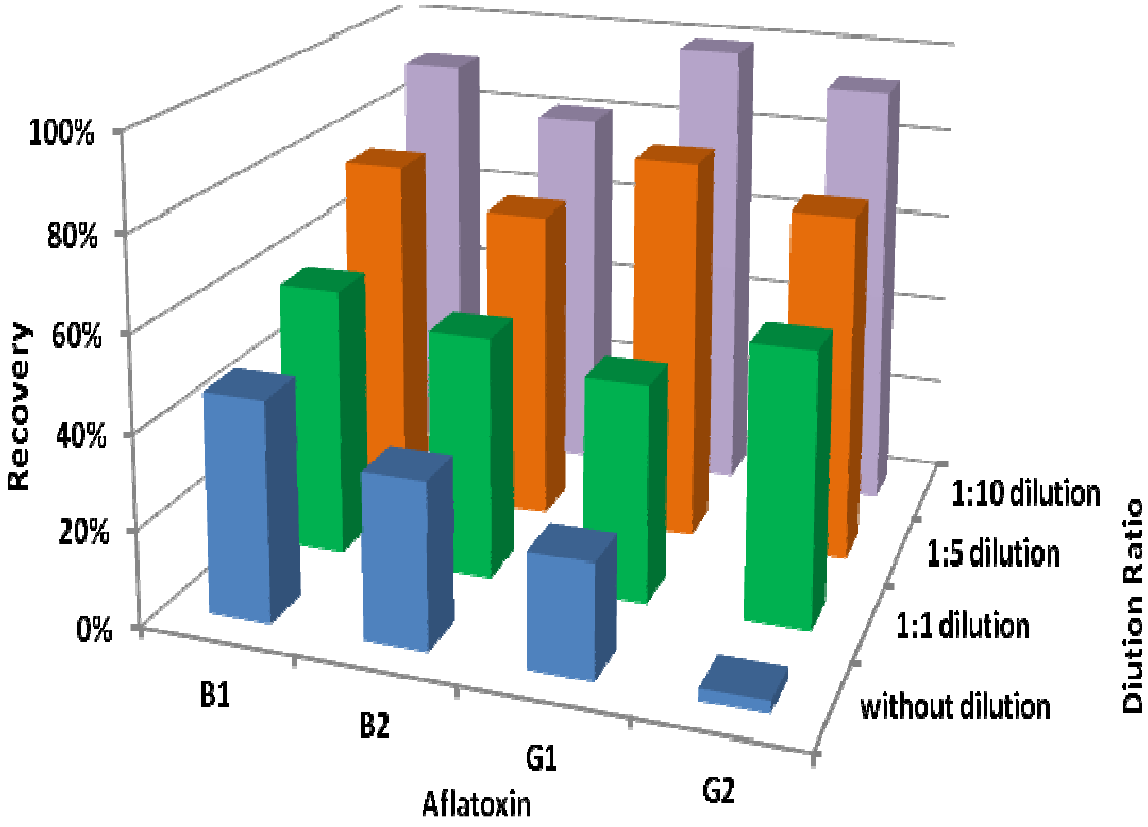
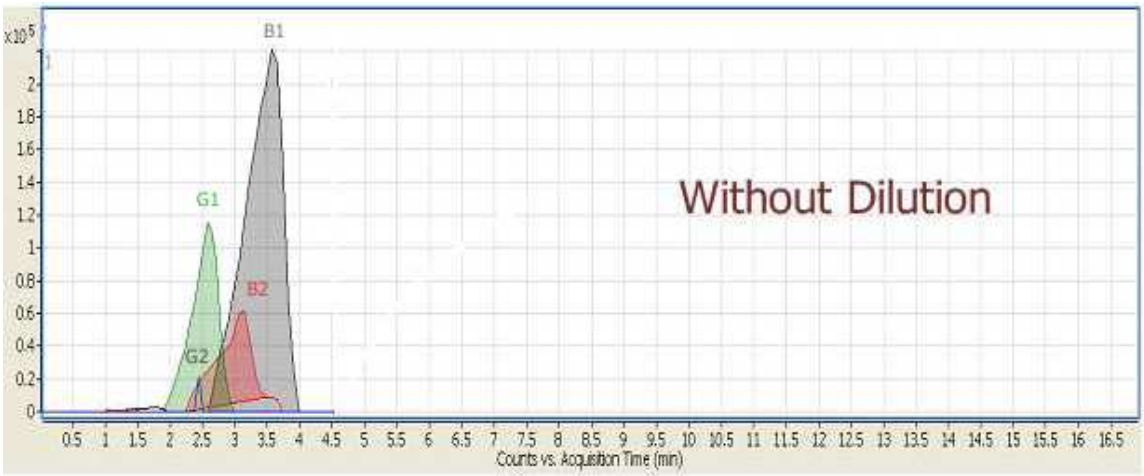
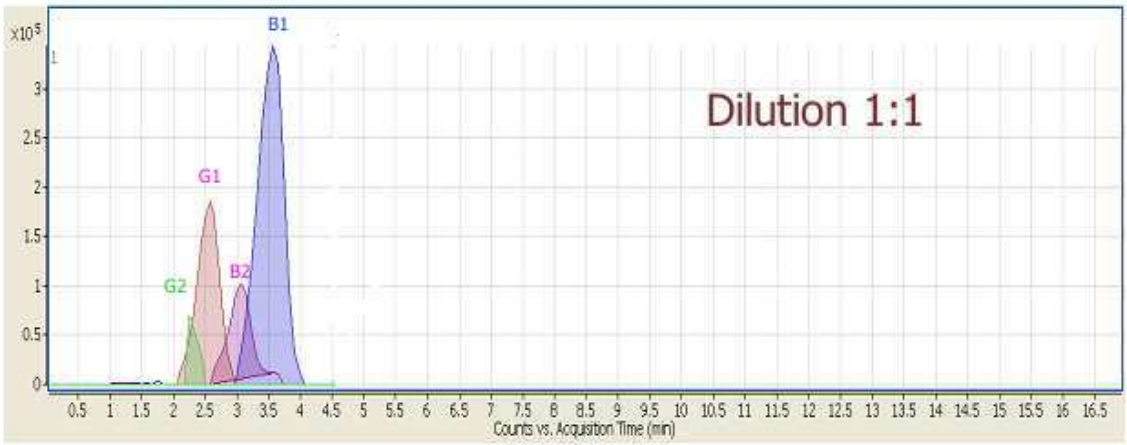
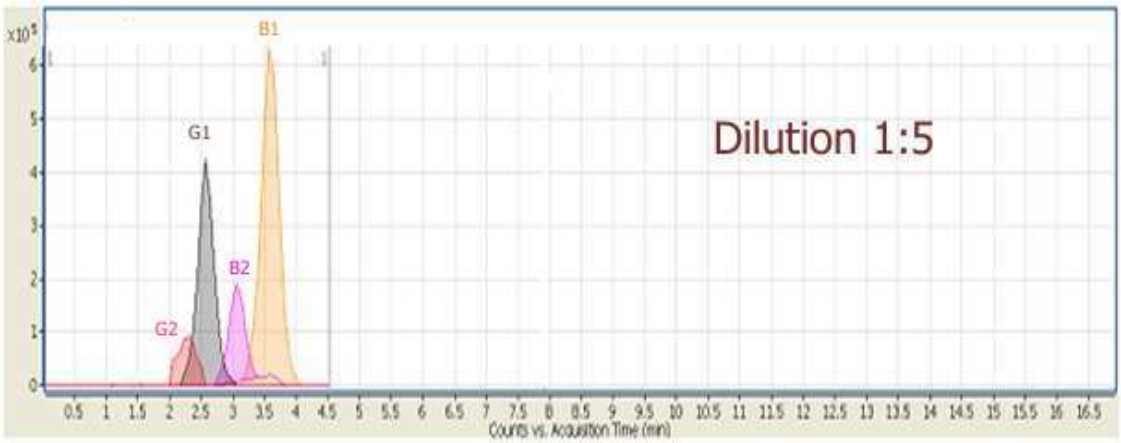


Figure 3.21: *Comparison of the percentage of recoveries of aflatoxin B1 and G1 spiked at 10 µg/kg and aflatoxin B2 and G2 at 3 µg/kg of wheat samples after dilution with different proportions of mobile phase*


B1
G1
B2
G2
Without Dilution
Counts vs. Acquisition Time (min)


B1
G1
B2
G2
Dilution 1:1
Counts vs. Acquisition Time (min)


B1
G1
B2
G2
Dilution 1:5
Counts vs. Acquisition Time (min)

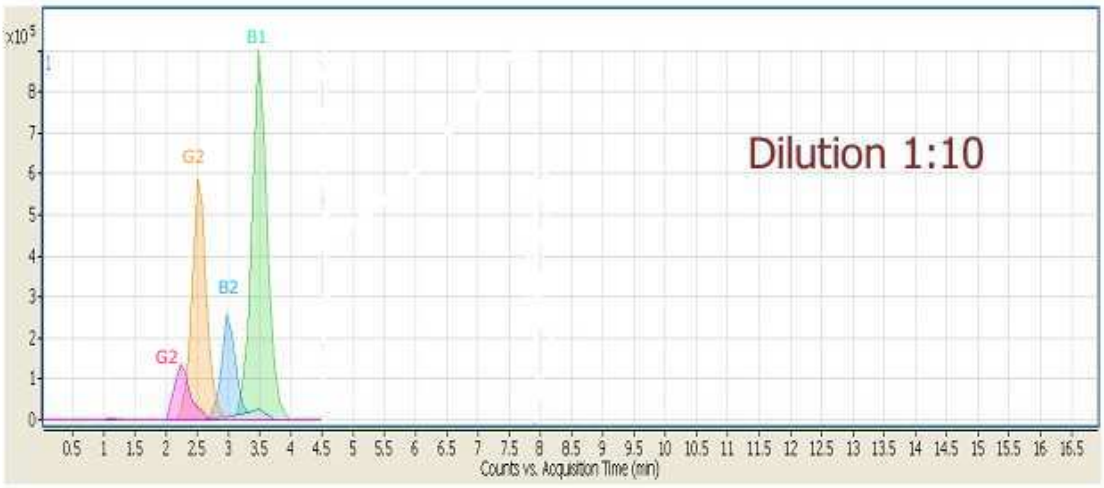


Figure 3.22: *Comparison of the chromatograms of aflatoxin B1 and G1 spiked at 10 µg/kg and aflatoxin B2 and G2 at 3 µg/kg of wheat samples after dilution with different proportions of mobile phase*

Figure 3.21 and Figure 3.22 showed that dilution of the sample extracts with the mobile phase by a ratio of 1:10 resulted in the removal of most of the matrix effects. Therefore, an external standard calibration can be applied in this procedure. Recoveries exceeding 80% were obtained for all the fortified extracts (Figure 3.21). Therefore, clean up procedures can be avoided thus saving time, effort and expenses with greater accuracy.

3.3.7. Method validation

This method was validated in terms of linearity, accuracy, intra-day precision, inter-day precision, limit of detection (LOD), and limit of quantification (LOQ). In order to verify the linearity, 7 concentrations of each aflatoxin was analyzed and the calibration curves were constructed as follows from 0.195 to 50 µg/kg for aflatoxin B1 and G1 and from 0.029 to 15.00 µg/kg for aflatoxin B2 and G2 using a least-squares regression analysis. The calibration curves were linear over the tested concentration range. The correlation

coefficients (r^2) of the calibration curves were greater than 0.998 for all the tested aflatoxins (Table 3.3).

Table 3.3
Linearity range, Equation, r^2 value and RSD of aflatoxins

Aflatoxin	Linearity Range (µg/kg)	Equation	r^2	RSD (%)
B1	0.195 – 50.000	y = 2345273.9615 * x + 028723.3146	0.9989	5.4
B2	0.029 - 15.000	y = 2310771.7336 * x + 302095.9501	0.9994	7.8
G1	0.195 – 50.000	y = 1795233.2615 * x + 877368.6504	0.9986	5.6
G2	0.029 - 15.000	y = 1887484.5475 *x - 70006.92280	0.9998	5.4

The accuracy of the results of this method is based on the recovery data. Recovery studies were carried out on different sample matrices. A mixed standard solution of aflatoxins was spiked into blank barley, wheat, soybean and corn samples. The samples were spiked in triplicates at three-fortification levels (9, 12, and 15 µg/kg for aflatoxin B1 and G1, and 2.7, 3.6, and 4.5 µg/kg for aflatoxin B2 and G2). The spiked samples were kept in a dark environment at room temperature overnight. Subsequently the fortified samples were ground and mixed at room temperature for 10 min, until a fine homogeneous powder was obtained. Then, 0.5 g of the ground fortified sample and 0.2 g of sodium chloride were weighed into a 250 mL glass flask followed by the addition of 40 mL of methanol: water (80:20, v/v). The mixture was stirred for three minutes at a high speed and filtered through a Whatman No. 1 filter paper. The filtrate was rinsed twice with 5 mL methanol. After that, the extracts were dried with anhydrous sodium sulfate and evaporated until dryness using a rotary evaporator at 45°C under vacuum. Finally, the residue was reconstituted with 0.5 mL methanol and diluted 10 times with the mobile phase (water containing 2 mM ammonium formate and 1% formic acid: methanol containing 1% formic

acid, 40%: 60%, v/v) and passed through a 0.2 µm disposable membrane filter prior to the LC-ESI-CID-MS/MS analysis. The obtained results are listed in Table 3.4.

Table 3.4
Mean of recoveries and RSDs of aflatoxins spiked into blank barley, wheat, soybean and corn samples at different spiking levels (n = 3)[a]

Aflatoxin	Spiking Levels (µg/kg)	Barley	Wheat	Soybeans	Corn
		Mean of Recovery (%) ± RSD (%)			
B1	9.0	82.40±10.11	105.00±8.45	98.54±13.85	108.15±10.06
	12.0	81.51±10.58	96.41±6.19	88.44±14.25	99.79±12.04
	15.0	83.61±11.49	93.25±9.76	80.61±10.06	82.82±14.58
B2	2.7	99.97±10.68	93.34±11.85	96.49±12.81	91.05±10.63
	3.6	87.01+16.06	88.39+8.64	83.79+13.71	87.98+13.56
	4.5	81.72+11.29	82.75+12.77	79.31+21.35	75.09+10.28
G1	9.0	105.55+11.49	84.88+14.91	81.77+13.35	105.63+11.69
	12.0	103.42+12.49	80.30+6.97	81.08+14.33	95.52+11.05
	15.0	100.44+12.13	85.02+13.71	81.55+10.98	83.28+11.03
G2	2.7	108.72+10.63	89.24+9.19	98.72+10.22	97.58+10.22
	3.6	82.87+14.66	100.63+10.95	74.02+14.60	105.40+15.08
	4.5	82.91+14.13	91.51+10.14	80.30+10.79	96.27+12.01

[a] n: is number of replicates

A high extraction efficiency was obtained by this method. The overall recoveries for aflatoxins were in the range of 74-108 % over the concentrations of 2.7, 3.6, 4.5 and 9 µg/kg for aflatoxin B2 and G2 and recoveries from 80-103 % were obtained for aflatoxin B1 and G1 at concentrations of 12 and 15 µg/kg. This fulfils the requirements established by the European Union for the determination of aflatoxin contaminants in food samples (European Commission, 2006a).

Intra-day precision and inter-day precision were evaluated by testing the RSD values of wheat samples spiked at 10 µg/kg with aflatoxin B1 and G1 and 3 µg/kg of aflatoxin B2 and G2. Five replicates for each concentration level were analyzed during each working day to test the intra-day precision and subsequently during five consecutive days

to test for inter-day precision. The intra-day precision and inter-day precision results for all aflatoxins in the wheat samples showed acceptable precision. The RSD values for the intra-day precision and inter-day precision were below 12.0% and 18.0 %, respectively (Table 3.5).

Table 3.5
The intra-day precision and inter-day precision of the aflatoxins expressed as RSD

Aflatoxin	Spiking Levels (µg/kg)	Intra-Day Precision (n = 5)[a]	Inter-Day Precision (n = 15)[a]
B1	10	6.9	11.4
B2	3	11.6	17.1
G1	10	6.9	10.1
G2	3	10.9	15.0

[a] n: number of replicates

The lowest concentration of aflatoxins standards producing a response of 3:1 signal to noise (S/N) ratio was calculated and considered as the LOD, while the LOQ was determined as the lowest concentration of the aflatoxin standards giving a response of 10:1 signal to noise (S/N) ratio (Table 3.6). Overall, the LOQ values obtained were lower than the maximum residue level (MRL) set by the EU which are 2 µg/kg for aflatoxin B1 and 4 µg/kg for total aflatoxins in cereals for human consumption (European Commission, 2006b).

Table 3.6
LOD and LOQ of aflatoxins

Aflatoxin	LOD (µg/kg)	LOQ (µg/kg)
B1	0.117	0.391
B2	0.141	0.469
G1	0.176	0.586
G2	0.211	0.703

This indicates the suitability of the proposed method for the determination of trace concentration of these aflatoxins in cereals with a high degree of confirmation, using the retention time, the accurate mass measurements of the TOF analyzer and the products ions, thus avoiding false-positive results.

3.3.8. Sample analysis

The developed method was applied to the determination of four aflatoxins in 28 samples of cereals (barley, wheat, soybeans and corn), which were randomly obtained from groceries and stores in Kuala Lumpur, Malaysia. None of the aflatoxins selected in this study were detected in these randomly collected samples (Table 3.7).

Table 3.7
Occurrence of aflatoxins in cereals and cereal products samples in the month of December 2010

Sample category	Total Number of Samples of the Particular Category	Number of Positive Samples	Incidence (Positive/Total Number of Samples of the Particular Category)	Incidence (Positive/ Number of Samples)	Aflatoxin (µg/kg)
barley	7	0	0.0%	0.0%	–[a]
Wheat	7	0	0.0%	0.0%	–[a]
Soybeans	7	0	0.0%	0.0%	–[a]
Corn	7	0	0.0%	0.0%	–[a]
Total	**28**	**0**	**0.0%**	**0.0%**	

[a]: Values were below the LOD of the adopted method

3.4. Conclusion

In this study, a simple, quick, and confirmatory method has been developed for the determination of aflatoxins in cereals. The use of ESI-CID-MS/MS analysis measured with

a hybrid QTOF-instrument uniquely offers the possibility of providing accurate mass data and can generate structural information on the analytes with minimal sample treatment and without derivatization. The sensitivity of the ESI interface could be significantly enhanced by optimizing the chromatographic conditions and the fragmentor voltage in the interface. Other factors such as the drying gas temperature and flow, sheath gas temperature have very little effects. By using the aflatoxin database table, aflatoxin were confirmed by their retention time, the accurate mass measurements of the TOF analyzer and the product ions, thus avoiding false-positive results. The results show that the extensive and expensive clean-up procedures required to reduce the matrix effects that cause ionization suppression could be avoided by dilution of the sample extracts at a 1:10 level with the mobile phase and analyzing by selected ion monitoring (SIM) in the MS detection. Excellent linearity, high recoveries, acceptable intra-day precision and inter-day precision with the LOQ values lower than the stipulated MRL were achieved indicating the suitability of the proposed method for the determination of aflatoxins in cereals.

4. SIMULTANEOUS DETECTION OF TYPE A AND TYPE B TRICHOTHECENES IN CEREALS BY LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION QUADRUPOLE TIME OF FLIGHT MASS SPECTROMETRY (LC-ESI-QTOF-MS/MS)

4.1. Introduction and scope of the work

Trichothecenes belong to a group of mycotoxins, which are produced by the *Fusarium* moulds. The primary source of trichothecene contamination in food and feedstuff is cereal commodities (maize, oats, barley and wheat) which have been infected by the *Fusarium* fungi (Lattanzio *et al.*, 2009; WHO, 2001; Zöllner & Mayer-Helm, 2006). A total of 190 different structures have been discovered, all sharing a common tetracyclic, sesquiterpenoid 12, 13-epoxytrichothec-9-ene ring system. They fall into four distinct groups namely from A through D, and characterized by specific structural features. Type-A trichothecenes include T-2 (T-2), HT-2 toxins (HT-2) and diacetoxyscirpenol (DAS). Type-B trichothecenes include deoxynivalenol (DON), nivalenol (NIV), fusarenon X (FUSX), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). Type-A and type-B trichothecenes exhibit acute toxicity, and when consumed, can result in vomiting and loss of appetite; while high concentrations of type-B trichothecenes can cause chronic intoxication, leading to extensive haemorrhage, and subsequent haematological toxicities (Berthiller *et al.*, 2005). In view of their toxicity and frequent occurrence, several countries have established regulations or recommendations for the control of DON, HT-2 and T-2 in food. The Food and Drug Administration (FDA) in the USA recommends maximum contamination levels of 1000 µg/kg for cereal products meant for human consumption, while the EU countries have set standards of between 100 and 1000 µg of DON per

kilogram for food, and between 400 and 5000 µg/kg for feedstuff (Zöllner & Mayer-Helm, 2006).

Sample preparation is often the most important part in the analysis of most mycotoxins, and relies largely on the physiochemical properties of the commodities that are contaminated with mycotoxins. Clean-up methods that are usually used for toxin isolation include solid-phase extraction (SPE) with a mixture of various solid phases such as charcoal–alumina–Celite 545, cation exchange resin, alumina–charcoal, in addition to multifunction column such as MycoSep # 225 and #227 column (Valle-Algarra *et al.*, 2005). Unfortunately, most of the methods applied for extracting these toxins are either time-consuming, such as SPE, or expensive and matrix-dependent, such as multifunction column. To simplify procedures for the sample preparation and to reduce the cost of analysis, the QuEChERS procedure has been adopted, since it is a quick and economical way to extract food contaminants. The most common methods for determining trichothecenes include gas chromatography (GC) with an electron capture detector (ECD) (Valle-Algarra *et al.*, 2005), gas chromatography coupled with mass spectrometry (GC-MS) (Ibáñez-Vea, Lizarraga, & González-Peñas, 2011). Recently, liquid chromatography coupled with mass spectrometry (LC–MS) and liquid chromatography with tandem mass spectrometry (LC–MS/MS) have become common (Dall'Asta, Sforza, Galaverna, Dossena, & Marchelli, 2004). It is easily amenable to the polar type-B trichothecenes and non-polar type-A trichothecenes without the need for derivatization.

The aim of this study is to design a simple, fast, economical and effective method of extracting the sample using the QuEChERS technique in addition to the implementation and validation of the optimized LC-ESI-QTOF-MS/MS method for a reliable, precise and accurate quantification and confirmation of the type-A and type-B trichothecenes in cereal and cereal products.

4.2. Experimental

4.2.1. Reagents and materials

Trichothecenes standards of NIV, DON, 15-AcDON, 3-AcDON, FUS X, DAS, HT-2 and T-2 at 100 mg/kg concentration were obtained from R-BIOPHARM (Deisenhofen, Germany). The standard stock solutions were all dissolved in 2 mL of acetonitrile. Subsequently, daily working standard solutions were prepared by diluting the stock solutions in water containing 5 mM ammonium acetate and methanol containing 5 mM ammonium acetate 50:50 (%, v/v). Glacial acetic acid (HPLC-grade) was purchased from Fisher Scientific (UK). Ammonium acetate, HPLC-grade, was purchased from Fluka (Darmstadt, Germany). Sodium chloride was purchased from BDH PROLABO (EC Countries). Anhydrous magnesium sulfate ($MgSO_4$) for QuEChERS was purchased from Agilent Technologies (USA). Water was purified by reverse osmosis with an electrodeionization (EDI) system (Maxima Ultra Pure Water, England). Methanol and acetonitrile, HPLC-grade, were obtained from Merck (Darmstadt, Germany). A nonsterile PTFE Syringe Filter with disposable membrane filter (0.22 μm) was purchased from Membrane Solutions (USA).

4.2.2. Sample preparation

Cereal samples were prepared in the following steps:

Step I: A thoroughly homogenized cereal sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 mL).

Sample recovery was done with (1.0 g) of the blank wheat samples at two different fortification levels; 0.5 mL of the trichothecene mixed standards were spiked at 100.0 and 500.0 µg/kg of the standard mix. The spiked samples were left overnight in the dark at

room temperature to allow the solvent to evaporate and for trichothecene absorption into the matrix. Then they were extracted via the following steps (II to IV).

Step II: 3.0 mL of 79:20:1 (%, v/v) acetonitrile/water/acetic acid mixture was added and the centrifuge tube was shaken for 1 min to ensure that the solvent has mixed thoroughly with the entire sample, for complete extraction of the analyte.

Step III: 0.8 g of anhydrous $MgSO_4$ and 0.2 g of NaCl were added into the mixture and the shaking procedure was repeated for 1 min to facilitate the extraction and partitioning of the eight trichothecenes into the organic layer.

Step IV: The extract was centrifuged for 5 min at 4000 rpm, and 0.5 mL of the upper organic layer was filtered through a 0.22 µm nylon syringe filter prior to LC-ESI-QTOF-MS/MS analysis.

4.2.3. Analytical procedure

The quantification of the analytes was carried out by performing low-energy collision induced tandem mass spectrometry (CID-MS/MS) using the multiple reaction monitoring (MRM) mode. The quantification of the trichothecenes were performed using an Agilent 6530 Q-TOF-MS/MS spectrometer coupled with an Agilent 1200 Series HPLC system. The HPLC system consists of a vacuum degasser (G1379B) with a thermostatted autosampler (G1330B) and binary pump (G1312B), and a thermostatted column compartment (G1316B). The QTOF-MS/MS instrument was equipped with an ESI source and the Agilent jet stream dual nebulizer.

4.2.4. Instrument conditions

The injection volume was fixed at 5 µL for the samples and standards. The sample extract was injected into a ZORBAX Eclipse XBD-C_{18}, 2.1 mm × 100 mm, 1.8 µm (P.N. 928700-

902) column at a flow rate of 0.2 mL/min running with 70% and 30% mobile phase A and B respectively. Mobile phase A consists of 1% acetic acid and 5 mM ammonium acetate in water and mobile phase B consists of 1% acetic acid and 5 mM ammonium acetate in methanol. The gradient was changed to 80% mobile phase B over 10 min, and then maintained for 3 mins. After 13 min of run time, the gradient was returned to 30% mobile phase B over 1 min. The column was washed for 6 min with water and conditioned for 4 min before the next injection. The mass spectrometer was operated in the positive and negative ESI mode. In this method, there were two periods when different polarities (0-10 min negative; 10-14 min positive) were employed. The optimum QTOF-MS/MS operating conditions are as follows: the drying gas and sheath gas temperatures were set at 150^{o}C and 350^{o}C, respectively, while the drying gas and sheath gas flow rates were set at 5 L/min and 12 L/min, respectively. The nebulizer pressure was set at 25 psi and the nozzle voltage was set at 0 V. Finally, the capillary voltage was set at 3500 V, while the fragmentor voltage was set at –60 V in the negative mode and +160 V in the positive mode.

4.2.5. Food samples

In the month of December 2010, 1-2 kg each of 25 samples of cereals and cereal products (wheat, wheat based noodles, rice, rice based noodles and corn) was obtained from groceries and stores in Kuala Lumpur, Malaysia. The samples were stored in the dark at room temperature (25–30^{o}C). The samples were ground and mixed at room temperature for 10 min until a fine and homogeneous powdered material was obtained. The powdered samples were then stored in plastic bags at 4^{o}C in a refrigerator prior to analysis

4.3. Results and discussion

4.3.1. QuEChERS method development

The preparation of the extract has a crucial impact on the accuracy of the results and there are several factors, which can affect performance in the original QuEChERS method such as the composition of the extraction solvent, the type and amount of the drying agents, extraction time and the dilution factor. All of these factors were taken into consideration and then optimized. The extraction solvent was found to be the most important factor and it heavily affects the extraction efficiency. However, the physicochemical properties of type-A and type-B trichothecenes are different, with the type-A trichothecenes being relatively non-polar compounds and the type-B trichothecenes are polar compounds. Hence, the selection of a suitable extraction solvent for both types of trichothecenes is difficult. From a review of the literature, six different extraction mixtures were tested in blank wheat samples spiked with 500 µg/L of the trichothecenes standards. The results using all these extraction solvents were then compared.

1- Extraction solvent 1: A mixture containing 79:20:1 (%, v/v) acetonitrile/ water/ acetic acid was employed (Sulyok, Krska, & Schuhmacher, 2010).
2- Extraction solvent 2: A mixture of 57:42.5:0.5 (%, v/v) acetonitrile/ water/ acetic acid was employed.
3- Extraction solvent 3: A mixture of 79:20:1 (%, v/v) methanol / water/ acetic acid was employed.
4- Extraction solvent 4: A mixture of 85:15 (%, v/v) methanol/acetonitrile was employed (Sospedra *et al.*, 2010).
5- Extraction solvent 5: A mixture of 20/80 (%, v/v) methanol/acetonitrile was employed
6- Extraction solvent 6: A mixture of 80/20 (%, v/v) methanol/water was employed.

The results as shown in Table 4.1 demonstrate that 57:42.5:0.5 (%, v/v) acetonitrile/ water/ acetic acid and 85:15 (%, v/v) methanol/acetonitrile combinations present good recoveries for relatively non-polar type-A trichothecenes, but not with the polar type-B trichothecenes such as NIV and DON. However, 79:20:1 (%, v/v) acetonitrile/water/acetic acid offers recoveries that are satisfactory for polar and non-polar analytes. In addition, it gave recoveries fulfilling the EU Commission Directive 2006/401/EC for analysis of mycotoxins in food samples (European Commission, 2006a). Therefore, mixtures of 79:20:1 (%, v/v) acetonitrile/ water/ acetic acid were applied in this study.

Table 4.1
Assessment of different extraction solvents in wheat (1.0 g) samples spiked at 500 µg/kg level (n = 5)[a]

Extraction Solvent	Trichothecene						
	NIV	DON	FUS X	ADON	DAS	HT-2	T-2
	Mean of Recovery (%) ± RSD (%)						
79:20:1 (%, v/v) acetonitrile/ water/ acetic acid	90.0±10.3	82.3±5.3	101.6±16.3	85.3±9.7	95.0±7.0	85.6± 9.8	96.4± 6.2
57:42.5:0.5 (%, v/v) acetonitrile/ water/ acetic acid	40.9±10.7	32.8± 11.2	90.7 ± 10.3	96.1 ± 13.8	97.5 ± 9.1	82.5 ± 15.2	81.8 ± 8.5
79:20:1 (%, v/v) methanol / water/ acetic acid	–[b]	–[b]	37.3 ± 12.7	–[b]	–[b]	–[b]	–[b]
85:15 (%, v/v) methanol/acetonitrile	57.0± 13.6	44.2± 8.7	84.0 ± 6.3	100.6 ± 9.5	73.7 ± 13.7	97.3 ± 12.3	79.1 ± 14.6
20/80 (%, v/v) methanol/acetonitrile	85.9± 8.2	–[b]	59.3 ± 12.2	80.5 ± 11.2	82.4 ± 6.7	74.7 ± 10.0	94.3 ± 5.9
80/20 (%, v/v) methanol/water	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]

[a] n: is number of replicates
–[b]: mean recovery value is <25%

4.3.2. Optimization of the Liquid chromatography (LC) conditions

Besides improving the resolution in the chromatographic system, the selection of the mobile phase based on consideration of the ionization efficiency by the ESI source, it would also give high sensitivity. By using the ESI source, solvents can greatly influence the generation of small droplets, which leads to increased evaporation of solvents and so enhance the sensitivity. The use of methanol instead of acetonitrile in the solvent mixtures resulted in better ionization and higher intensities for all analytes, probably due to the proton affinity of some of the isomeric forms of acetonitrile generated, especially for DON, T-2 and HT-2.

The effects of acetic acid, formic acid, ammonium acetate and ammonium formate on the analyte response were tested by using various combinations. After addition of either ammonium acetate or acetic acid, no significant increase in sensitivity was observed. On the other hand, the addition of a mixture of acetic acid (1%) and ammonium acetate to the mobile phase, led to a significant increase in the response for all the targeted analytes, due to an improvement in the peak shape and better ionization. As for the choice between using a mixture of ammonium acetate/acetic acid (1%) and ammonium formate/formic acid (1%), the results indicated that when ammonium formate was chosen, the ionization of all the selected analytes was significantly reduced. Whereas, the use of ammonium acetate at a concentration of 5 mM and acetic acid (1%), was found to be sufficient to generate ammonium $[M+NH_4]^+$ or sodium $[M+Na]^+$ adduct ions in the positive mode for the type-A trichothecenes and bromo $[M+Br]^-$, acetate $[M+CH_3COO]^-$ or chloro $[M+Cl]^-$ adduct ions in the negative mode for the type B-trichothecenes. At higher concentrations (≥10 mM), ionization suppression was observed.

The mass spectra of the type-B trichothecenes (Figure 4.1 to Fig 4.4) showed the base peak of the bromo adduct ion $[M+Br]^-$ (NIV of m/z value 391.0396), acetate adduct

ion [*M*+CH3COO]$^-$ (DON of m/z value 355.1403 and FUS X of m/z value 413.1446) or chloro adduct ion [*M*+Cl]$^-$ (ADON of m/z value 373.1062), while those of type-A trichothecenes (Figure 4.5 to Fig 4.7), on the other hand, showed the base peak of the sodium adduct ion [*M*+Na]$^+$ (DAS of m/z value 389.1571) and ammonium adduct ion [*M*+NH_4]$^+$ (HT-2 of m/z value 422.2435 and T-2 of m/z value 484.2534).

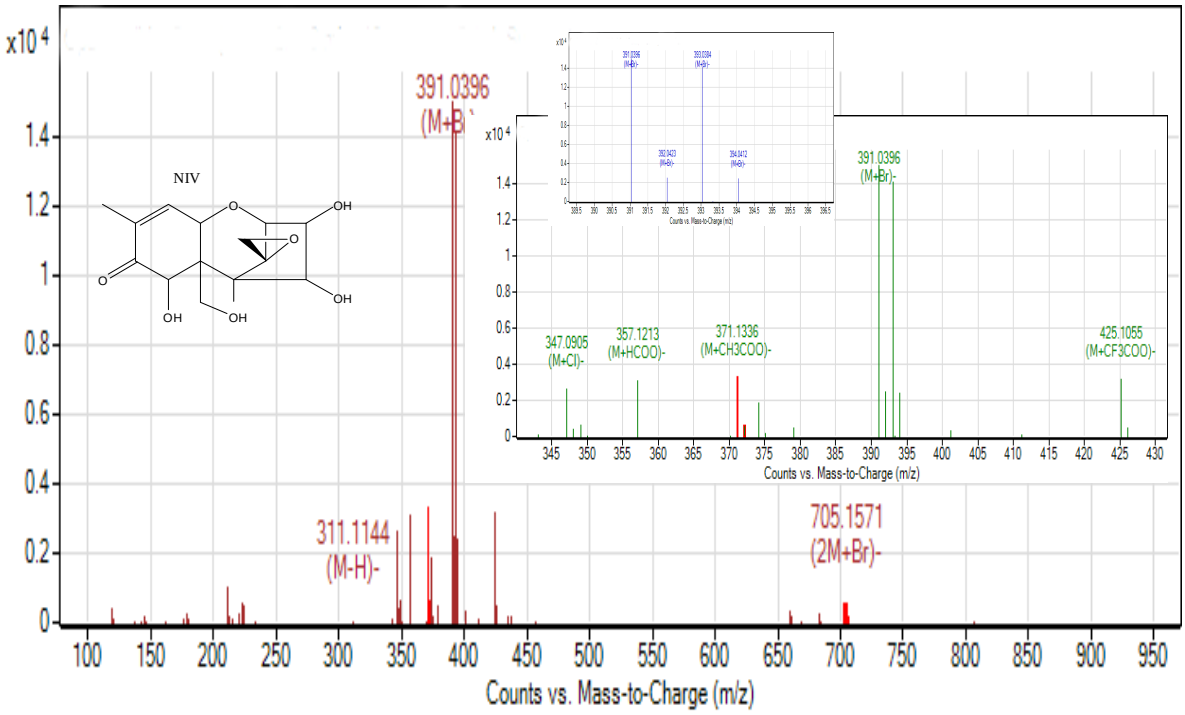


Figure 4.1: *Full scan ESI (-) production mass spectra and the structures of NIV*

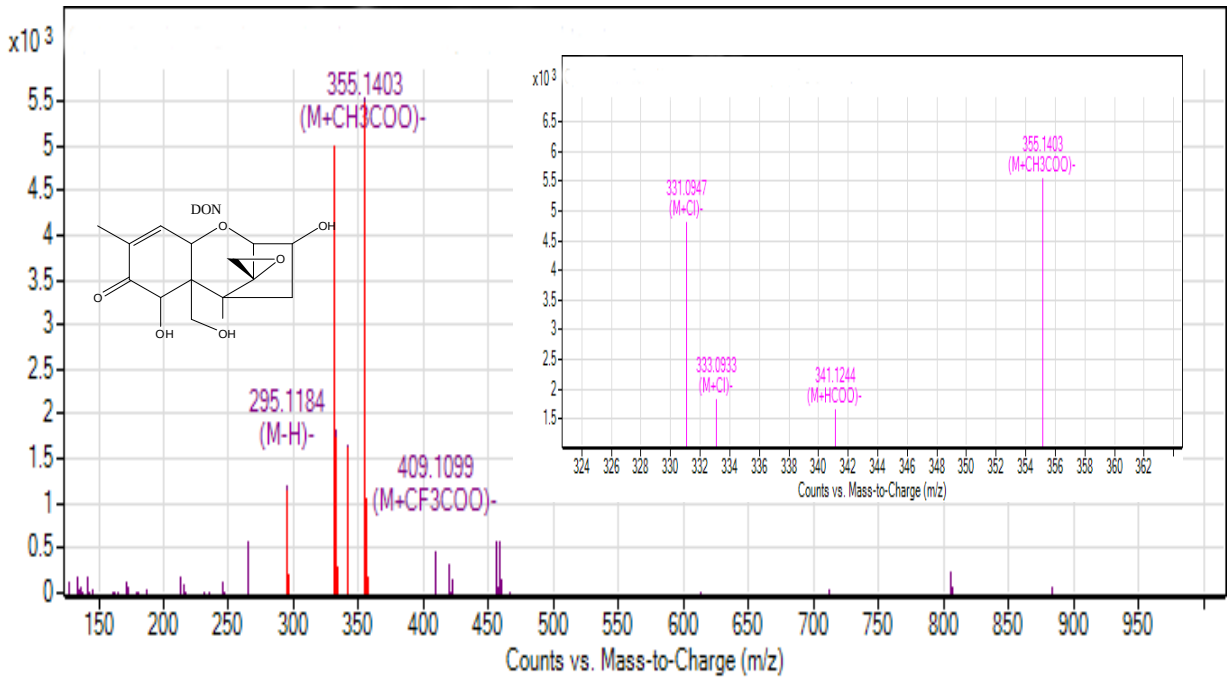


Figure 4.2: *Full scan ESI (-) production mass spectra and the structures of DON*

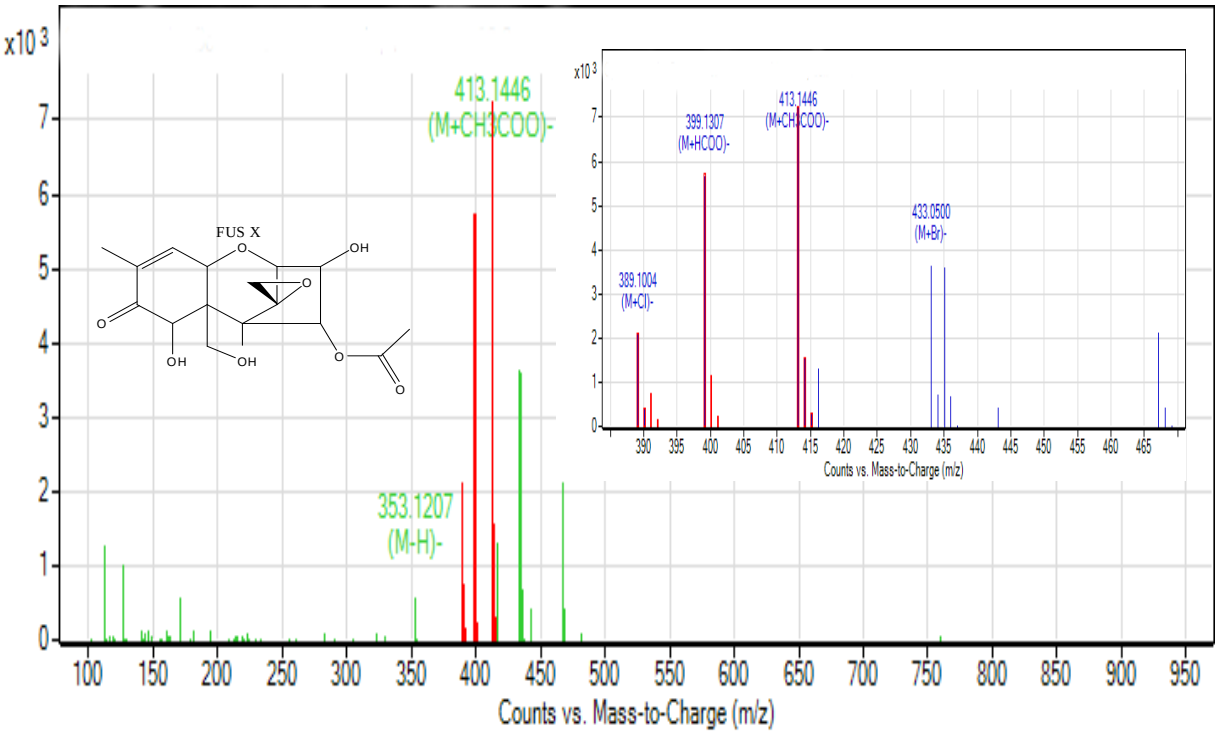


Figure 4.3: *Full scan ESI (-) production mass spectra and the structures of FUS X*

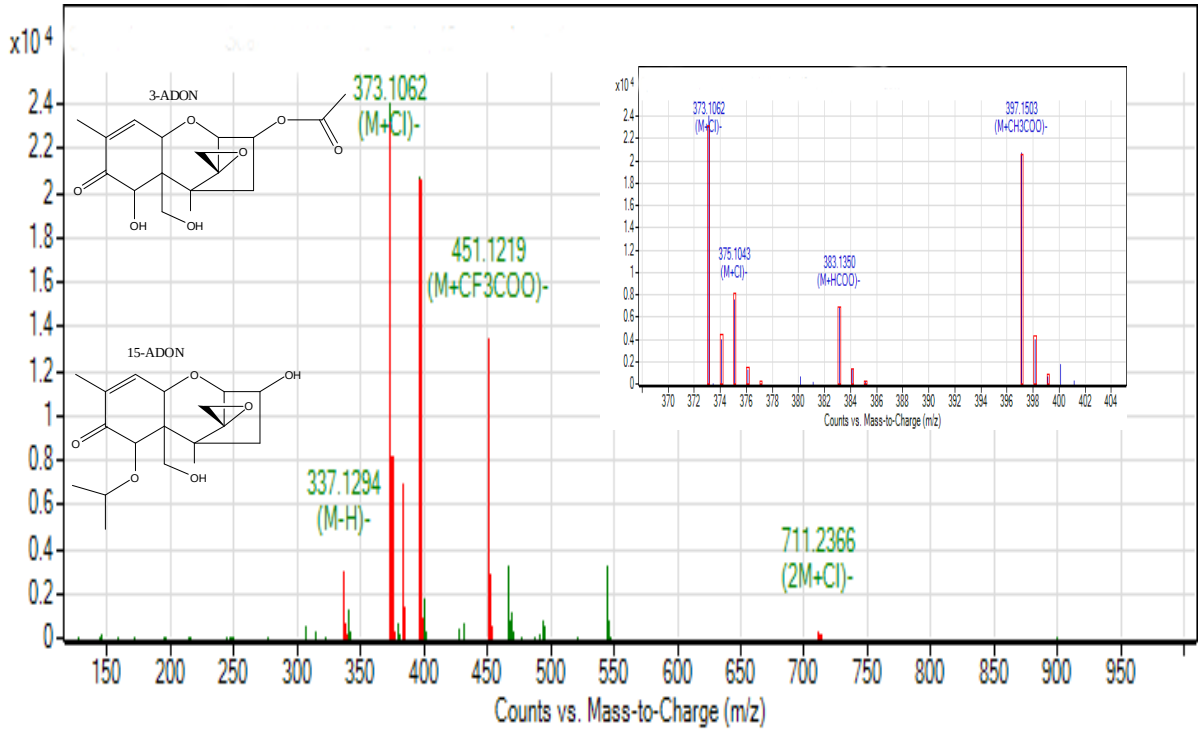


Figure 4.4: *Full scan ESI (-) production mass spectra and the structures of 3-ADON and 15-ADON*

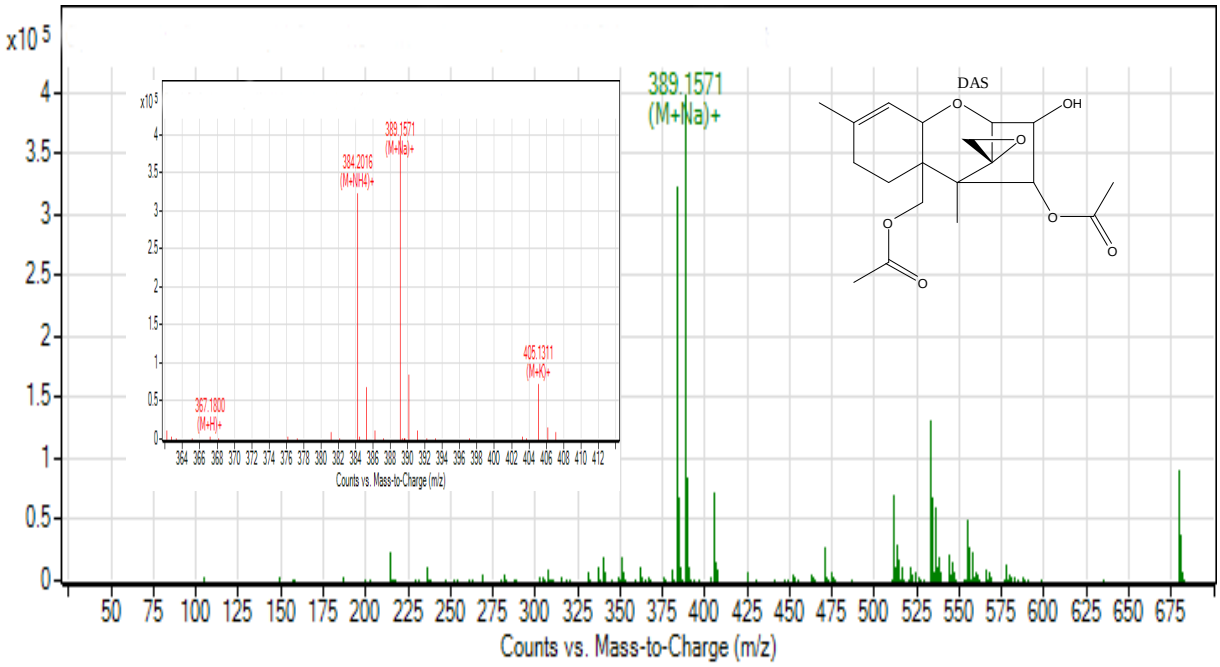


Figure 4.5: *Full scan ESI (+) production mass spectra and the structures of DAS*

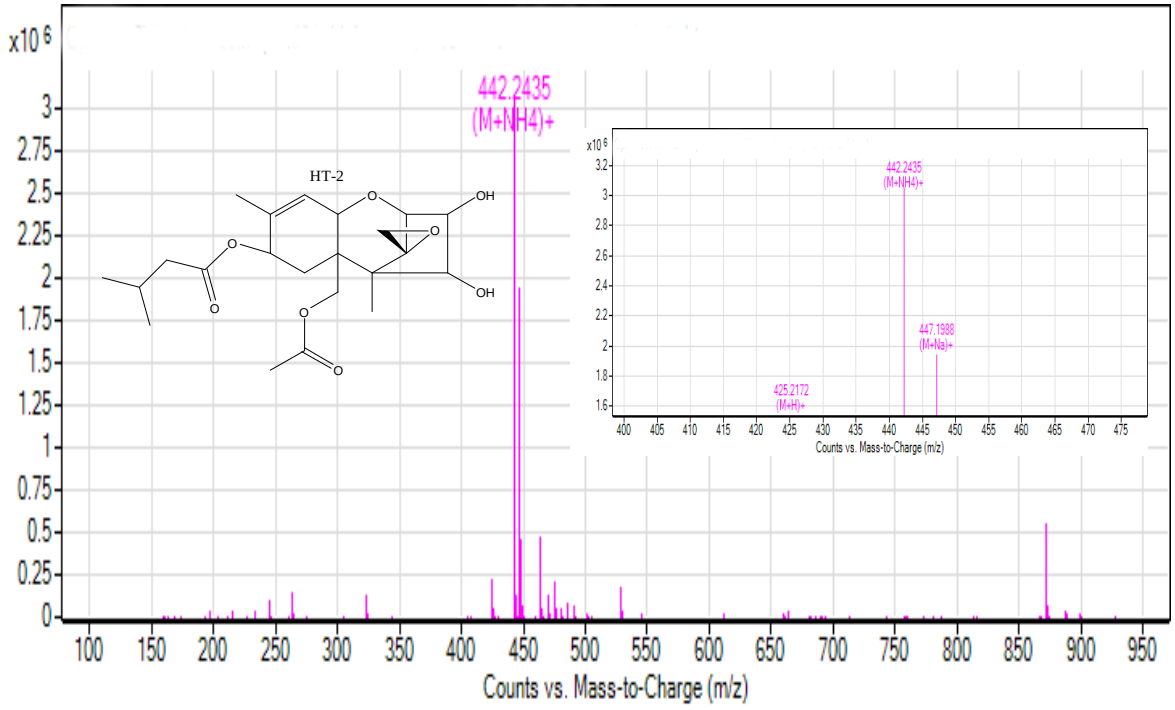


Figure 4.6: *Full scan ESI (+) production mass spectra and the structures of HT-2*

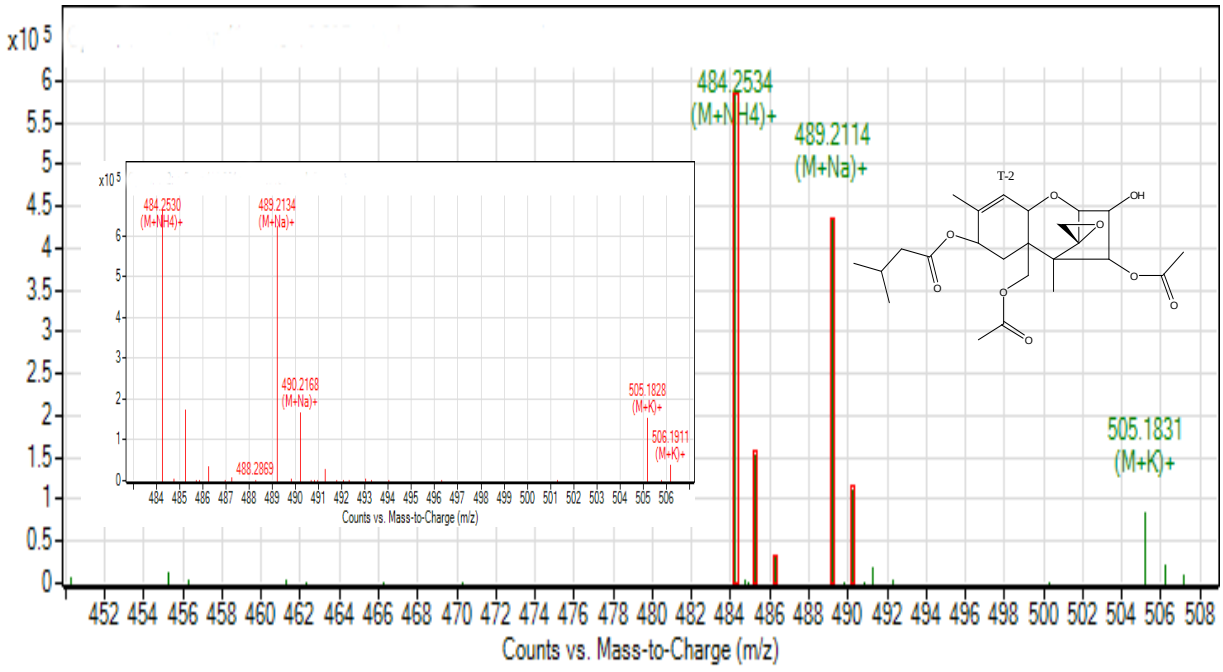


Figure 4.7: *Full scan ESI (+) production mass spectra and the structures of T-2*

The mass spectra of NIV (Figure 4.1) showed a higher abundance of the bromo, acetate, formate and chloro adduct ions with $[M+Br]^-$ of 391.0396, $[M+CH3COO]^-$ of 371.1336, $[M+HCOO]^-$ of 357.1213 and $[M+Cl]^-$ of 347.0905 m/z values respectively, compared to the molecular ion $[M-H]^-$ m/z value 311.1144. Figure 4.2 showed the mass spectra of DON which have intense acetate and chloro adduct ions with m/z value of $[M+CH3COO]^-$ 355.1403 and $[M+Cl]^-$ 371.1336 respectively, compared to the molecular ion $[M-H]^-$ m/z value 295.1184. The mass spectra of FUS X (Figure 4.3) also showed intense acetate adduct ion, $[M+CH3COO]^-$ of m/z value 413.1446 and the formate adduct ion $[M+HCOO]^-$ of m/z value 399.1307, compared to the molecular ion $[M-H]^-$ m/z value 353.1207. Whereas, the mass spectra of ADON (Figure 4.4) shows a relatively intense chloro and acetate adduct ions of $[M+Cl]^-$ of m/z 373.1062 and $[M+CH3COO]^-$ of m/z 397.1503 values respectively, compared to the molecular ion $[M-H]^-$ of m/z value 337.1294.

On the other hand, the mass spectra of DAS (Figure 4.5) showed a higher abundance of sodium and ammonium adduct ions of $[M+Na]^+$ of m/z 389.1571 and $[M+NH_4]^+$ of m/z 384.2016 values respectively, compared to the molecular ion $[M+H]^+$ of m/z value 367.1800. Additionally, for both HT-2 and T-2 were observed (Figure 4.6 and Figure 4.7) except the ammonium adduct ion $[M+NH_4]^+$ (HT-2 of m/z value 442.2435 and T-2 of m/z value 484.2534) were more abundant than the sodium adduct ion $[M+Na]^+$ (HT-2 of m/z value 447.1988 and T-2 of m/z value 489.2114) and the molecular ion $[M+H]^+$ (HT-2 of m/z value 425.2172) and potassium adduct $[M+K]^+$ (T-2 of m/z value 505.1831). However, the molecular ion $[M+H]^+$ of T-2 was not observed.

The presence of the sodium or ammonium adduct ions were observed for type-A trichothecenes and the bromo, acetate or chloro adduct ion for type-B trichothecenes (Figure 4.1 to Fig 4.7). This may be because of the presence of cation and anion impurities in the HPLC solvents and furthermore, sodium ions are more abundant than ammonium

impurities in methanol. However, the molecular ions are also present in the mass spectrum and they can be used for quantification.

For the gradient elution conditions, the main task was to determine the composition of solvent A and solvent B as the initial mobile phase. Several initial compositions of the mobile phase were studied, ranging from 5% to 40% of solvent B. The results showed that good peak shapes and high sensitivity were achieved with the decrease of solvent B content in the initial composition.

However, when the initial gradient started at 40% of solvent B, overlapping peaks were obtained for DON and NIV. Considering the combined factors of separation efficiency, run time and sensitivity, the use of 30% of solvent B was selected as the initial composition of the mobile phase (Figure 4.8).

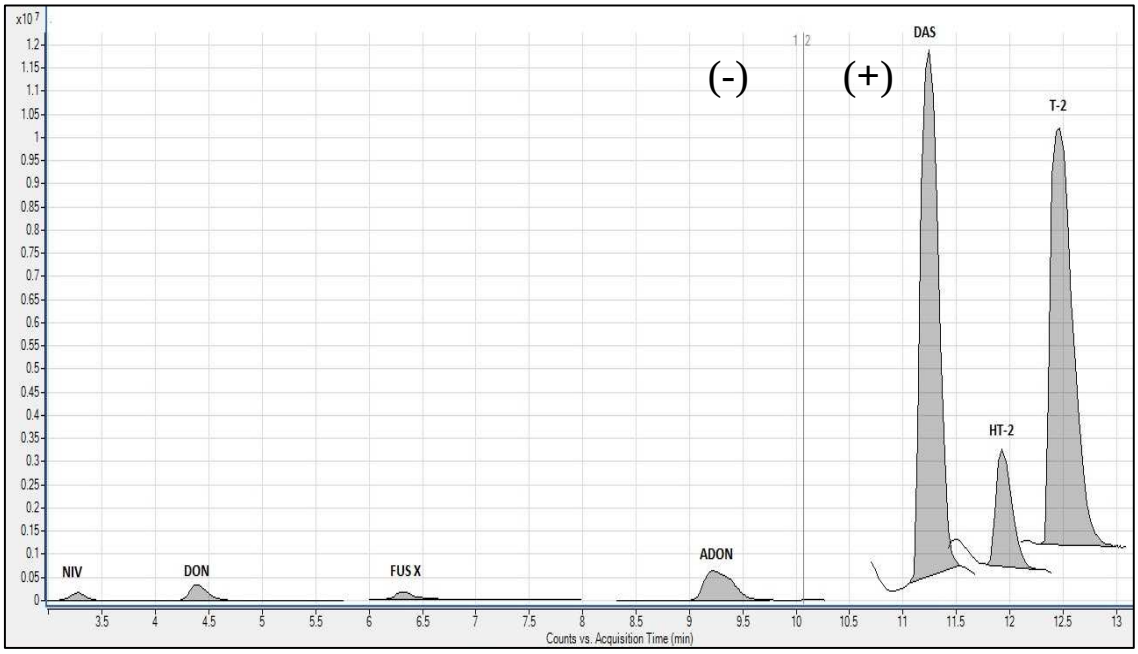


Figure 4.8: *Extract ion chromatogram (EIC) (LC/QTOF-MS/MS) of separation of a trichothecenes mixture solution containing 600 µg/kg for each toxin. Vertical line illustrates change of ionization polarities from negative to positive (10 min)*

In the case of 3-ADON and 15-ADON, besides having the same MS/MS spectrum, separation under these conditions were not achieved. Hence, both isomers were determined as total ADON (Cavaliere *et al.*, 2005). Other parameters such as flow rate, injection volume, and column temperature were optimized and the following parameters were selected as the optimum conditions: 0.20 mL/min as flow rate, 40^{O}C as column temperature and 5 µL as the injection volume.

4.3.3. Optimization of the ESI parameters

Both the positive and negative modes were investigated for ion acquisition in the MS. NIV, DON, FUS X, and ADON showed higher signal intensities in the negative ionization mode, whereas DAS and T-2 undergo better ionization in the positive mode. HT-2 can be detected in both positive and negative ion mode, with a slight increase in sensitivity and higher stability in the positive ion mode compared to the negative mode.

The instrumental parameters (drying and sheath gas flow rates and temperatures, fragmentor and capillary voltage) were optimized to provide the best possible sensitivity. However, it was found previously (refer to chapter 3) that the effects of all these parameters did not significantly affect the signal from the analytes. However, the fragmentor voltage, which played an important role in both the sensitivity and fragmentation, do influence the sensitivity. The fragmentor voltage (cone voltage or orifice voltage) is the difference between the voltage applied to the cone and that applied to the skimmer. Increasing the potential difference between the two would increase the kinetic energy and thus the velocity of the charged analyte droplets with more ions being transferred towards the skimmer resulting in a significant enhancement of the transmission efficiency and sensitivity. For this reason, the fragmentor voltage value was studied in the range from 20 to 400 V under optimized source conditions.

Figure 4.9 to Figure 4.10 show that, fragmentor voltages greater than 60 V led to extensive fragmentation even for the reference masses for type-B trichothecenes, while for type-A trichothecenes, extensive fragmentation was observed at even higher fragmentor voltages (≥180V). Voltage values in the region of 160 V provided minimal fragmentation and adequate sensitivity for quantification for type-A trichothecenes, while voltage values of approximately 60 V provided minimal fragmentation and adequate sensitivity for quantification for type-B trichothecenes.

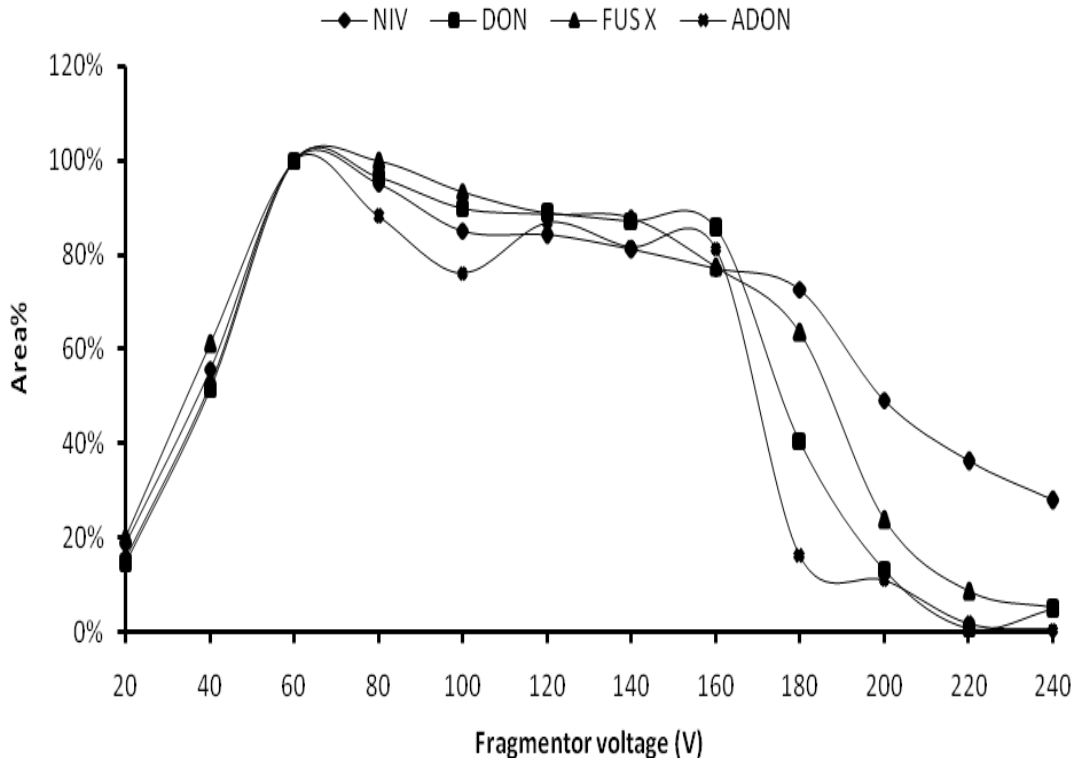


Figure 4.9: *Effects of the fragmentor voltage on the peak area% of type -B trichothecenes*

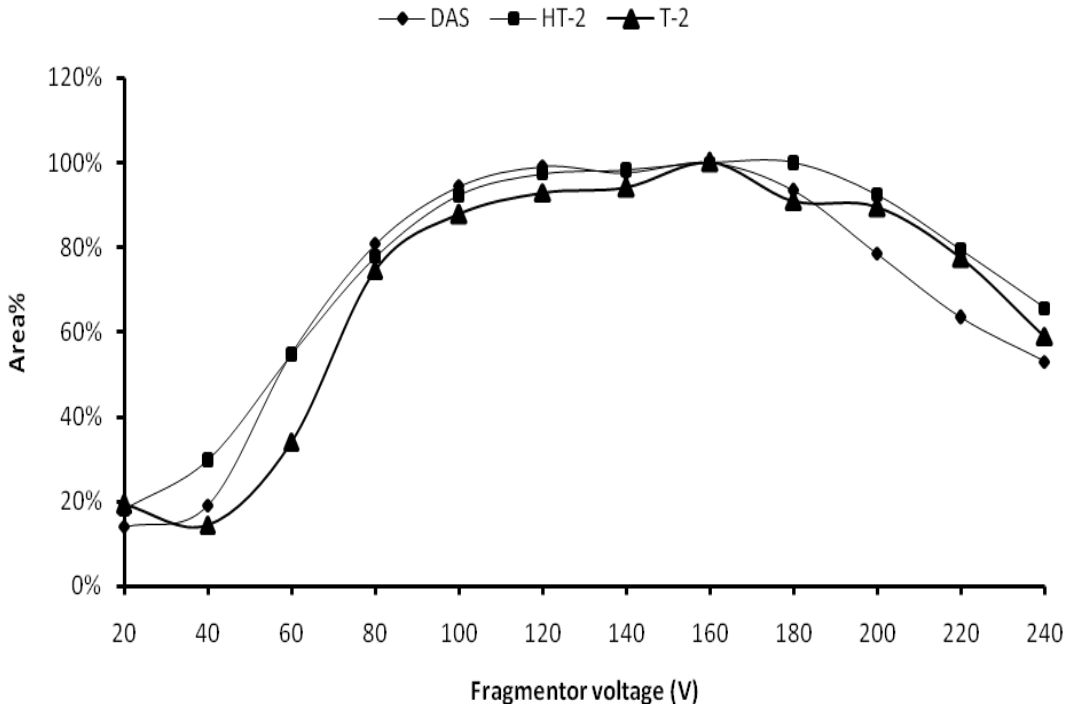


Figure 4.10: *Effects of the fragmentor voltage on the peak area% of type-A trichothecenes*

4.3.4. Accurate mass measurements

In order to achieve accurate mass information of the molecular ion, the effect of the accuracy of the mass measurements of the selected trichothecenes was evaluated at different concentration levels in the range of 150-1200 μg/L. In this way, the accurate mass information for the molecular ion was obtained. However, accurate mass measurements are also affected by overlapping /unresolved chromatogram peaks. Hence, the reason for separation of chromatogram peaks. The results for the determination of the mass accuracies of the molecule ion are as summarized in Table 4.2.

Table 4.2
Accurate mass information of the measured trichothecenes

Trichothecene	Molecular Ion	Molecular Formula	Observed Mass	Calculated Mass	Difference (ppm)
NIV	$[M- H]^-$	$C_{15}H_{20}O_7$	312.1210	312.1209	0.25
DON	$[M- H]^-$	$C_{15}H_{20}O_6$	296.1260	296.1260	0.03
FUS X	$[M- H]^-$	$C_{17}H_{22}O_8$	354.1312	354.1315	-0.62
ADON	$[M- H]^-$	$C_{17}H_{22}O_7$	338.1366	338.1366	0.15
DAS	$[M+ H]^+$	$C_{19}H_{26}O_7$	366.1678	366.1679	-0.05
HT-2	$[M+ H]^+$	$C_{22}H_{32}O_8$	424.2095	424.2097	-0.41
T-2	$[M+ H]^+$	$C_{24}H_{34}O_9$	466.2204	466.2203	0.18

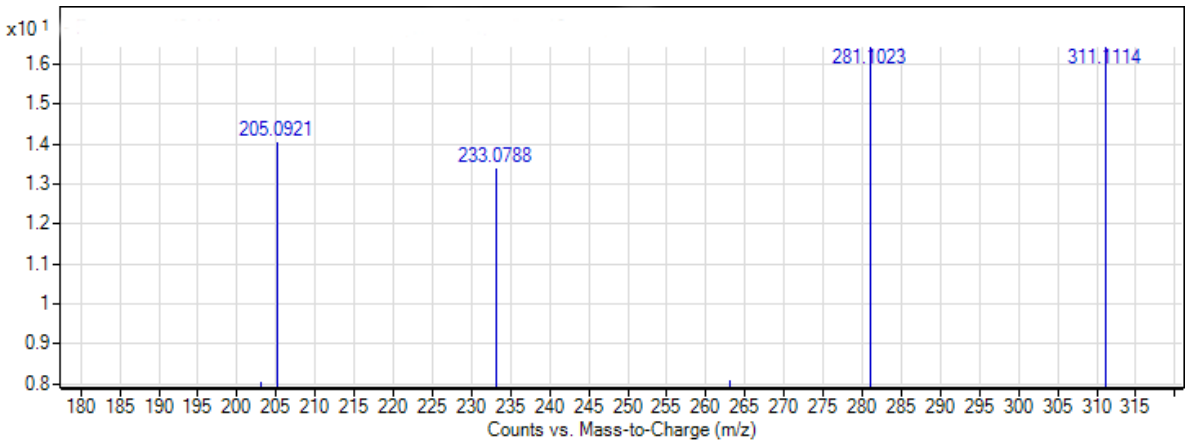
It can be observed from Table 4.2 that, there were no significant differences in the mass accuracy between the observed mass and the exact mass calculated, since the differences were between 0.03 ppm of DON and 0.62 ppm of FUS X. Therefore, it can be deduced that the accurate mass measurements have capability for unmistakable confirmation of these analytes at different concentration levels.

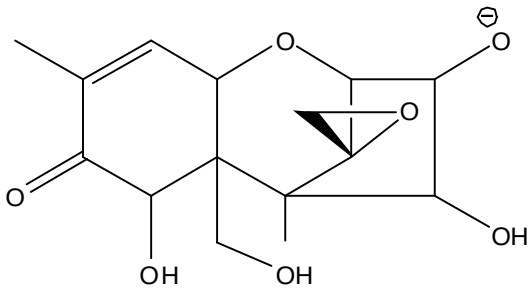
4.3.5. Selection of Product Ions

The selection of product ions was carried out by varying the collision energy between 5 and 45 V, at increments of 5 V.

Figure 4.11 to Figure 4.17 show the chemical structures, the MS/MS spectra and the corresponding molecular weights of NIV, DON, 15-AcDON, 3-AcDON, FUS X, DAS, HT-2 and T-2, with arrows indicating the formation of the product ions used in the quantification against the optimal collision energy. Two product ions were selected, the

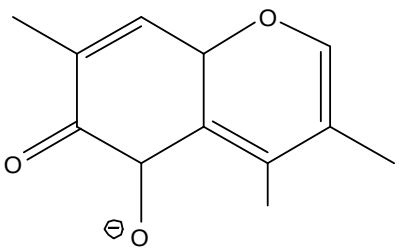
more intense as the quantifier ion and the other as the qualifier ion. Furthermore, the selection of the product ions was carried out for confirmation of the identity of the analyte.



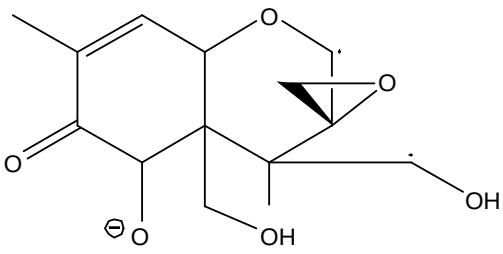


Chemical Formula: $C_{15}H_{19}O_7^-$
Exact Mass: 311.1136

Collision Energy 40 V

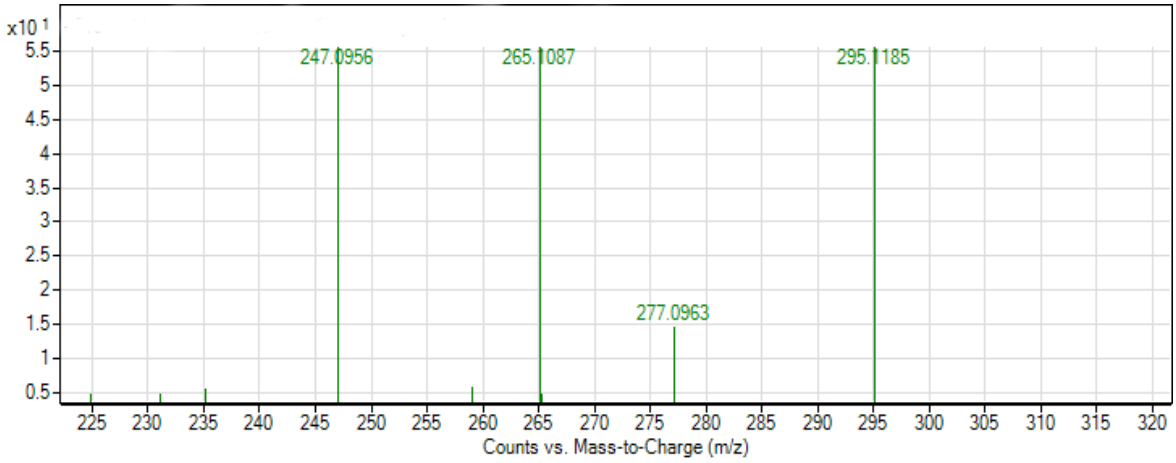


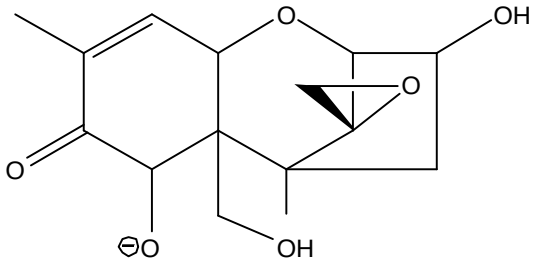
Exact Mass: 205.0870



Exact Mass: 281.1031

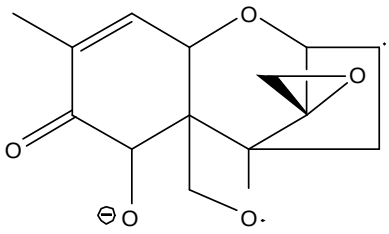
Figure 4.11: *The mass spectra and the proposed fragmentation scheme of NIV*



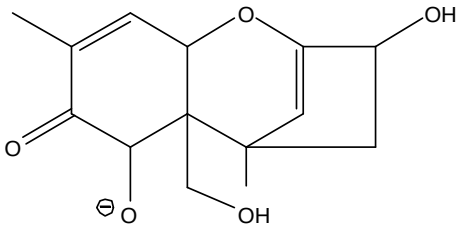


Chemical Formula: $C_{15}H_{19}O_6^-$
Exact Mass: 295.1187

Collision Energy 15 V

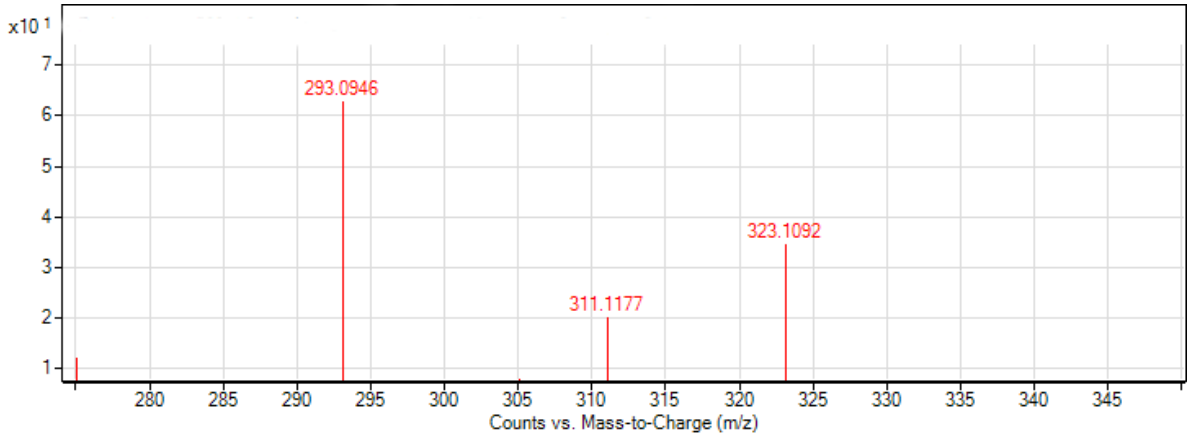


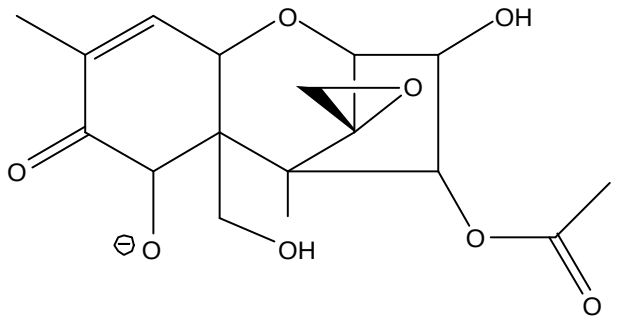
Exact Mass: 277.1081



Exact Mass: 265.1081

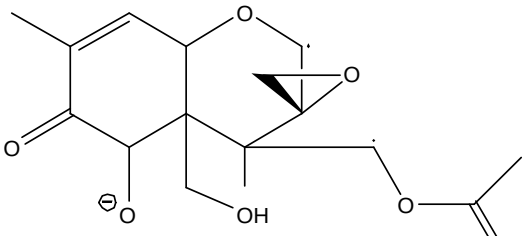
Figure 4.12: *The mass spectra and the proposed fragmentation scheme of DON*



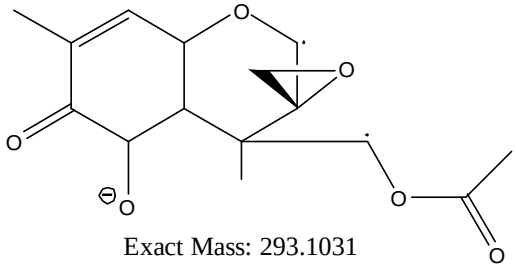


Chemical Formula: $C_{17}H_{21}O_8^-$
Exact Mass: 353.1242

Collision Energy 40 V

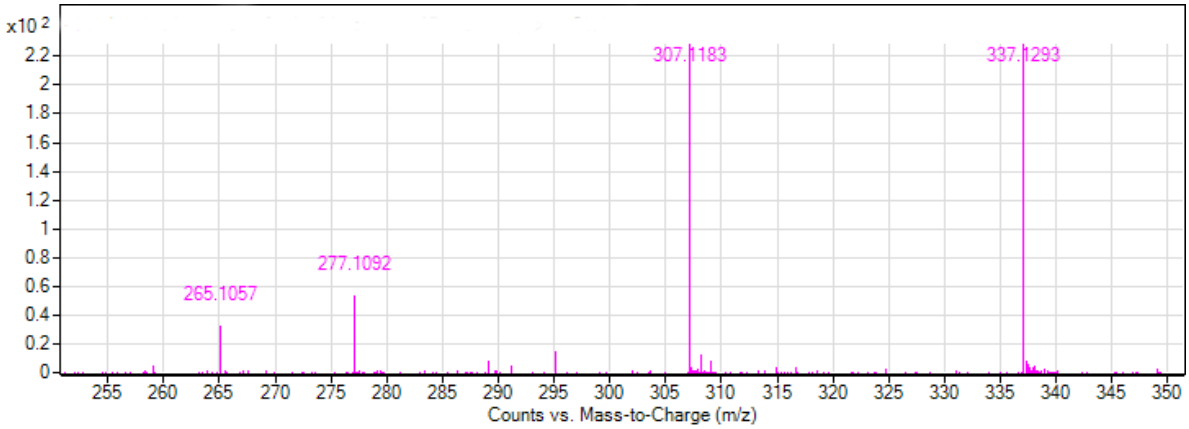


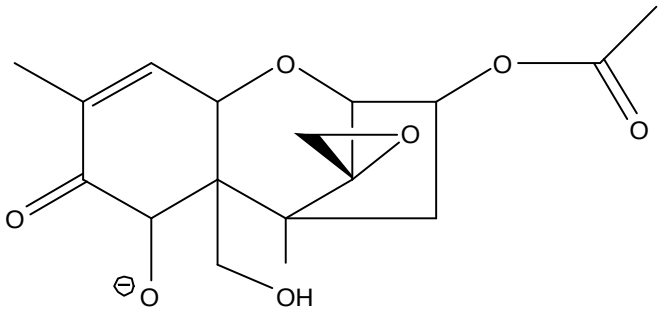
Exact Mass: 323.1136



Exact Mass: 293.1031

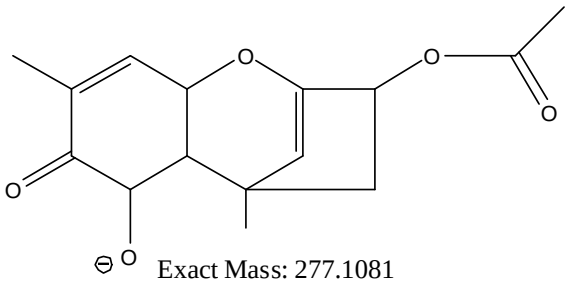
Figure 4.13: *The mass spectra and the proposed fragmentation scheme of FUS X*



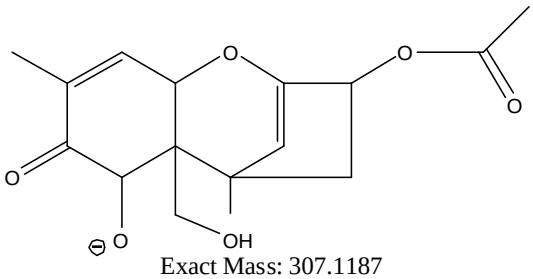


Chemical Formula: $C_{17}H_{21}O_7^-$
Exact Mass: 337.1293

Collision Energy 20 V

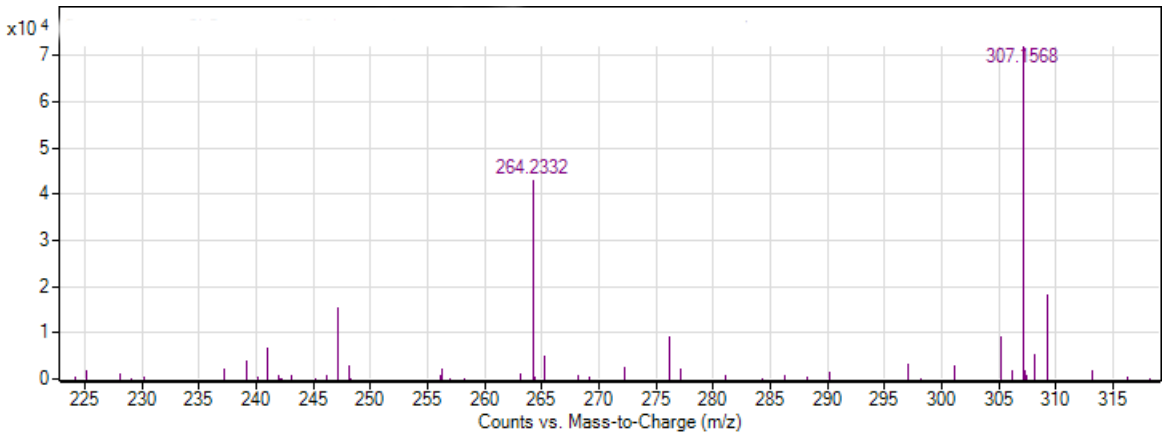


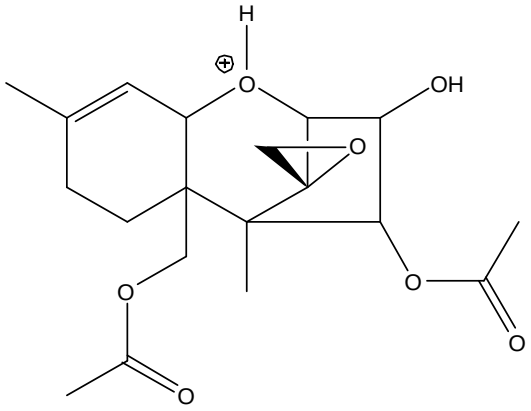
Exact Mass: 277.1081



Exact Mass: 307.1187

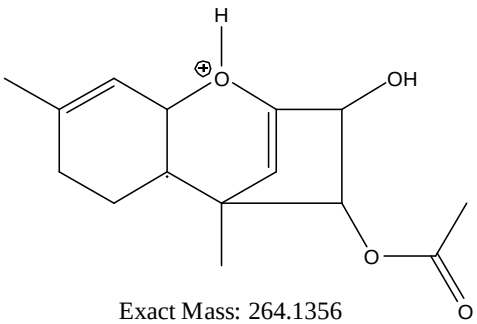
Figure 4.14: *The mass spectra and the proposed fragmentation scheme of 3-ADON*



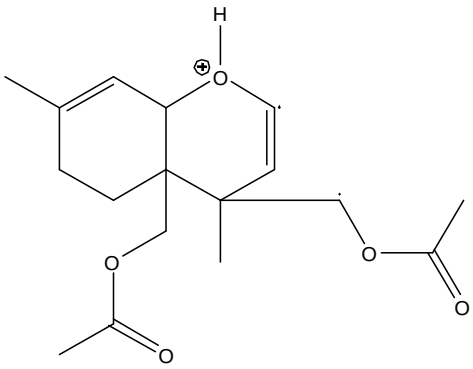


Chemical Formula: $C_{19}H_{27}O_7^+$
Exact Mass: 367.1751

Collision Energy 15 V

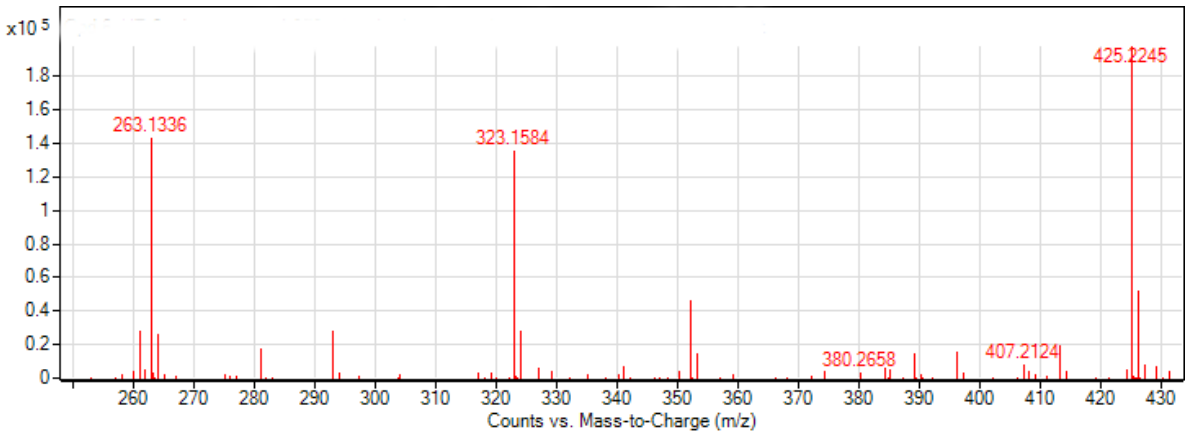


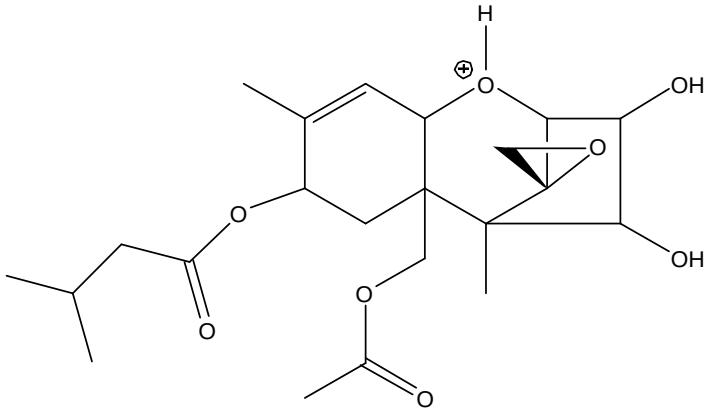
Exact Mass: 264.1356



Exact Mass: 307.1540

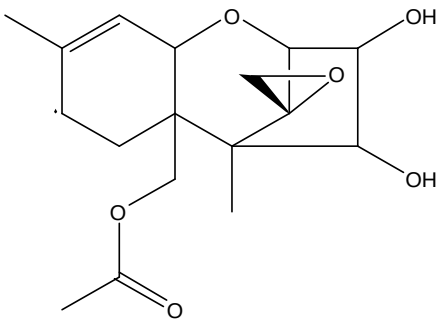
Figure 4.15: *The mass spectra and the proposed fragmentation scheme of DAS*



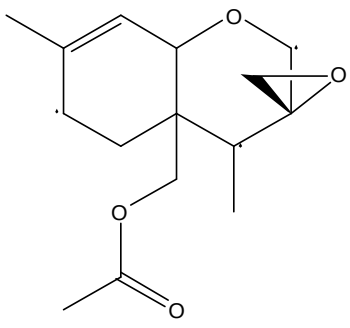


Chemical Formula: $C_{22}H_{33}O_8^+$
Exact Mass: 425.2170

Collision Energy 15 V

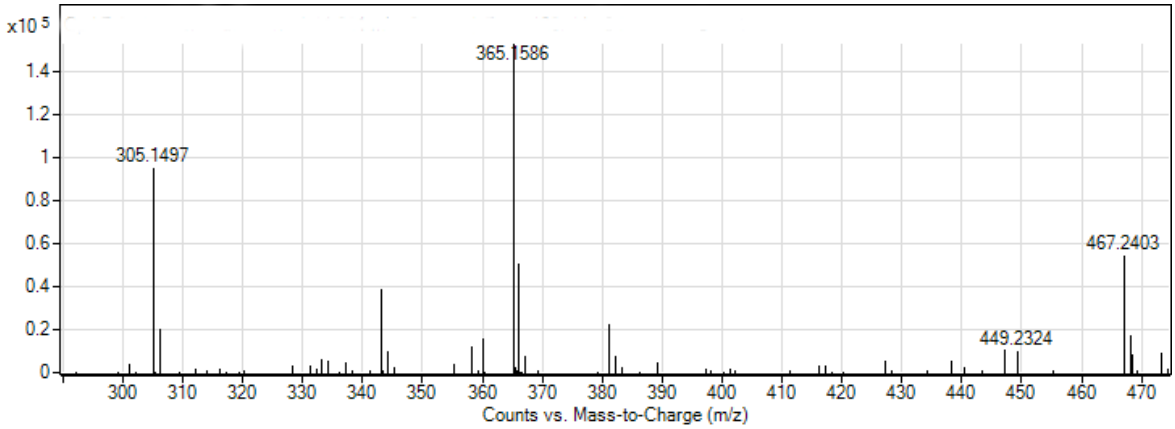


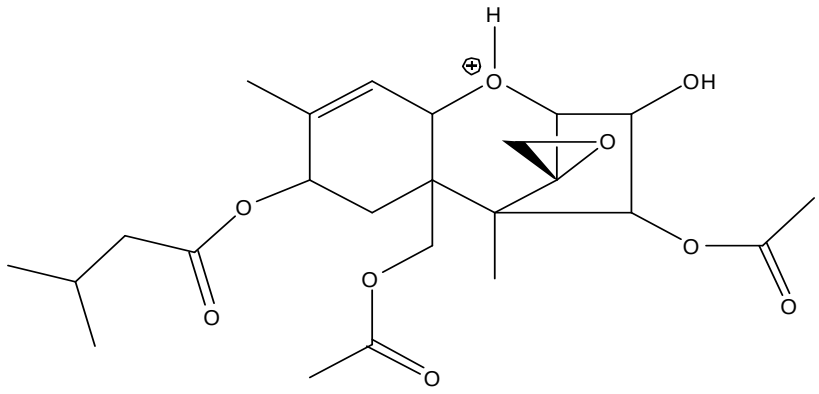
Exact Mass: 323.1495



Exact Mass: 263.1283

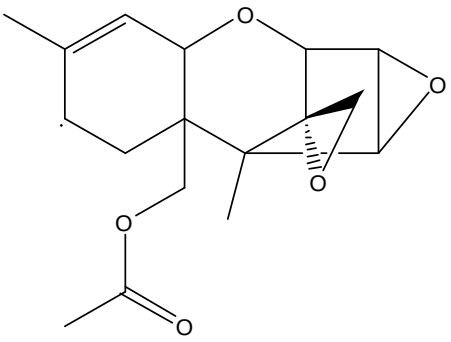
Figure 4.16: *The mass spectra and the proposed fragmentation scheme of HT-2*



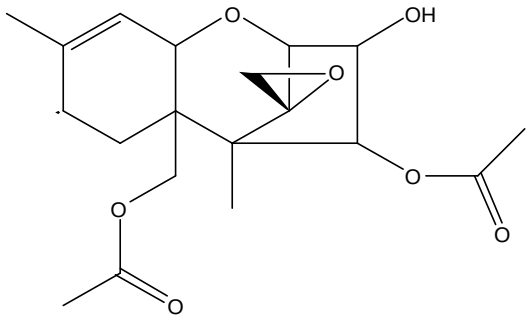


Chemical Formula: $C_{24}H_{35}O_9^+$
Exact Mass: 467.2276

Collision Energy 15 V



Exact Mass: 305.1389



Exact Mass: 365.1600

Figure 4.17: *The mass spectra and the proposed fragmentation scheme of T-2.*

4.3.6. Trichothecene database table

A trichothecene database table was created by injecting the eight(8) type-A and type-B trichothecenes in the LC-ESI-QTOF-MS/MS, working in the TOF-MS mode, at a concentration of 600 µg/L µg/kg. This table can be continuously updated to extend the trichothecene database table to include new mycotoxins.

Retention times add an extra degree of confidence in the identification of the unknown compound having the same empirical formula and the same exact mass. The repeatability and reproducibility of the retention times as measured by their RSD values ranged from 0.6% to 1.1% and 2.5% to 4.5%, respectively.

By using the database table, the automatic screening method was carried out by investigating the retention time, the observed mass and the exact mass of each compound that were collected on a Microsoft Excel sheet. In this way, the analytes were confirmed by their retention time, the accurate mass measurements of the TOF analyzer and the product ions (Table 4.3).

Table 4.3
Trichothecene database table

Molecular Formula	**Trichothecene**	**Retention Time** (min)	**Observed Mass** (m/z)	**Calculated Mass** (m/z)	**Difference** (ppm)	**Product Ions** (m/z)
$C_{15}H_{20}O_7$	NIV	3.3	312.1210	312.1209	0.25	281.1031 205.0870
$C_{15}H_{20}O_6$	DON	4.4	296.1260	296.1260	0.03	265.1081 277.1081
$C_{17}H_{22}O_8$	FUS X	6.3	354.1312	354.1315	-0.62	293.1031 323.1136
$C_{17}H_{22}O_7$	ADON	9.2	338.1366	338.1366	0.15	307.1187 277.1081
$C_{19}H_{26}O_7$	DAS	11.1	366.1678	366.1679	-0.05	307.1540 264.1356
$C_{22}H_{32}O_8$	HT-2	11.7	424.2095	424.2097	-0.41	263.1283 323.1495
$C_{24}H_{34}O_9$	T-2	12.4	466.2204	466.2203	0.18	365.1600 305.1389

4.3.7. Method validation

This was an in-house validated method, in terms of linearity, accuracy, intra-day precision, inter-day precision, limit of detection (LOD), and limit of quantification (LOQ). The linearity was tested using a standard solution of the trichothecenes in the concentration range of 75 and 200 µg/L. Table 4.4 shows that good linear relationships with correlation coefficients greater than 0.97 for all the targeted analytes were obtained. Calibrations with standard solutions were used for quantification, because moderate signal suppression was noticeable for all the analytes. Furthermore, the ANOVA test, did not give any significant difference at p = 0.05.

Table 4.4
Linear regression equation , r^2 value, LOD and LOQ of trichothecenes

Trichothecene	Equation (concentration range 75-1200 µg/kg)	r^2	LOD (µg/kg)	LOQ (µg/kg)
NIV	y = 432.9520 * x + 14992.5101	0.9991	35.0	116.6
DON	y = 512.0872 * x -25579.3895	0.9914	31.3	104.2
FUS X	y =2253.4553 * x + 44116.9630	0.9820	1.90	6.40
ADON	y = 2414.1887 * x – 105359.7636	0.9919	17.8	59.3
DAS	y = 37449. 6731 * x – 629015.6099	0.9974	14.2	47.2
HT-2	y =3606.8426 * x – 201684.9410	0.9901	28.4	94.8
T-2	y = 28273.0811 * x – 1548667.1141	0.9731	11.4	37.9

The accuracy was tested by the determination of the recoveries of the trichothecenes in clean wheat, wheat products (wheat-based noodles), rice, rice products (rice-based noodles), and corn samples spiked at 100.0 and 500.0 µg/kg of the trichothecenes standards and were analyzed in triplicates (Table 4.5). The recoveries obtained ranged from 61.9 % to 110.97%, with a RSD value of less than 12%. The

recoveries for relatively non-polar trichothecenes (DAS and HT-2) were slightly more significant than the polar analytes (NIV and DON).

Table 4.5
Mean of recoveries and RSDs (n=5)[a] of trichothecenes spiked into blank wheat, wheat product, rice, rice product and corn samples at two spiking levels

Matrix	Spiking Level (µg/kg)	Trichothecene						
		NIV	DON	FUS X	ADON	DAS	HT-2	T-2
		Mean of Recovery (%) ± RSD (%)						
Wheat	100	77.2±10.3	77.4±8.4	105.9±6.2	110.1±8.1	95.3±9.2	110.9±10.8	109.0±7.1
	500	80.7±4.5	82.4±4.9	95.0±9.9	107.9±4.0	106.6±5.0	106.8±5.5	95.3±4.4
Wheat product	100	78.1±9.1	76.4±8.7	99.4±4.5	105.3±4.3	107.0±7.8	90.5±2.8	110.1±9.8
	500	74.5±6.8	80.4±6.9	108.7±5.6	106.1±4.0	104.0±8.5	105.7±9.9	105.9±3.6
Rice	100	77.3±9.4	76.9±8.5	116.8±9.6	109.5±6.3	94.4±7.1	99.6±7.3	90.6±8.3
	500	79.9±9.4	72.5±6.2	104.4±6.4	108.0±4.5	106.4±9.2	103.6±4.5	103.6±9.4
Rice product	100	84.7±6.9	70.2±8.6	82.8±7.4	104.8±8.2	90.0±9.8	86.1±7.2	96.8±10.2
	500	95.4±7.9	66.3±5.9	84.4±9.5	110.9±11.3	91.4±8.2	97.0±9.4	95.4±9.1
Corn	100	61.9±8.5	73.3±9.4	96.5±10.8	92.7±11.1	74.0±7.1	71.6±10.0	84.9±10.0
	500	65.7±4.2	78.8±8.5	99.1±5.0	95.2±7.9	80.6±9.4	87.2±5.7	89.3±5.4

[a] n: is number of replicates

The sensitivity was determined by estimating the LOD and LOQ. LODs and LOQs were estimated experimentally as the lowest concentration giving a response of three- and ten-times, respectively, the base-line noise. The LOD of type- A and type-B trichothecenes were 6.1–8.3 and 12.5–18.7 µg/kg, respectively (see the details in Table 4.4).

Intra-day precision was evaluated by assaying five replicates of blank wheat sample at a spiked level of 500µg/L trichothecenes on the same day. For the inter-day precision, five replicates of blank wheat sample at a spiked level of 500 µg/L trichothecenes were analyzed on three consecutive days. The intra-day precision and inter-day precision were calculated and tabulated in Table 4.6. The intra-day precision (n = 5) are between 1.1 and 6.7%, while the inter-day variation (n=15) values are between 4.8 and 14.3%. These values determined are lower than the acceptable maximum of 15%, confirming the good reproducibility and repeatability of this technique.

Table 4.6
Intra- and inter-day precision of trichothecenes

Trichothecene	**Intra-Day Precision (n = 5)[a]** (500 µg/kg)	**Inter-Day Precision (n = 15)[a]** (500 µg/kg)
NIV	2.6	5.1
DON	4.6	6.8
FUS X	6.7	14.3
ADON	1.1	4.8
DAS	2.3	7.3
HT-2	3.8	10.5
T-2	4.7	5.9

[a] n: is number of replicates

Considering the data for method validation, the current LC-ESI-CID-MS/MS analysis measured with a hybrid QTOF-instrument and sample preparation procedures

employed can be regarded as selective, precise and robust. Furthermore, the determination of trace concentration of these trichothecenes in cereals and cereal products was with a high degree of confirmation using the retention time, the accurate mass measurements of the TOF analyzer and the products ions, thus avoiding false-positive results.

4.3.8. Sample analysis

The developed method was applied to the determination of eight (8) type-A and type-B trichothecenes in 25 samples of cereals and cereal products (wheat, wheat based noodles, rice, rice based noodles and corn), which were randomly obtained from groceries and stores in Kuala Lumpur, Malaysia. None of the trichothecenes investigated in this study were detected in these randomly selected samples (Table 4.7).

Table 4.7
Occurrence of trichothecenes in cereals and cereal products samples in the month of December 2009

Sample category	Total Number of Samples of the Particular Category	Number of Positive Samples	Incidence (Positive/ Number of Samples of the Particular Category)	Incidence (Positive/ Total Number of Samples)	Trichothecene (µg/kg)
Wheat	5	0	0.0%	0.0%	– [a]
Wheat product	5	0	0.0%	0.0%	– [a]
Rice	5	0	0.0%	0.0%	– [a]
Rice product	5	0	0.0%	0.0%	– [a]
Corn	5				
Total	**25**	**0**	**0.0%**	**0.0%**	

[a]: Values were below the LOD of the adopted method

4.4. Conclusion

A simple, rapid and confirmatory method has been developed for the determination of type-A and type- B trichothecenes in cereals. The use of QTOF-MS/MS uniquely offers the possibility of providing accurate mass data and can generate structural information of the analytes with minimal sample treatment and without derivatization. The sensitivity of the instrument could be significantly enhanced by optimizing the chromatographic conditions and the fragmentor voltage in the ESI interface. Extensive and expensive clean-up procedures could be replaced by adopting the QuEChERS procedure without prior dSPE step. By using the trichothecene database table, trichothecenes were confirmed by their retention time, the accurate mass measurements of the TOF analyzer and the products ions, thus avoiding false-positive results. The extraction solvent was found to be the most important factor and strongly affects the extraction efficiency. Excellent linearity, high recoveries, acceptable repeatability and reproducibility with the LOQ values lower than the stipulated MRL values were achieved indicating the suitability of the proposed method for the determination of trichothecenes in cereals.

5. QuEChERS-HPLC METHOD FOR AFLATOXIN DETECTION OF DOMESTIC AND IMPORTED FOOD IN JORDAN

5.1. Introduction and scope of the work

Aflatoxins are a group of highly toxic and carcinogenic substances, which occur naturally, and can be found in food substances. The most common food commodities affected by aflatoxins are cereals (corn, wheat, barley, maize, oats and rye), nuts (hazelnut, peanut and pistachio nut), dried fruits (fig) and spices (chili powder) (Ardic *et al.*, 2008; Bacaloni *et al.*, 2008; Battilani *et al.*, 2008). Aflatoxins pose a potential threat to human and animal health through the consumption, contact or inhalation of foodstuffs and feedstuffs prepared from these commodities. As a result of the adverse health effects of mycotoxins, their levels have been strictly regulated especially in food and feed samples. While the European Union in general has set a much lower allowable residue level than the US Food and Drug Administration, they are generally in the μg/kg level for most of these compounds (FAO, 2004). In order to ensure compliance with the international regulations, it is necessary to have a sensitive, reliable and an accurate method for the determination of mycotoxins in different food commodities.

High performance liquid chromatography with fluorescence detection (HPLC-FLD) is considered as the most commonly used instrument of these methods for quantification of aflatoxins, because of its accuracy, high sensitivity and ease in automation (Kaniou-Grigoriadou, Eleftheriadou, Mouratidou, & Katikou, 2005). However, it requires one or more clean-up steps involving liquid–liquid extraction (LLE), solid-phase extraction (SPE)

or immunoaffinity columns (IAC) clean-up and in addition, aflatoxin B1 and G1 suffer from fluorescence quenching in the HPLC mobile phase. Therefore, pre-column or post-column derivatization is necessary to enhance the fluorescence intensity of aflatoxin B1 and G1 which is needed to achieve the low limits of detection (low-ppb level).

Various derivatization methods including precolumn derivatization with trifluoroacetic acid (TFA) (Móricz, Fatér, Otta, Tyihák, & Mincsovics, 2007), postcolumn derivatization with iodine (Fallah, Jafari, Fallah, & Rahnama, 2009), electrochemical derivatization with potassium bromide (Tavčar-Kalcher, Vrtač, Pestevšek, & Vengušt, 2007) and electrochemical derivatization using the Kobra cell (Kaniou-Grigoriadou *et al.*, 2005) have been developed. While, precolumn derivatization technique presents several significant drawbacks such as being laborious, time consuming and because of the high polarity and relative instability of these derivatives (Tan, Chu, Shen, & Yu, 2009), postcolumn derivatization with postcolumn photochemical reactor method for enhanced detection (PHRED) has been successfully used to overcome these drawbacks (AOAC Official Method 2005.08).

The aim of this study is focused on optimizing the sample pretreatment conditions of the developed Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method and the optimization of the chromatographic conditions of an HPLC-FLD method with postcolumn photochemical derivatization for quantification of four(4) aflatoxins B1, B2, G1 and G2 in food. Sample pretreatment conditions such as the extraction solvent; the type and amount of drying agent; the extraction time; and the solvent-sample ratio were optimized. The developed QuEChERS-HPLC method was then validated and compared with the fluorometric determination method. The methods were then used for the analysis

of the selected aflatoxins in a total of 669 domestic and imported food samples in Jordan using a QuEChERS-HPLC method.

5.2. Experimental

5.2.1. Reagents and materials

Certified mixed standard solutions consisting of aflatoxin B1, B2, G1 and G2 were obtained from Supelco (Bellafonte, PA, USA). Subsequently, daily working standard solutions were prepared by diluting the stock solutions in methanol. Water and acetonitrile (HPLC-grade) were purchased from VWR International (EC). Methanol (HPLC-grade) was purchased from Labscan (Dublin, Ireland). Sodium chloride was purchased from Acros Organics (New Jersey, USA). Anhydrous magnesium sulfate ($MgSO_4$) for QuEChERS was purchased from R&M Chemicals (Essex, UK). A nonsterile PTFE Syringe Filter with a disposable membrane filter (0.45 μm) was purchased from Whatman GmbH (Dassel, Germany).

5.2.2. QuEChERS-HPLC method

5.2.2.1. Sample preparation

Samples were prepared using the previous QuEChERS method with some modifications.

Step I: A thoroughly homogenized food sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 mL).

Sample recovery was done with 1.0 g of the clean wheat, corn, rice, pistachio nut, peanut and almond spiked with 5.0, 10.0 and 50.0 μg/kg of aflatoxin B1, G1 and 1.5, 3.0 and 15.0 μg/kg of aflatoxin B2, G2. The spiked samples were left overnight in the dark at room

temperature to allow the solvent to evaporate and for aflatoxin absorption into the matrix. Then they were extracted via the following steps (II to IV).

Step II: 3.0 mL of 60:40 (%, v/v) methanol/ acetonitrile mixture was added and the centrifuge tube was shaken for 1 min to ensure that the solvent has mixed thoroughly with the entire sample, for the complete extraction of the analyte.

Step III: 0.80 g of anhydrous $MgSO_4$ and 0.18 g of NaCl were added into the mixture and the shaking procedure was repeated for 1 min to facilitate the extraction and partitioning of the four(4) aflatoxins into the organic layer.

Step IV: The extract was centrifuged for 5 min at 4000 rpm, and 0.5 mL of the upper organic layer was filtered through a 0.45 µm nylon syringe filter prior to HPLC analysis.

5.2.2.2. HPLC analysis

The HPLC analysis was performed using a Perkin Elmer Series 200 (Liantrisant, UK) system consisting of pump with a quaternary configuration, a vacuum degasser, a column oven, a fluorescence detector and coupled with an autosampler equipped with a 200 µL sample loop. A post column PHRED from Aura Industries (NY, USA) with a low-pressure mercury lamp (λ = 254 nm) and a knitted reactor coil (25 m x 0.25 mm) was applied to enhance the detection of aflatoxin B1 and G1. The chromatographic separation was performed with a Nucleodur 250-5 C_{18} 250 mm × 4.6 mm x 5 µm chromatographic column and was purchased from Macherey-Nagel (Duren, Germany). The sample extracts were analyzed isocratically using 65:25:10 water/ methanol/acetonitrile mixture as the mobile phase. The column was kept in a column oven at 45^{o}C at a flow rate of 1.0 mL/min to

achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 10 μL for both the sample and standard solutions.

5.2.3. Fluorometric Determination

5.2.3.1. Sample preparation

For each sample, ten grams were extracted in a blender with 50 mL methanol: water (60:40, v/v) and 2.0 g of sodium chloride for 1 min in a Waring blender at high speed. After filtration through a fluted filter paper (24 cm, Vicam, Watertown, MA, USA), a 20 mL of the filtrate was diluted with 20 mL distilled water and was mixed vigorously. The mixture was then filtered a second time using a microfiber filter paper (11 cm, Vicam, Watertown, MA, USA) and 10 mL of filtrate (equivalent to 1.0 g sample) was applied to the immunoaffinity column (Aflatest, Vicam, Watertown, MA, USA). The column was washed twice with 5 mL of distilled water and the aflatoxins were eluted to the monoclonal antibodies by passing 1 mL of HPLC grade methanol through column at a rate of 1–2 drops/ second.

5.2.3.2. Measurement

The Fluorometric determination was performed by a VICAM Series 4 (G8002) Fluorometer optical system (MA, USA) equipped with a high intensity pulsed Xenon lamp with selected fluorescence excitation and emission filters. A linear calibration of the instrument was developed by subsequently reading three quinine sulfate dihydrate reference standards (Aflatest-MTM; VICAM). The red and the green mycotoxin calibration vials were used to set the higher and the lower calibration points and the last vial contains the yellow mycotoxin calibration standard, following the VICAM instruction manual. Exactly

1 mL of the eluate was mixed vigorously with 1 mL of the ***AflaTest*** Developer. The aflatoxin content was then obtained from the fluorometric measurements. The detection limit of the method was 1.0 µg/kg.

5.2.4. Food samples

A total of 669 samples of domestic and imported samples were supplied to the Jordan food and drug administration in 2011 and analyzed. The samples consisted of 274 samples corresponding to cereals; eighty seven to peanuts and peanut butters; seventy eight to nuts (walnut, cashew, pine, hazelnut and almond); forty six to sesame seeds; sixty one to pistachio nuts; fifty one to seeds (sunflower, water melon, etc); fifty nine to green coffee and the remaining samples were related to spices. Approximately 1-4 kg of each type of sample was obtained. The samples were stored in a dark place at room temperature (20–25°C). The samples were ground and mixed at room temperature till a fine and homogeneous powdered material was obtained. The powdered samples were then stored in plastic bags at 4°C in a refrigerator prior to analysis.

5.3. Results and discussion

5.3.1. QuEChERS-HPLC method

5.3.1.1. Optimization of HPLC conditions

Different combinations of water/ methanol/ acetonitrile such as 60:20:20, 65:25:10, 70:20:10 and 75:17:8 (%, v/v) were tested as the mobile phase in order to optimize the resolution of the aflatoxin standard mixture and to enhance the sensitivity. Aflatoxin G1 and B2 were not separated by the first mobile phase composition (60:20:20 (%, v/v)). On the other hand, broad peaks and long retention times were produced using the third and

fourth mobile phase systems, when the water composition exceeded 70%. In contrast, the second mobile phase offered an adequate separation between the aflatoxin G2, G1, B2 and B1 peaks with reasonable and acceptable retention times (Figure 5.1).

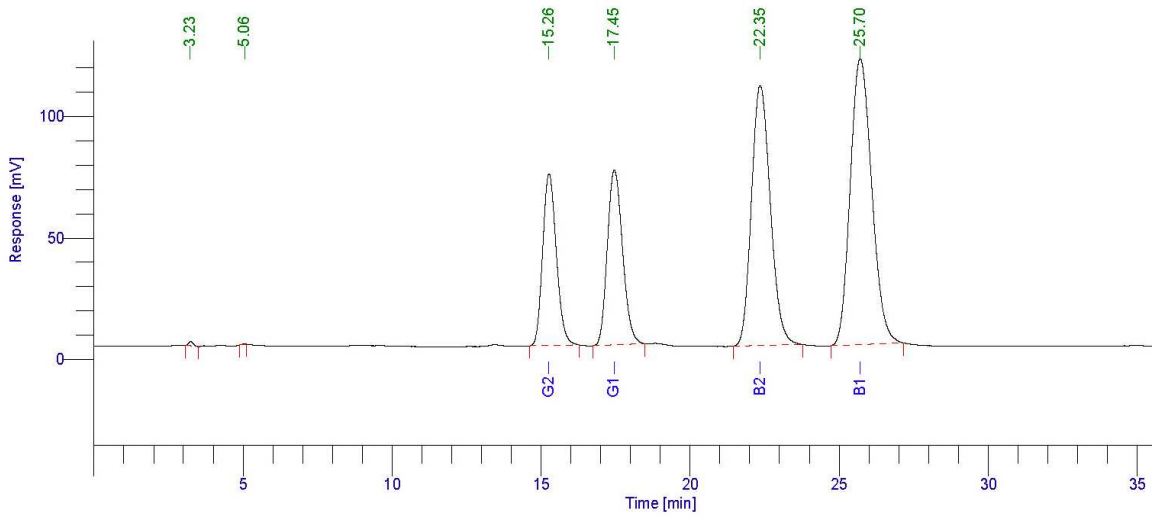


Figure 5.1: *HPLC chromatogram of aflatoxin standard solutions containing 100 µg/L of afatoxin B1 and G1 and 30 µg/L of aflatoxin B2 and G2*

However, the bottleneck of a chromatographic system is the column, where the actual separation of the analyte mixture occurs. Column selection depends strongly on prior knowledge of the physicochemical properties of the analytes and of the matrix. The column type and its length were optimized by investigating various HPLC columns under the same chromatographic conditions in order to obtain the best chromatogram separation in the shortest analysis time. The investigated columns are:

A. ACE 5, 5 µm Phenyl 250 mm × 4.6 mm, from Hichrom (UK),

B. Nucleodur, 5µm 250-5 C_{18} 250 mm × 4.6 mm, from Macherey-Nagel (Duren, Germany),

C. Brownlee Analytical, 5 μm C_{18} 250 mm × 4.6 mm, from PerkinElmer (Shelton, USA),

D. Spherisorb 5, 5 μm ODS-1 C_{18} 150 mm × 4.6 mm, from Phenomenex (USA),

E. Nucleodur 100-5, 5 μm C_{18} 100 mm × 4.6 mm, from Macherey-Nagel.

Table 5.1 showed that the column has a significant effect on the retention time and sensitivity of aflatoxins. Aflatoxin G1 standard cannot be detected by the ACE 5, 5 μm Phenyl 250 mm × 4.6 mm, (column A). In the case of 100 mm column length (column E), their retention times were reduced to less than 20 min but was accompanied with an increasing risk of interferences and false positive results. While column D suffers from a significant decrease in the resolution between aflatoxin G2 and G1 (Rs ≈ 1.1), column C has poor sensitivities for all analytes with a tailing peak for aflatoxin B2 (0.79 at 5% height). However, column B shows high efficiency (HETP= 2.3- 3.9E-05 mm) and the best resolution (Rs > 4.2) but with longer retention times for the aflatoxins (≈ 50 min) (Table 5.1).

Table 5.1
Retention time, area %, tailing factor, resolution and theoretical plate number / column length (HETP) of aflatoxins

	Column	Aflatoxin	Retention Time (min)	Area %	Tailing Factor	Resolution	HETP (mm)
A	**ACE 5,**	**G2**	26.1	82.4%	1.001	-	2.1E-05
	5 µm, Phenyl,	**G1**	-	-	-	-	-
	250 x 4.6 mm	**B2**	36.8	100.0%	0.800	-	1.8E-05
		B1	49.6	100.0%	0.969	8.91	1.5E-05
B	**Nucleodur,**	**G2**	21.1	100.0%	1.080	-	3.9E-05
	5 µm, C_{18},	**G1**	27.7	82.3%	1.094	5.80	3.1E-05
	250 x 4.6 mm	**B2**	33.2	99.0%	1.007	4.16	3.2E-05
		B1	43.8	95.3%	1.043	6.77	2.3E-05
C	**Brownlee**	**G2**	13.6	81.6%	1.157	-	6.3E-05
	Analytical	**G1**	17.7	54.7%	1.067	4.43	4.6E-05
	5 µm, C_{18},	**B2**	21.1	81.6%	0.788	3.22	3.6E-05
	250 x 4.6 mm	**B1**	27.5	87.1%	0.981	5.20	3.2E-05
D	**Spherisorb**	**G2**	13.3	100.0%	1.138	-	3.0E-05
	5 µm, C_{18},	**G1**	14.2	100.0%	1.023	1.09	2.8E-05
	150 x 4.6 mm	**B2**	17.4	79.6%	0.898	3.27	1.8E-05
		B1	22.0	89.7%	1.098	4.20	2.2E-05
E	**Nucleodur**	**G2**	8.1	97.8%	1.095	-	5.5E-05
	5 µm, C_{18},	**G1**	10.6	81.8%	1.078	3.01	4.0E-05
	100 x 4.6 mm	**B2**	12.9	74.8%	0.824	2.44	3.4E-05
		B1	16.8	92.1%	1.058	3.66	2.8E-05

However, by increasing the column temperature to 45^{o}C, the retention time was reduced to 30 min without affecting the resolution. The selectivity of the isocratic mixture of 65:25:10 (%, v/v) water/ methanol/ acetonitrile at 45^{o}C column temperature are considered satisfactory as it enables aflatoxin quantification in the analyzed food commodities with higher selectivity and sensitivity within a reasonable run time. The repeatability and reproducibility of the retention times as measured by their relative standard deviation (RSD) ranged from 1.0% to 2.0% and 1.8% to 4.3%, respectively. In order to evaluate whether the aflatoxin mix standards could be distinguished and separated

from interfering substances in the sample matrix, a naturally contaminated pistachio nut sample was pretreated using the QuEChERS method and separated using a Nucleodur 250-5. The chromatograms demonstrating the selectivity of the procedure are shown in Figure 5.2.

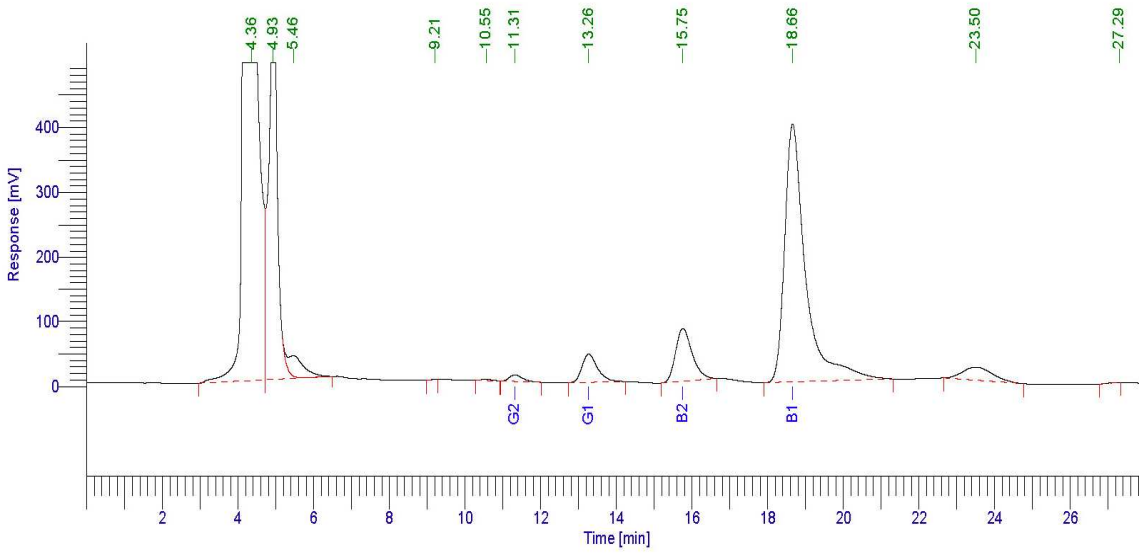


Figure 5.2: *Representative HPLC chromatogram of a naturally contaminated pistachio nut sample*

From the chromatograms of the naturally contaminated pistachio nut sample, it is evident that the peaks of the aflatoxin standard are well separated from the peaks of the interfering substances in the sample matrix and reasonable retention times were obtained.

For the fluorescence detection, a spectrum of aflatoxin standard solution in the HPLC mobile phase was tested to optimize the detection of aflatoxins and to obtain the best fluorescence signals in terms of signal-to-noise ratio and sensitivity. Various

emission/excitation wavelengths were applied to obtain the best values in order to enhance the detection for aflatoxins, as shown in Figure 5.3.

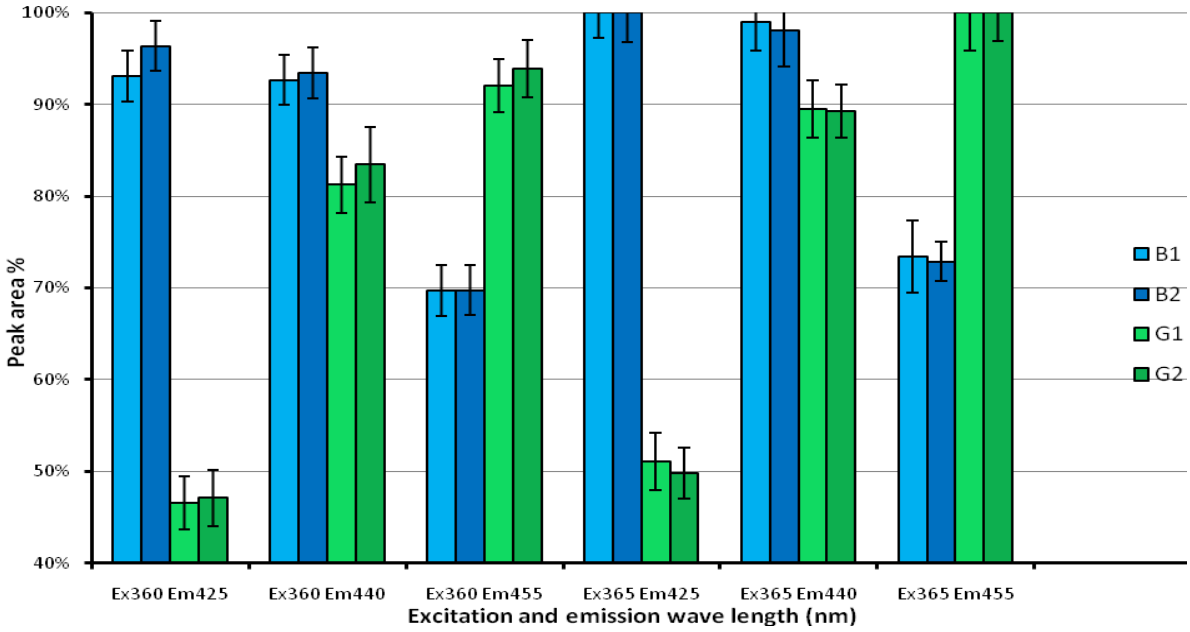


Figure 5.3: *Effect of excitation and emission wavelength (nm) on the chromatographic peak areas of aflatoxins B1, B2, G2 and G1*

When the emission wavelength of aflatoxins was set at 425 nm, the best fluorescence signals in terms of signal-to-noise ratio and sensitivity were obtained for aflatoxin B1 and B2, while simultaneously obtaining the lowest fluorescence signals for aflatoxin G1 and G2. In contrast, when the emission wavelength of aflatoxins was set at 455 nm, the strongest fluorescence signals were obtained for aflatoxin G1 and G2, while B1 and B2 produced the weakest fluorescence signals. Setting the excitation wavelength at different values produced different fluorescence signal strengths for the aflatoxins. However, by setting the excitation wavelength at 365 nm, strong fluorescence signals were obtained for all aflatoxins.

Therefore, the 365nm excitation/455nm emission wavelength combination was selected as the most suitable for aflatoxin G1and G2, likewise the 365 nm excitation/425 nm emission wavelength combination was chosen for aflatoxin B1 and B2. The above-mentioned modifications resulted in improved sensitivity. Alternatively, it was found that the wavelength setting at 365 nm excitation and 440 nm emission combinations could be used as the compromise wavelength for all aflatoxins, since it gave reasonable fluorescence signals.

5.3.1.2. Sample pretreatment optimization

For the mycotoxin determination, the sample pretreatment has a crucial impact on the accuracy of the results, especially when complex matrices such as cereals and nuts are analyzed for the very low levels of aflatoxins, which may be in the food. To attain this goal, a simple QuEChERS extraction procedure was adopted before the chromatographic determination. The extraction solvent, type and amount of drying agent, the extraction time and the solvent sample ratio affecting the efficiency of extraction, were all taken into consideration and optimized.

5.3.1.2.1. Determination of the optimum duration and method of extraction

After addition of the extraction solvents to the falcon tube, the tube was closed and manually shaken vigorously for 1 minute, according to the original QuEChERS procedure, to disperse the solvent and analytes. The effects of the duration and method of extraction in the aflatoxin recoveries were investigated by hand-shaking the falcon tube for 1 min in addition to vortexing the falcon tube between 1 min to 4 min. Manual shaking of the falcon

tube for more than 1 min was not applied, as it was laborious. All experiments were carried out using the same procedure and were tested in a blank peanut sample that had been spiked with 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2. After that, the recovery results were compared.

Figure 5.4 shows that the extraction times affect the recoveries of aflatoxins. For the first stage of extraction, the recoveries increased by increasing the vortex time with up to 15% enhancement for all analytes. However, the recoveries remained almost the same after 2 min of extraction. This is because there is a large concentration gradient for the solutes in the peanut sample and the extraction solvent interacts with the sample at the initial stage of extraction resulting in higher extraction rates. Additionally, the constituents located in the surface layers of the particles are more readily accessible than those in the deeper regions, which hardly diffuse out and hence slow down the rate of extraction.

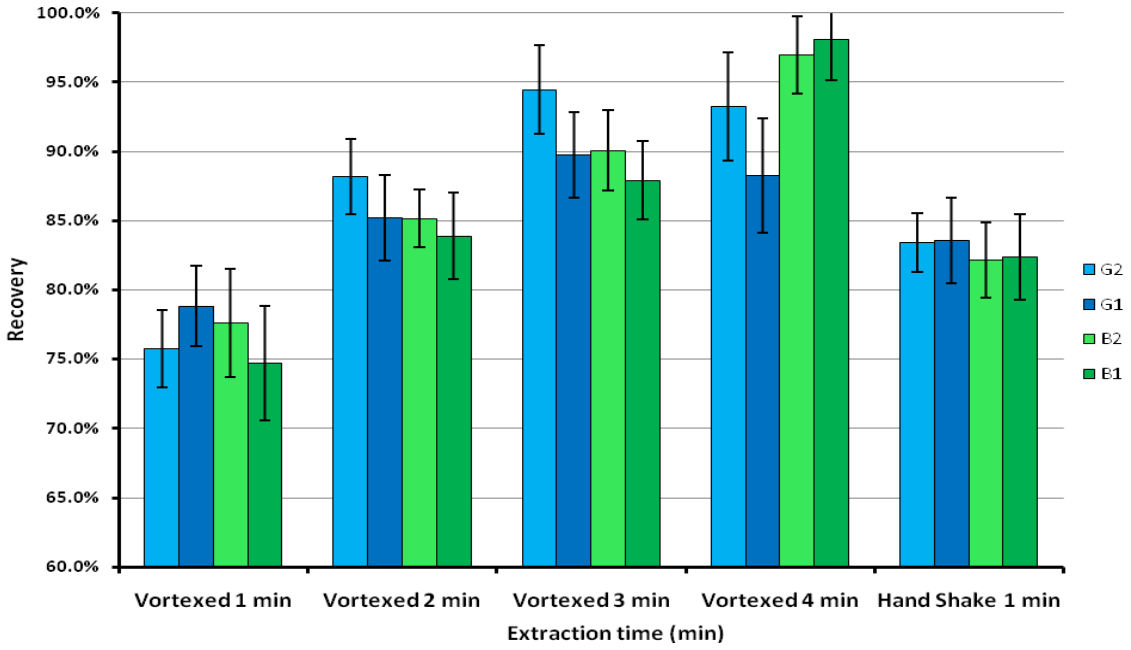


Figure 5.4: *Effects of duration and type of extraction on the recovery of aflatoxin B1, B2, G2 and G1*

Figure 5.4 indicates that manual shaking of the falcon tube for 1 min is more efficient than vortexing for the same period. This may be because hand shaking the falcon tube speeds up the extraction in addition to aiding in dispersing the solvent and analytes throughout the sample, thus ensuring that the solvent interacted well with the entire sample. However, manual shaking of the falcon tube for 1 min resulted in lower recoveries, which do not comply with the EU regulations. Therefore, a vortex mixer was employed, as it complies with the EU regulations in addition to being time and an effort-saving procedure.

5.3.1.2.2. Determination of the appropriate type and amount of drying agent

A drying agent is an inorganic hygroscopic (readily absorbs water from the air) salt. Several salts are used routinely in the QuEChERS procedure. The most common is anhydrous $MgSO_4$. Other inorganic sulfate salts such as sodium sulfate (Na_2SO_4) may also be used in the same way. These salts are added to the extracts with a combination of sodium chloride (NaCl) to remove excess water and unwanted contaminants from the extracted samples as well as to provide a separate and distinct phase to facilitate the penetration of the mycotoxin into the organic layer.

In order to investigate the effects of adding different drying agents, blank peanut samples were spiked with 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2, and analyzed after the addition of either anhydrous $MgSO_4$ or anhydrous Na_2SO_4 and the results from both analyses were compared. The results in Figure 5.5 show that anhydrous $MgSO_4$ is a far more effective drying agent than anhydrous Na_2SO_4 and gave higher recoveries for both the targeted aflatoxins. An explanation for this observation is that

anhydrous $MgSO_4$ is a fine powder while anhydrous Na_2SO_4 has a larger particle size, so that the drying power of $MgSO_4$ is greater together with its substantially higher capacity.

However, since anhydrous $MgSO_4$ is a highly exothermic compound, it is added to the extracts and not vice versa to avoid elevating the temperature in the falcon tube, which may lead to analyte decomposition and subsequently a reduction in the recoveries. For this reason, it is recommended to add ice into the falcon tube before adding the anhydrous $MgSO_4$ to absorb the released heat. This effect was investigated by comparing two blank peanut samples that were spiked with 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2. The first sample was placed overnight in a freezer at -18^{o}C and the second sample was left at room temperature. Both of them were analyzed and the results were compared (Figure 5.5).

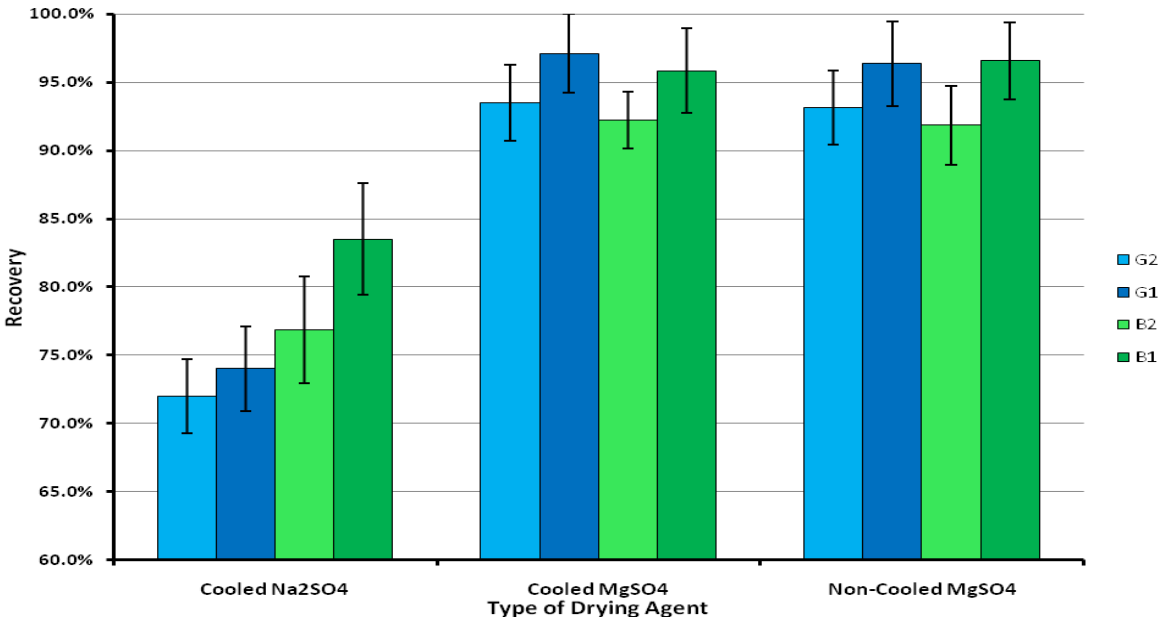


Figure 5.5: *Comparison between drying agent type as well as comparison between a cooled falcon tube and non-cooled falcon tube effect on the recovery of aflatoxin B1, B2, G2 and G1*

Figure 5.5 indicates that recoveries for the selected aflatoxins were identical; this may be that the heat released after addition of anhydrous $MgSO_4$ was not sufficiently high enough to affect the analytes stability. Therefore, it is not necessary for an additional pre-cooling step of the falcon tubes.

The amount of anhydrous $MgSO_4$ required depends on the amount of water in the extraction solvents as well as in the samples. Therefore, the effect of the amount of anhydrous $MgSO_4$ in the recoveries was investigated by adding different amounts of anhydrous $MgSO_4$ from 0.5 to 4.0 g together with a constant amount of NaCl (Figure 5.6).

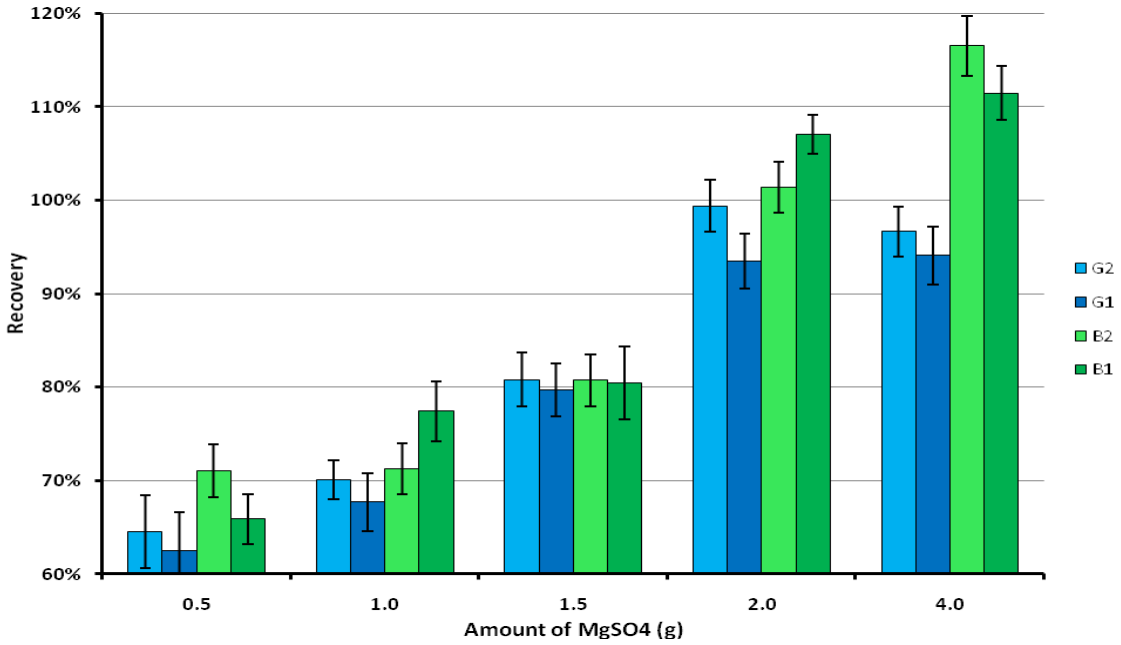


Figure5.6: *Effects of addition of various amounts of anhydrous $MgSO_4$ on the recovery of aflatoxin B1, B2, G2 and G1*

Figure 5.6 indicated that the recoveries were increased as the amount of anhydrous $MgSO_4$ was increased from 0.5 to 4.0 g. However, after addition of 2.0 g of anhydrous $MgSO_4$, it was found that the recoveries were not further increased for the majorities of the

selected analyte. From these observations, it was determined that the addition of 2.0 g of anhydrous $MgSO_4$ is sufficient for the extracts without an additional pre-cooling of the falcon tube.

5.3.1.2.3. Effect of Dilution on Sample Extraction

Dilution of the sample extract is usually the method of choice to eliminate and reduce the matrix effects; dilution will also increase the extraction efficiency by decreasing the number of competing molecules per drop area to enhance the release of the analyte from the matrix. In addition, sample dilution shifts the equilibrium towards the free form of the analyte from the "bound" form with the matrix components. However, further dilution may adversely affect the sensitivity and lead to poorer detection limits. Therefore, it is necessary to look for an optimal dilution. In this study, the effect of dilution of the sample extracts was investigated by adding different amounts of extraction solvents ranging from a dilution ratio of 1:2 to 1:5 (Figure 5.7).

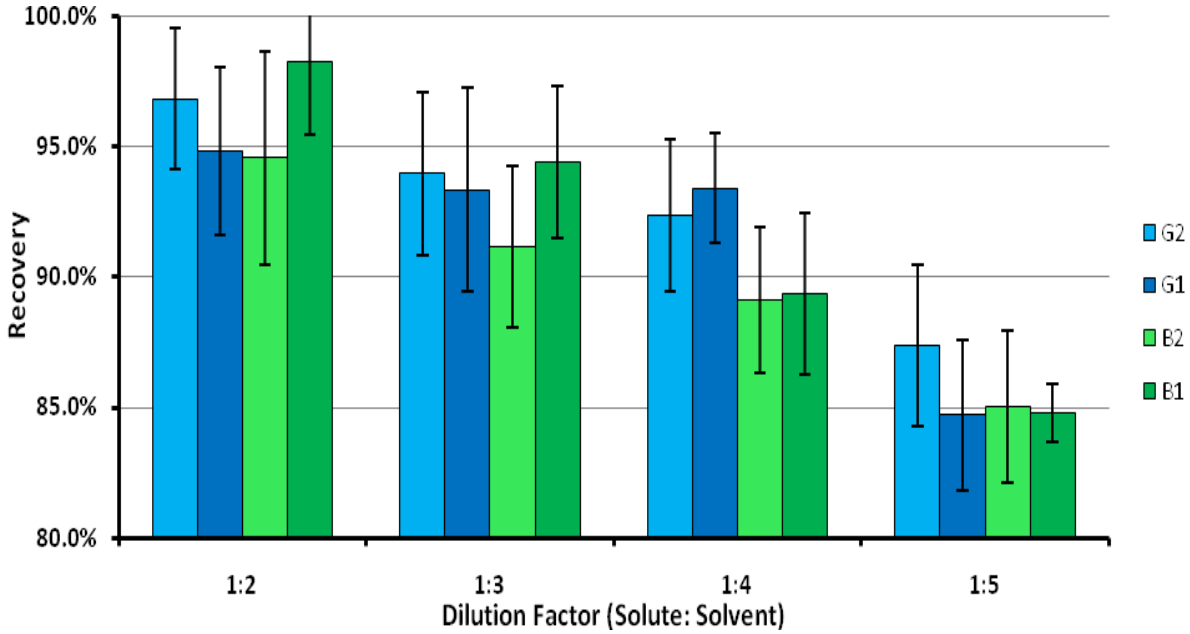


Figure 5.7: *Effects of dilution of the sample extract on the recovery of aflatoxin B1, B2, G2 and G1*

From Figure 5.7, it was found that for all the investigated dilution ratios, the recoveries for all target compounds fall within the acceptable range (between 85 to 98%) as well as complying with the EU regulations. It was found that increasing the amount of extraction solvents has a limited effect on the recoveries. Based on these data, dilution in the ratio of 1:3 was chosen as a good compromise between increased extraction efficiency and loss of sensitivity, and it was thus adopted for the subsequent experiments.

5.3.1.2.4. Optimization of the Extraction solvent

Optimization of the effect of extraction solvent was investigated by using the design of the experiment (DOE) technique which utilizes the response surface methodologies (RSM) by applying mathematical and statistical techniques (Correia *et al.*, 2004). The basic assumption is that there is a possible interaction effect of factors which depend solely on

the component proportions of the mixture and not on the component quantities; thus, the response of the property is entirely determined by the proportions of the total. Therefore, it is necessary to select the appropriate mixtures from which the response surface might be calculated; and having the response surface, a prediction of how such changes will affect the properties of the mixture can be obtained for any mixture by changing the mixture composition. The optimal extraction mixture of aflatoxins from food extracts was analyzed using the JMP® 9.0.0 software (JMP®, 2010).

From a review of the literature, the use of different volume proportions of water, methanol and acetonitrile were frequently employed as the solvent mixture to extract aflatoxins (Ardic *et al.*, 2008; Bacaloni *et al.*, 2008; Elbert *et al.*, 2008; Leong, Ismail, Latif, & Ahmad, 2010). Therefore, the DOE technique was applied to select the best combination of the three solvents (water, methanol and acetonitrile) to optimize the recovery of the aflatoxins in the food extracts by spiking a blank peanut sample with 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2. The ABCD design with 21 experimental points was performed in duplicate randomly at all points. The experimental data were then fitted to a quadratic polynomial model. An overlay contour tertiary plot (mixture profiler) of the aflatoxins recovery with 21 experimental points was then constructed as depicted in Figure 5.8.

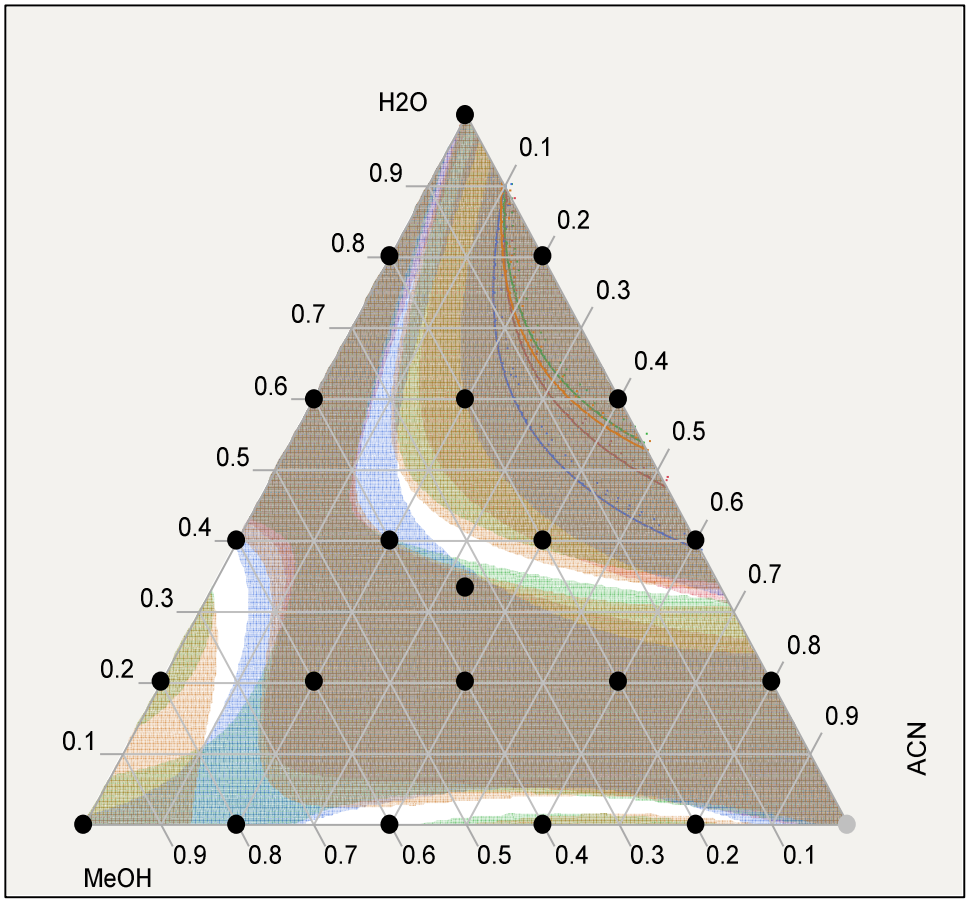


Figure 5.8: *An overlay contour plot (mixture profiler) of aflatoxins recovery with 21 experimental points (the black dot point). The non-colored area as shown indicates the desirability of aflatoxins recovery (85-105%).*

The non-colored area in the plot in Fig 5.8 indicates the desirability of aflatoxins recovery (85-105%). To get the maximum desirability recoveries of the investigated analytes, the prediction profiler was applied, which shows the principal effects of the factors on the response. The diagram was constructed with assigned units to give immediate values for comparison (Figure 5.9 and Figure 5.10).

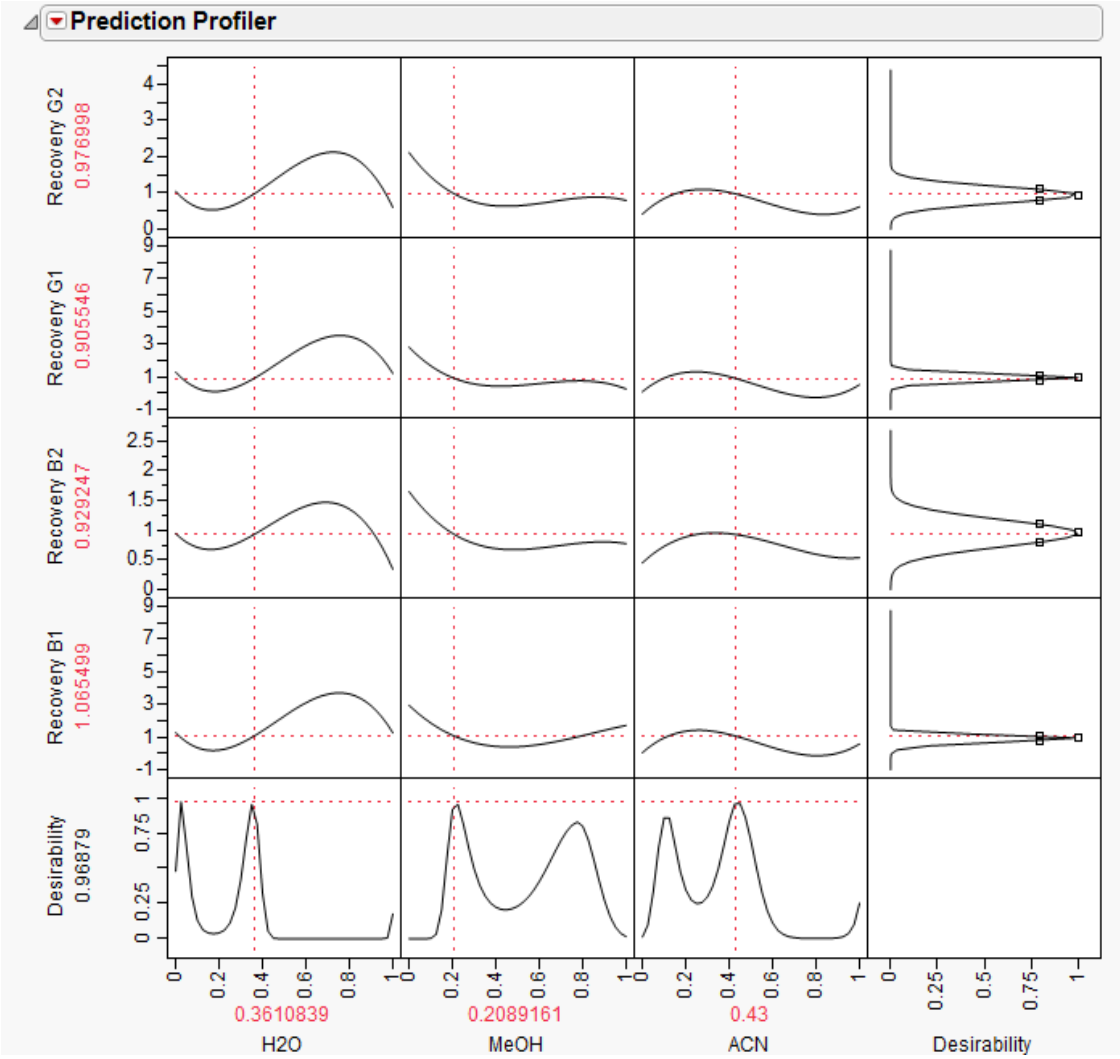


Figure5.9: *The profiler in "Maximum Desirability in Profiler for Mixture Analysis," displays optimal settings of 0.36 for water, 0.21 for methanol and 0.43 for acetonitrile, which give an estimated recovery between 91% and 101% of peanut samples spiked at 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2*

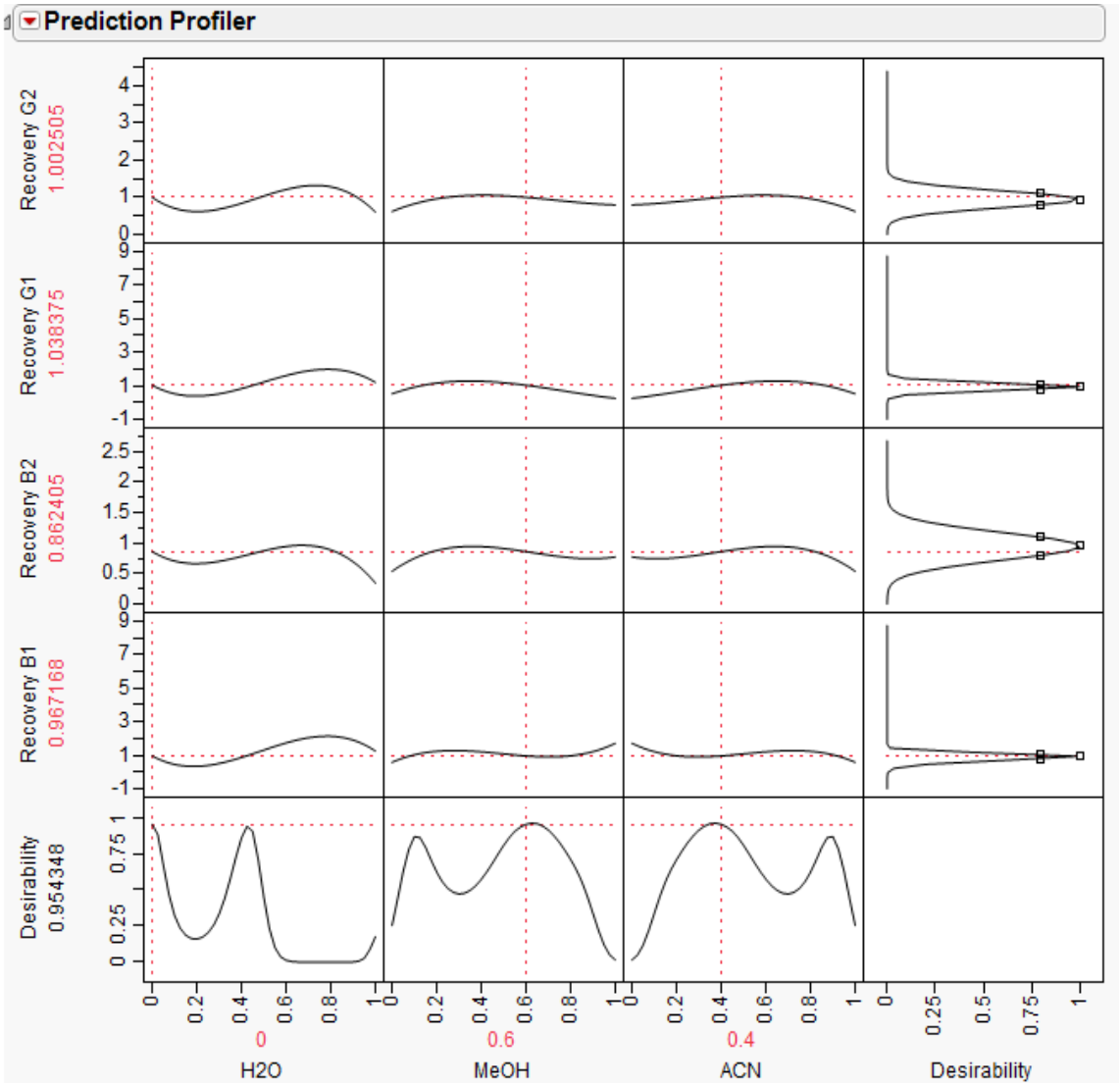


Figure5.10: *The profiler in "Maximum Desirability in Profiler for Mixture Analysis," displays optimal settings of 0.60 for methanol and 0.40 for acetonitrile, which give an estimated recovery between 86% and 104% of peanut samples spiked at 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2*

Figure 5.9 and 5.10 indicated that high extraction efficiency of the selected analyte was achieved when using 43:21:36 (%, v/v) water/ methanol/acetonitrile and 60:40 (%, v/v) methanol/acetonitrile. The second composition was selected; since this combination gave the desired responses as well as not containing water. In the presence of large amounts of water, anhydrous $MgSO_4$ tend to form lumps that can harden rapidly and reduce the

supernatant layer. In addition, the water content cannot be evaporated easily and be reconstituted in a different solvent (mobile phase) compared to either methanol or acetonitrile. Therefore, mixtures of 60:40 (%, v/v) methanol/acetonitrile were applied in this study, since it gave recoveries fulfilling the EU Commission Directive 2006/401/EC for the analysis of mycotoxins in food samples (European Commission, 2006a).

5.3.1.3. Method validation

The in-house developed method was validated in terms of linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), intra-day precision and inter-day precision. The linearity was tested using a standard solution of the aflatoxins in the concentration range of 0.059 to 30.000 µg/kg for aflatoxin B2 and G2 and from 0.195 to 100.000 µg/kg for aflatoxin B2 and G2 using a least-squares regression analysis. Table 5.2 shows that good linear relationships with correlation coefficients greater than 0.993 for all the targeted analytes were obtained. Calibrations with standard solutions were used for quantification, because the ANOVA test did not show any significant difference ($P < 0.05$).

Table 5.2
Linearity range, Equation and r^2 value of aflatoxins

Aflatoxin	Linearity Range (µg/kg)	Equation	r^2
B1	0.195 – 100.00	Y = (22078.573601) +(67517.549908) X	0.99396
B2	0.059 – 30.00	Y = (8866.164274) +(151599.561135) X	0.99648
G1	0.195 – 100.00	Y = (14087.825485) +(25425.320385) X	0.99446
G2	0.059 – 30.00	Y = (11493.592180) +(71338.219538) X	0.99536

Figure 5.11 shows the HPLC chromatogram of aflatoxin standard solutions containing 3.125 µg/L of aflatoxin B1 and G1 and 0.938 µg/L of aflatoxin B2 and G2.

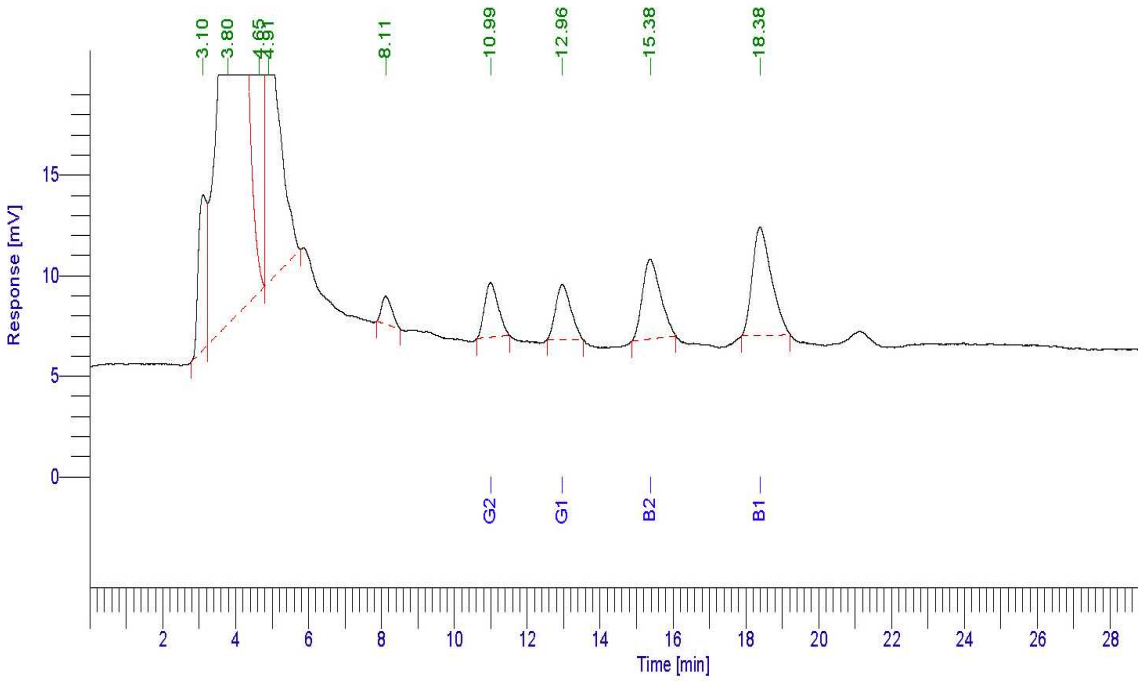


Figure 5.11: *HPLC chromatogram of aflatoxin standard solutions containing 3.125 µg/L of aflatoxin B1 and G1 and 0.938 µg/L of aflatoxin B2 and G2*

The accuracy was tested by the determination of the recoveries of the aflatoxins in lean peanut samples spiked in triplicates at three-fortification levels (5, 10, and 50 µg/kg for aflatoxin B1 and G1, and 1.5, 3.0, and 5.0 µg/kg for aflatoxin B2 and G2) (Table 5.3).

Table 5.3
Mean of recoveries and RSDs (n=5)[a] of aflatoxins spiked into clean wheat, corn, rice, pistachio nut, peanut, almond and sesame seed samples at three spiking levels using QuEChERS-HPLC method

Aflatoxin	Spiking Level (µg/kg)	Matrix						
		Wheat	**Corn**	**Rice**	**Pistachio Nut**	**Peanut**	**Almond**	**Sesame Seed**
		Mean of Recovery (%) ± RSD (%)						
G2	1.5	75.7±7.6	78.6±5.8	96.3±5.9	98.3±8.2	80.5±5.3	76.3±3.4	106.7±12.0
	3.0	74.5±4.4	82.3±6.7	104.4±4.9	100.1±8.1	95.3±9.2	75.9±6.2	102.5±13.9
	15	85.4±5.6	92.1±4.9	94.3.8±4.5	102.9±7.6	97.5±7.8	98.0±9.9	107.5±9.5
G1	5	73.1±9.8	83.2±2.7	102.8±8.3	104.4±6.1	95.5±4.6	81.5±4.5	86.0±8.3
	10	87.7±9.0	82.9±7.2	91.9±10.8	100.0±10.4	92.2±5.7	86.0±6.2	102.6±9.7
	50	81.6±9.8	91.5±6.9	105.7±7.8	96.1±9.8	98.0±6.7	90.4±6.1	103.4±8.2
B2	1.5	73.8±9.4	84.0±8.5	90.0±13.2	84.0±7.7	77.8±7.7	71.9±5.6	102.7±11.9
	3.0	82.1±5.7	73.3±8.4	101.9±6.8	90.1±8.1	82.4±4.6	80.6±6.2	103.4±13.8
	15	87.5±7.4	81.0±6.2	103.6±4.5	98.0±4.5	93.5±4.4	86.2±6.4	106.7±12.0
B1	5	75.4±4.5	78.7±7.2	97.4±4.8	81.2±5.0	76.3±6.3	80.8±9.8	86.1±14.2
	10	71.8±7.4	84.2±8.4	104.9±10.8	95.6±8.1	89.4±7.2	80.7±6.2	107.7±10.8
	50	84.1±4.5	84.7±5.9	97.0±9.4	97.6±5.1	97.0±8.6	87.7±9.5	97.0±9.4

[a] n: is number of replicates

The recoveries obtained ranged between 76.3% and 98.0%, with RSD values of less than 10%. Alternatively, the method was tested for other matrices: corn, rice, pistachio nut, almond and sesame seed samples. The recovery results ranged between 71.8% and 107.7%, with RSD values of less than 15% at concentrations of 1.5 and 3.0 µg/kg for aflatoxin B2 and G2 as well as 5.0 and 10.0 µg/kg for aflatoxin B1 and G1. While the recoveries at the concentrations of 15.0 µg/kg for aflatoxin B2 and G2 and 50.0 µg/kg for aflatoxin B1 and G1were from 81.0 to 107.5 %, with RSD values of less than 13%. These results fulfilled the requirements established by the European Union for the determination of aflatoxin contaminants in food samples (European Commission, 2006a).

The sensitivity of the method was estimated by the LOD and LOQ. The LOD was verified as the lowest concentration giving a response of three-times the average of the base-line noise obtained from non-contaminated aflatoxin peanut samples spiked with a mixed standard stock solution containing the four(4) investigated aflatoxins. The LOQ was verified as the lowest concentration giving a response of six-times the average of the base-line noise obtained from blank peanut samples spiked with a mixed standard stock solution containing the four(4) investigated aflatoxins.

The LOD values obtained (see Table 5.4) are between 0.16 µg/kg for aflatoxin B2 and 1.06 µg/kg for aflatoxin G1. While the LOQs for aflatoxin B1 are lower than 2.00µg/kg, which is below the maximum permitted level as per the European regulations (2.00µg/kg for aflatoxin B1 and 4.00µg/kg for total aflatoxins in foodstuffs for direct human consumption with the exception of infant food) (European Commission, 2006b).

Table 5.4
LOD and LOQ of aflatoxins

Aflatoxin	LOD (µg/kg)	LOQ (µg/kg)
B1	0.17	0.57
B2	0.05	0.18
G1	0.35	1.17
G2	0.06	0.20

The intra-day precision and inter-day precision were evaluated by testing the RSD values of blank peanut samples spiked at 10 µg/kg with aflatoxin B1 and G1 and 3 µg/kg of aflatoxin B2 and G2. Five replicates for each concentration level were analyzed during each working day to test the intra-day precision and subsequently during five consecutive days to test for inter-day precision. The intra-day precision and inter-day precision results for all aflatoxins in the peanut samples showed acceptable precision. The RSD values for the intra-day precision and inter-day precision were below 12.0% and 18.0 %, respectively (Table 5.5).

Table 5.5
The intra-day precision and inter-day precision of aflatoxins expressed as RSD values

Aflatoxin	Spiking Level (µg/kg)	Intra-Day Precision (n = 5)[a]	Inter-Day Precision (n = 15)[a]
B1	10	6.9	11.4
B2	3	11.6	17.1
G1	10	6.9	10.1
G2	3	10.9	15.0

[a] n: is number of replicates

5.3.2. Fluorometric method

For the quantitative analysis of aflatoxins in food samples, the fluorometric method gave unsatisfactory results. This method has a poor precision since both positive and negative biases were detected (Table 5.6). In addition, it suffers from matrix effects as the results varied when the composition of the matrix was changed. However, the intra-day precision and inter-day precision were acceptable with RSD values for both the naturally contaminated food and the spiked sample of less than 9 % and 8%, respectively.

Table 5.6
Mean of recoveries and RSD values (n=5)[a] of total aflatoxins spiked into clean wheat, corn, rice, pistachio, peanut, almond and sesame samples at two spiking levels using fluorometric method

Spiking Level (µg/kg)	**Matrix**						
	Wheat	**Corn**	**Rice**	**Pistachio Nut**	**Peanut**	**Almond**	**Sesame Seed**
	Mean of Recovery (%) ± RSD (%)						
5	118.8±6.9	137.5±8.5	140.2±2.4	82.5±5.2	123.8±6.7	175.0±4.1	118.8±1.3
10	83.1±4.5	118.8±5.5	190.8±6.7	221.9±2.1	96.9±4.6	106.3±6.7	336.3±4.7

[a] n: is number of replicates

A very significant factor in the validation of this method was the detection of matrix effects since both false positive and false negative results were noted (Table 5.6), with a positive and negative bias detected in the fluorometric method. The fluorometric method is a useful rapid screening technique capable of analyzing a large number of samples in a relatively short period. However, it would be necessary to confirm the positive results by other chromatographic methods.

5.3.3. Sample analysis

The developed method was applied for the analysis of commercial products supplied from the Jordanian food and drug administration. Only 20 samples showed aflatoxin contamination. Most of these contaminations involved peanuts (8 contaminations) originating from Egypt. Aflatoxins are also frequently reported in pistachio nuts (6 contaminations) originating from Syria and two contaminations were detected in sesame seeds originating from India, as well as, two contaminations were detected in raw peanut butter originating from both China and India. Finally, one contamination was reported in almonds (in shell) originating from Syria as well as one in sunflower seeds originating from the United States. For cereals, coffee and spices, none of the samples showed any detectable concentration of aflatoxins. The contaminated peanuts and pistachio nuts are all without shell. This may be because the micro-environment beneath the shell with the protection of the kernels is less susceptible to cross-contamination and mold growth, with consequently less toxin production.

The sharp increase of contamination by aflatoxins has caused concern to the authorities, since approximately 2.99% of 669 domestic samples and imported samples examined between April and November 2011 are contaminated by aflatoxins at levels higher than the levels permitted in Jordanian Regulations set by Jordan Institution for Standards & Metrology (JISM).

These results have shown that contamination by aflatoxins are two times higher than what was reported in previous studies (1.38 %) since 37 out of 2745 samples examined between January 2009 and March 2011 showed aflatoxin contamination (Table 5.7 and Figure 5.12), which used thin layer chromatography (TLC) as a screening method and fluorometric determination as the quantification method. TLC has been the most widely

employed method for both qualitative and quantitative determination of aflatoxins since 1960s. It has been adopted as the official method for aflatoxins in food by the AOAC since 1990 (Rahmani *et al.*, 2009) Despite its advantages such as low cost, useful and an easy technique, it is time-consuming, has extremely low sensitivity for aflatoxins B1, B2, G1 and G2 (Var *et al.*, 2007) and gives unsatisfactory accuracy in quantification. Therefore, many researchers have used HPLC methods to achieve accurate quantification (Herzallah, 2009; Micheli, Grecco, Badea, Moscone, & Palleschi, 2005). However, fluorometric determination, as mentioned earlier, suffers from matrix effects, so that it generally used as a rapid screening technique followed by more accurate chromatographic methods. Therefore, the differences in sample preparation and measurement methods may be responsible for these variations.

Table 5.7
Occurrence of aflatoxins in commercial Jordanian foods from January, 2009 to November , 2011

Sample category	Total Number of Samples of the Particular Category	Number of Positive Samples	Incidence (Positive/Total Number of Samples of the Particular Category)	Incidence (Positive/Total number of Samples)	Total Aflatoxins (µg/kg)
Fluorometric Method (January, 2009 – March, 2011)					
Cereals[b]	792	4	0.5%	0.1%	23- 110
Peanut and Peanut Butter	536	19	3.5%	0.7%	32- 1200
Nuts[c]	412	0	0.0%	0.0%	–[a]
Sesame Seed	156	0	0.0%	0.0%	–[a]
Pistachio Nut	287	11	3.8%	0.4%	35- 2065
Seeds[d]	290	4	1.4%	0.1%	23- 150
Coffee	272	0	0.0%	0.0%	–[a]
Spices[e]	66	0	0.0%	0.0%	–[a]
Total	**2745**	**38**		**1.4%**	
QuEChERS HPLC Method (April, 2011- November, 2011)					
Cereals[b]	274	0	0.0%	0.0%	–[a]
Peanut and Peanut Butter	87	10	11.5%	2.6%	18- 400
Nuts[c]	78	1	1.3%	0.3%	–[a]
Sesame Seed	46	2	4.3%	0.5%	100- 1280
Pistachio Nut	61	6	9.8%	1.5%	21- 1645
Seeds[d]	51	1	2.0%	0.3%	760
Coffee	59	0	0.0%	0.0%	–[a]
Spices[e]	13	0	0.0%	0.0%	–[a]
Total	**669**	**20**		**3.0%**	

[a]: Values were below the LOD of the adopted method

[b]: rice, corn, corn and potato starch, wheat, semolina, flour,..etc

[c]: walnut, cashew nut, almond, pine nut and hazelnut

[d]: pumpkin seed, watermelon seed and sunflower seed

[e]: cardamom and chili

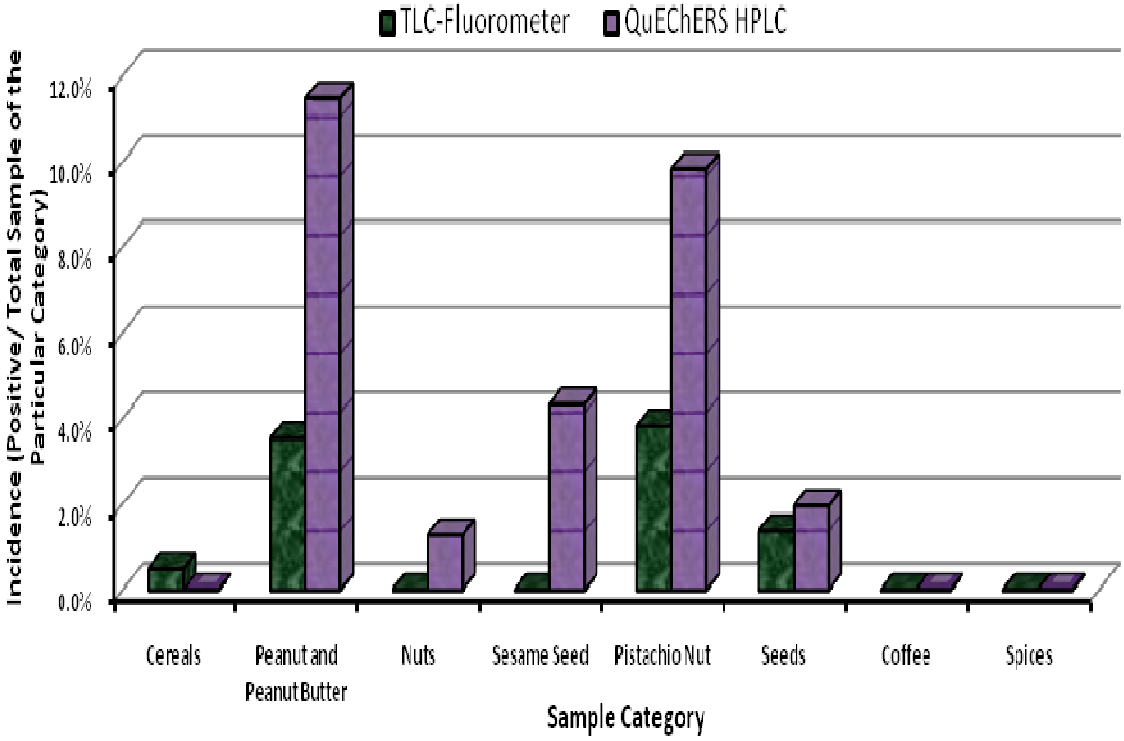


Figure 5.12: *Comparison between TLC-Fluorometer method and QuEChERS- HPLC method on incidence of contamination for various sample categories*

Figure 5.12 and Table 5.7 revealed a wide variation among sample categories in aflatoxin contamination across the 669 samples analyzed. There is a significant difference ($P > 0.05$) in aflatoxin contaminations between different types of sample categories. Approximately 11.5% of peanuts and peanut butters examined were contaminated with aflatoxins in the range of 18- 400 µg/kg; 9.8% of pistachio nuts examined contained aflatoxins at concentrations of 21- 1645 µg/kg; 4.3% of sesame seeds examined contained aflatoxins at concentrations of 100 and 1280 µg/kg; 2.0% of nuts examined contained aflatoxins at concentrations of 1680 µg/kg. However, samples of cereals, coffee and spices were found to be non-contaminated with aflatoxins. This may be because there were contaminated with other types of mycotoxins or the contaminations were below the LOD of the adopted method.

5.4. Conclusion

A simple, rapid, inexpensive and effective sample preparation procedure using QuEChERS procedure has been developed. The developed sample preparation procedure involved the extraction/partition of samples with 60:40 (%, v/v) methanol/acetonitrile mixture without employing a prior dSPE clean-up. Therefore, it can be recommended as an alternative to the time-consuming solid phase extraction or liquid–liquid extraction as well as to the more expensive immunoaffinity columns and multifunction column for aflatoxins determination in food. The extraction solvent was found to be the most important factor and it heavily affects the extraction efficiency. Other factors such as the type and amount of drying agents, the extraction time and the solvent-sample ratio have little effects. On the other hand, it was found that the wavelength setting at 365nm excitation and 440nm emission could be used as the optimum wavelength for all aflatoxins. Excellent linearity, high recoveries, acceptable repeatability and reproducibility with the LOQ values lower than the stipulated MRL were achieved indicating the suitability of the QuEChERS HPLC-FLD method for the determination of aflatoxins in various food commodities. On the other hand, the fluorometric method has a poor precision since both positive and negative biases were detected. Therefore, for the fluorometric method, it would be necessary to confirm the positive results by other chromatographic methods. Finally, it would be necessary to scrutinize the sharp increase of aflatoxins contaminations on the pistachio and peanut samples imported from Syria and Egypt, respectively, given the reported results from this and other studies.

6. QuEChERS extraction and HPLC-FLD determination of Ochratoxin A in cereals and cereal Products

6.1. Introduction and scope of the work

Ochratoxins are secondary metabolites produced from moulds particularly *Aspergillus* and *Penicillium* (Atkins & Norman, 1998; Ghali *et al.*, 2009). Among the family of ochratoxins, which consists of three members, A, B, and C, the most toxic and most commonly detected member in foodstuff are ochratoxin A (OTA) (Atkins & Norman, 1998; Peraica *et al.*, 1999). The primary source of ochratoxin contamination in food and feedstuff is cereal commodities (maize, oats, barley and wheat) in addition to groundnuts, dried fruits and coffee beans, which have been infected by the *P. verrucosum* and *A. ochraceus*.

The International Agency for Research on Cancer (IARC) has classified ochratoxin A (OTA) as a group 2B carcinogen, as it has been shown to be nephrotoxic, hepatotoxic, immunosuppressive, teratogenic and have carcinogenic effects on animals and humans (IARC, 1993). As a result of its toxicity and frequent occurrence, several countries have established legal regulations or recommendations to control mycotoxin contamination of various food products (FAO, 2004). The European Commission has published the Commission Regulation (EC) No. 1881/2006 setting maximum limits for OTA in the unprocessed cereals as 5 µg/kg and in the case of unprocessed cereal products it is 3 µg/kg (European Commission, 2006b).

However, the analytical determination of OTA in the food matrix is a difficult task, because of the complexity of the matrix and the low concentrations of OTA that are usually present. Therefore, the use of appropriate extraction and clean-up procedures in addition to sensitive, reliable and an accurate quantification method for the determination of OTA in foods are necessary. Various methods have been developed for the determination of OTA in food and feed comity as qualitative methods including enzyme linked immunosorbent assay (ELISA)(Flajs, Domijan, Ivic, Cvjetkovic, & Peraica, 2009) and fluorometer or as quantitative methods including high performance liquid chromatography with fluorescence detection (HPLC-FLD) (Flajs *et al.*, 2009; Ghali *et al.*, 2009) and liquid chromatography with tandem mass spectrometry (LC–MS/MS) (Reinsch, Töpfer, Lehmann, Nehls, & Panne, 2007). To reduce the sample handling and toxic wastes and consequently to maximize the recovery of the analytes and to attain quick sample turnaround time, the QuEChERS procedure was adopted. The aim of this study is to implement and validate the optimized QuEChERS-HPLC method for the determination of OTA in cereal samples at trace levels.

6.2. Experimental

6.2.1. Reagents and materials

The OTA analytical standard was obtained from Sigma-Aldrich (Malaysia). A working solution was prepared in acetonitrile and stored at -20 °C in amber glass vials over a period of two months. The external standard solutions used for the calibration curve for the HPLC experiments were prepared by further dilution of the working solution with the mobile phase.

HPLC-grade acetonitrile and methanol used for the mobile phase were purchased from Merck (Darmstadt, Germany), whereas analytical grade acetonitrile and methanol used for extraction were purchased from Fischer Scientific (Leicestershire, UK). HPLC-grade glacial acetic acid was purchased from Fischer Scientific (Leicestershire, UK). Water was purified by reverse osmosis followed by an electrodeionization (EDI) system (Maxima Ultra Pure Water, England). The 0.45 μm disposable membrane filters were purchased from Cronus Filter (UK). Anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) were purchased from Agilent Technologies (USA).

6.2.2. Sample preparation

The samples were prepared similar to the previous QuEChERS method with some modification.

Step I: A thoroughly homogenized cereal sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 mL).

Sample recovery was done with (1.0 g) of the clean wheat, wheat products (wheat-based noodles), rice, rice products (rice-based noodles), and corn samples spiked with 10.0, 20.0 and 40.0 μg/kg of the OTA standards. The spiked samples were left overnight in the dark at room temperature to allow the solvent to evaporate and for OTA absorption into the matrix. Then they were extracted via the following steps (II to IV).

Step II: 3.0 mL of 20:70:10 (%, v/v) water/ acetonitrile/ acetic acid mixture was added and the centrifuge tube was shaken for 1 min to ensure that the solvent has mixed thoroughly with the entire sample, for complete extraction of the analyte.

Step III: 0.8 g of anhydrous $MgSO_4$ and 0.2 g of NaCl were added into the mixture and the shaking procedure was repeated for 1 min to facilitate the extraction and partitioning of the OTA into the organic layer.

Step IV: The extract was centrifuged for 5 min at 4000 rpm, and 0.5 mL of the upper organic layer was filtered through a 0.45 µm nylon syringe filter prior to HPLC analysis.

6.2.3. HPLC analysis

The HPLC analysis were carried out with a Shimadzu LC-20AT system (Kyoto, Japan) consisting of degasser, tertiary pump, auto sampler, column oven and a fluorescence detector. The chromatographic separation was performed with a C_{18} 150 mm × 4.6 mm Spherisorb 5 ODS-1 (particle size 5µm) chromatographic column and was purchased from Phenomenex (USA). The cereals extracts were analyzed isocratically using 6% acetic acid in water and acetonitrile 25:75 (%, v/v) mixture as the mobile phase. The column was kept in a column oven at 30^{o}C at a flow rate of 1.0 mL/min. The injection volume was maintained at 10 µL for both the sample and standard solutions.

6.2.4. Food samples

In the month of December 2010, 1-2 kg each of 25 samples of cereals and cereal products (wheat, wheat based noodles, rice, rice based noodles and corn) was obtained from groceries and stores in Kuala Lumpur, Malaysia. The samples were stored in the dark at room temperature (25–30^{o}C). The samples were ground and mixed at room temperature for 10 min until a fine and homogeneous powdered material was obtained. The powdered samples were then stored in plastic bags at 4^{o}C in a refrigerator prior to analysis.

6.3. Results and discussion

6.3.1. Optimization of analytical conditions

To give the best separation in the chromatograms and to enhance sensitivity, the column type and its length, the mobile phase composition and excitation/ emission wavelength were investigated in this study. The heart of a chromatographic system is the column, where the actual separation of the analyte mixture occurs. Column selection depends strongly on prior knowledge of the samples to be analyzed, as well as the matrix. For example, non-polar and moderately polar compounds require conventional C_{18} column while polar compounds requires strong retention power column. OTA is a non-polar compound; therefore, conventional C_{18} has been applied in this study. Another criterion that was studied is the column length. Compared to the 25-cm column, the total run time was shorter in the case of the 15-cm column. This reduces the mobile phase consumption and analysis time required for routine work.

Different combinations of water/ acetonitrile/ acetic acid such as (73:25:2, 48:50:2, 23:75:2 and 13:85:2) were tested as the mobile phase in order to optimize the resolution of the OTA standard peak and to enhance the sensitivity. Broad peaks and long retention times were produced using the first and second mobile phase systems with high water composition. In contrast, OTA was not separated from the front peaks by the last mobile phase composition (13:85:2). The third mobile phase offered an adequate separation between the OTA and front peaks within 4 minutes retention times. The column temperature effect was also studied by varying the temperature from 20^{o}C to 50^{o}C with the objective of increasing the resolution. Furthermore, the OTA standard that was spiked into the blank wheat sample was applied to ensure that the OTA mix standards could be

distinguished and separated from interfering substances in the sample matrix. The chromatograms demonstrating the selectivity of the procedure are shown in Figure 6.1.

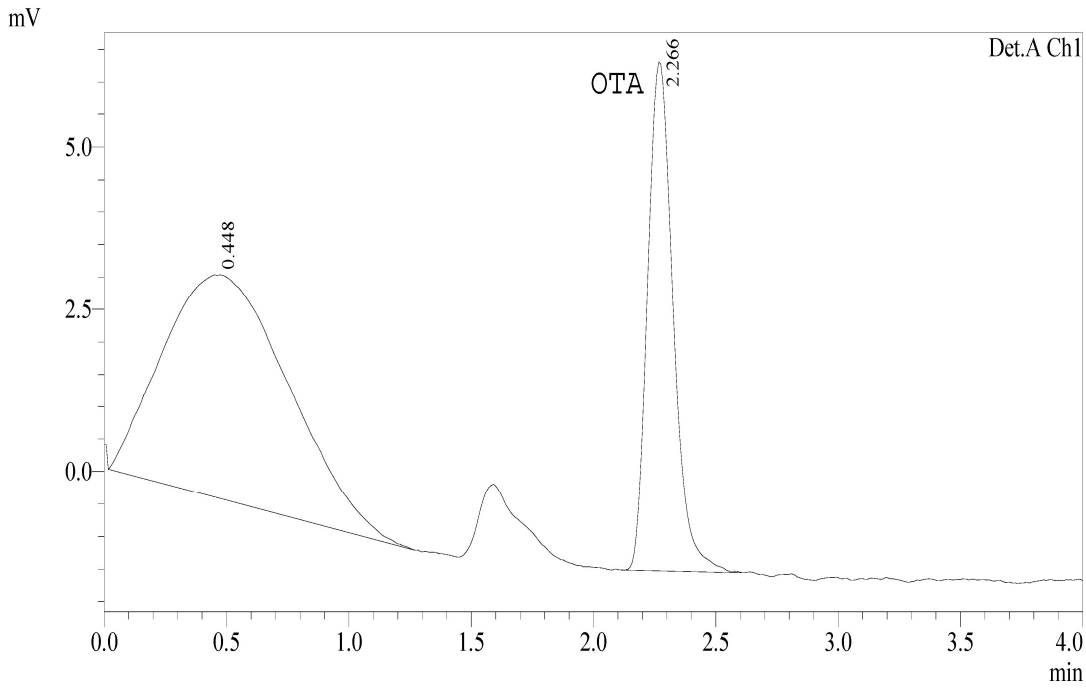


Figure6.1: *Representative HPLC chromatogram of blank wheat sample spiked at 10 μg/kg of OTA*

The chromatograms of the blank wheat sample that was spiked with 10 µg/kg of OTA showed that the peak of OTA is well-separated from interfering substances in the sample matrix with short retention times. The selectivity of the isocratic mixture of water/ acetonitrile/ acetic acid (23:75:2) at 30^{o}C column temperature is considered satisfactory as it enables OTA quantification in the analyzed food commodities with higher selectivity and sensitivity within a reasonable run time (see Figure 6.1). The repeatability and reproducibility of the retention times as measured by their relative standard deviation (RSD) ranged is 0.02% and 0.13%, respectively.

For the fluorescence detection, a spectrum of the OTA standard solution in the HPLC mobile phase was tested to optimize the detection of OTA and to obtain the best fluorescence signals in terms of signal-to-noise ratio and sensitivity. Various emission/excitation wavelengths were monitored and compared to obtain the best values in order to enhance the detection for OTA, as shown in Figure 6.2. When the emission wavelength of OTA was set at 465 nm, the best fluorescence signals in terms of signal-to-noise ratio and sensitivity were obtained for OTA. However, by setting the excitation wavelength at 333 nm, strong fluorescence signals were obtained for OTA. Therefore, the 333nm excitation/465nm emission wavelength combination was selected for all the subsequent experiments. The above-mentioned modifications resulted in improved sensitivity.

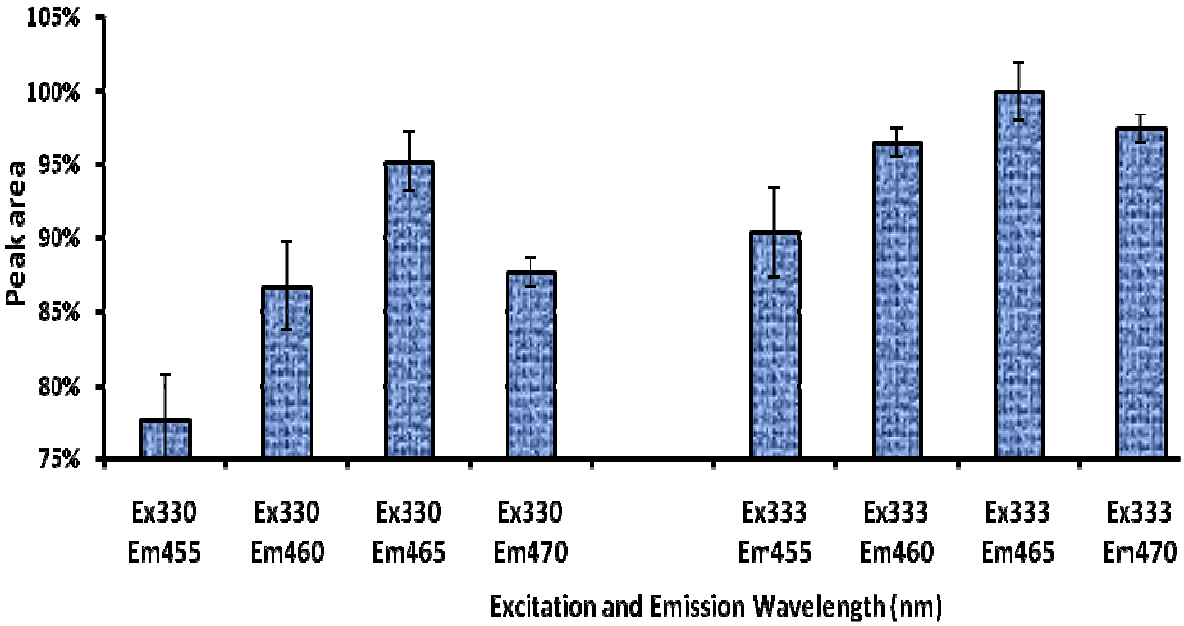


Figure6.2: *Effect of excitation and emission wavelength (nm) on the chromatographic peak area of OTA*

6.3.2. Sample pretreatment optimization

Sample pretreatment has a crucial impact on the accuracy of the results. The extraction solvent, the type and amount of drying agents, the extraction time and the solvent to sample ratio can affect the efficiency of extraction, therefore, there are taken into consideration and optimized. The extraction solvent was found to be the most important factor that heavily affects the extraction efficiency. Optimization of the effects of extraction solvent was investigated by using the design of the experiment (DOE) technique which utilizes the response surface methodologies (RSM) by applying mathematical and statistical techniques (Correia *et al.*, 2004). The basic assumption is that there is a possible interaction effect of factors which depend solely on the component proportions of the mixture and not on the component quantities; thus, the response of the property is entirely determined by the proportions of the total. Therefore, it is necessary to select the appropriate mixtures from which the response surface might be calculated; and having the response surface, a prediction of how such changes will affect the properties of the mixture can be obtained for any mixture by changing the mixture composition. The optimal extraction mixture of OTA from cereal extracts was analyzed using the JMP® 9.0.0 software (JMP®, 2010).

From a review of the literature, the use of different volume proportions of water, acetonitrile and acetic acid were frequently employed as the solvent mixture to extract OTA (Frenich *et al.*, 2009; Ghali *et al.*, 2009; Sulyok *et al.*, 2006). Therefore, the DOE technique was applied to select the best combination of the three solvents (water, acetonitrile and acetic acid) to optimize the recovery of the OTA in the cereal extracts by spiking a wheat sample with 10 µg/kg of OTA. The ABCD design with 9 experimental points was performed in duplicate randomly at all points. The experimental data were then fitted to a quadratic polynomial model. An overlay contour tertiary plot (mixture profiler)

of the OTA recovery with 9 experimental points was then constructed as depicted in Figure 6.3.

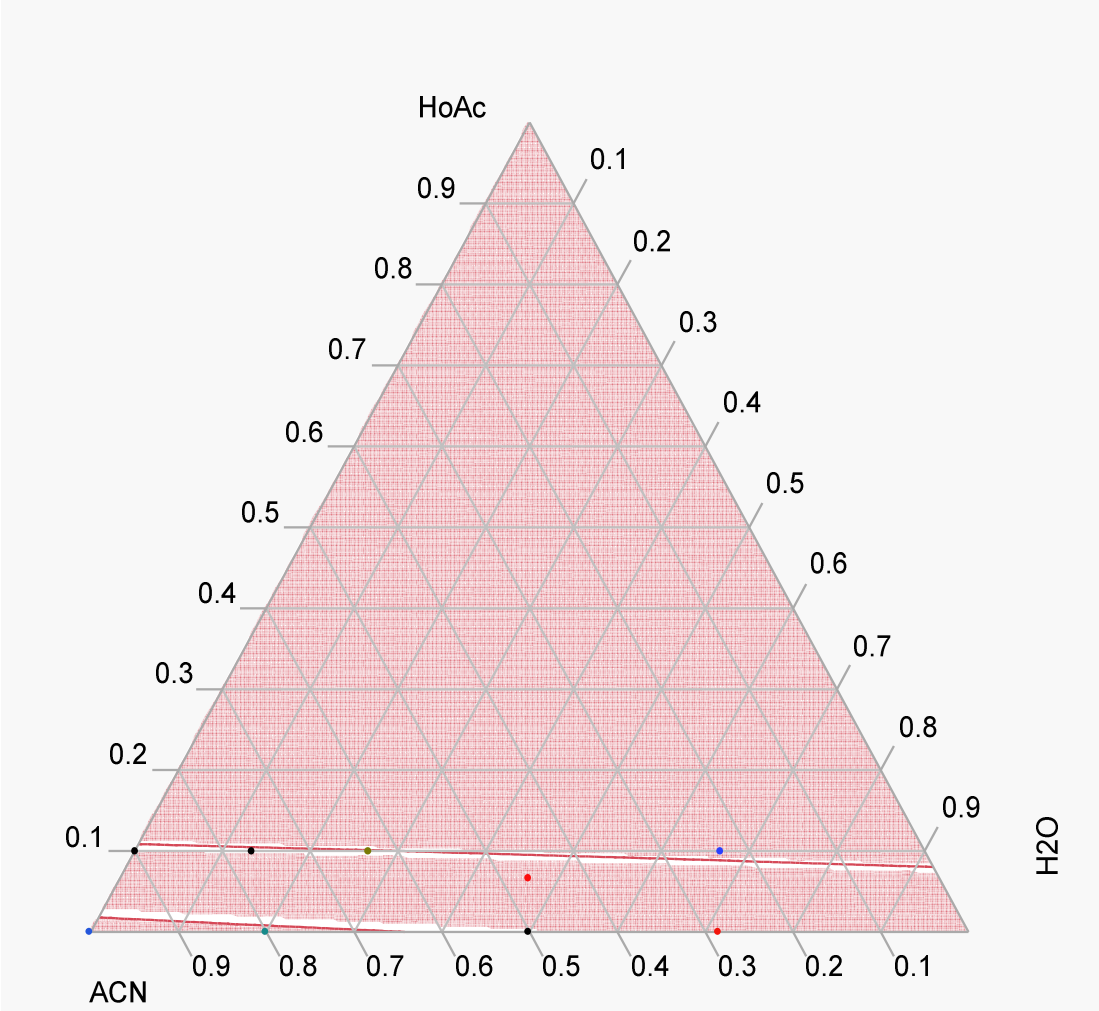


Figure 6.3: *An overlay contour plot (mixture profiler) of OTA recovery with 9 experimental points. The non-colored area as shown indicates the desirability of OTA recovery (85-105%).*

The non-colored area in the plot in Fig 6.3 indicates the desirability of aflatoxins recovery (85-105%). To get the maximum desirability recoveries of the investigated analytes, the prediction profiler was applied, which shows the principal effects of the

factors on the response. The diagram was constructed with assigned units to give immediate values for comparison (Figure 6.4 and Figure 6.5).

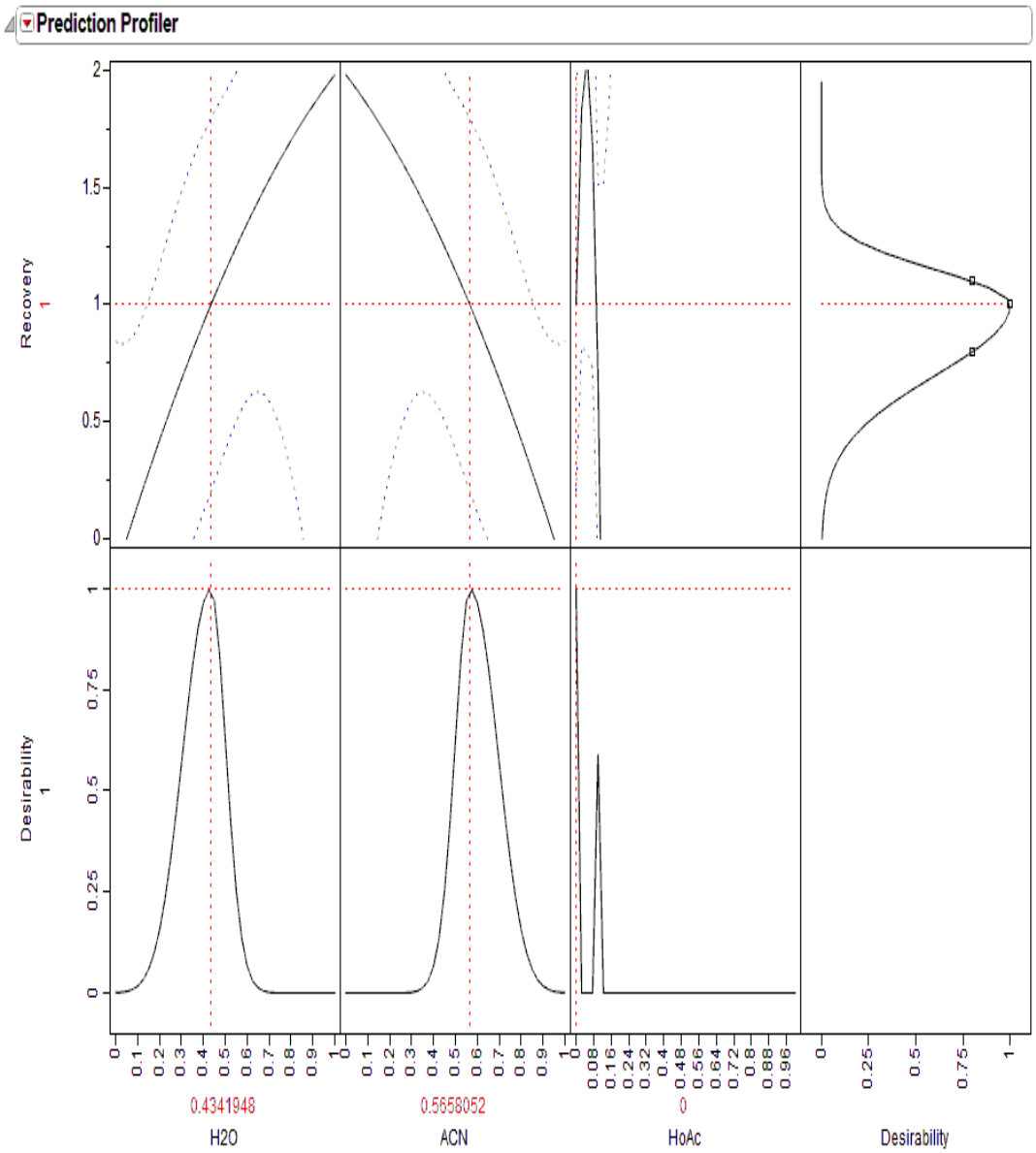


Figure6.4: *The profiler in "Maximum Desirability in Profiler for Mixture Analysis," displays optimal settings (rounded) of 0.43 for water, 0.57 for acetonitrile, and 0.0 for acetic acid, which give an estimated recovery of 1 (100%) of blank wheat samples spiked at 10.0 µg/L of OTA.*

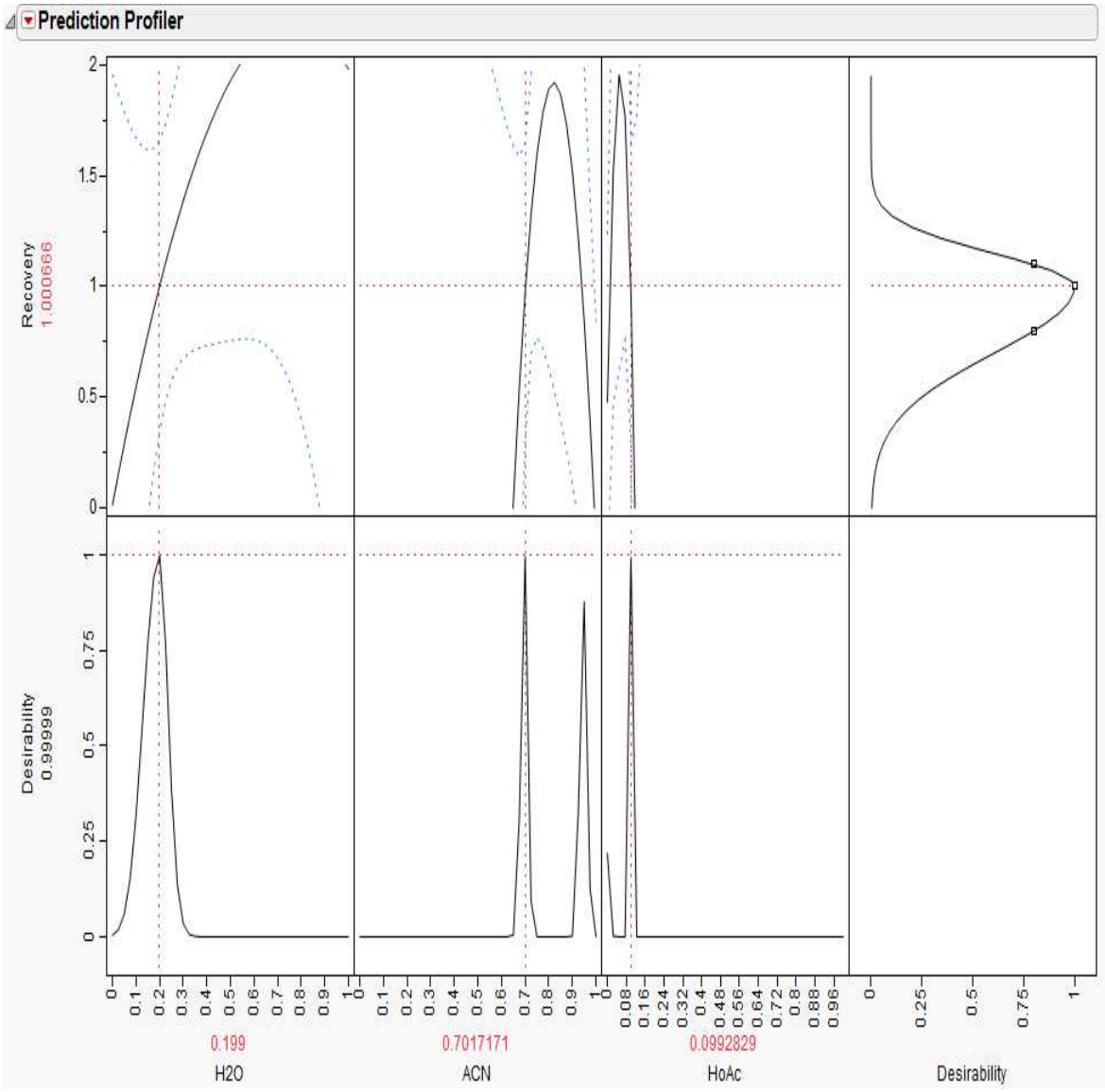


Figure6.5: *The profiler in "Maximum Desirability in Profiler for Mixture Analysis," displays optimal settings (rounded) of 0.20 for water, 0.70 for acetonitrile, and 0.10 for acetic acid, which give an estimated recovery of 1 (100%) of blank wheat samples spiked at 10.0 µg/L of OTA.*

Figure 6.4 and 6.5 indicated that high extraction efficiency (100% recovery) of the selected analyte was achieved when using 43.4:65.6 (%, v/v) water/acetonitrile and 20.0:70.0:10.0 (%, v/v) water/acetonitrile/acetic acid. The first one was avoided, since in the presence of high amounts of water, anhydrous $MgSO_4$ tend to form lumps that can

harden rapidly and reduce the supernatant layer. In addition, the water content cannot be evaporated easily and be reconstituted in a different solvent (mobile phase) compared to either methanol or acetonitrile. Therefore, mixtures of 20:70:10 (%, v/v) water/ acetonitrile/ acetic acid were applied in this study, since it gave recoveries fulfilling the EU Commission Directive 2006/401/EC for analysis of mycotoxins in food samples (European Commission, 2006a).

6.3.3. Method validation

This was an in-house validated method, in terms of linearity, accuracy, intra-day precision, inter-day precision, limit of detection (LOD), and limit of quantification (LOQ). The linearity was tested in triplicate using a standard solution of the OTA in the concentration range of 3.75 and 120 µg/L. Good linear relationships with correlation coefficients of 0.992 for the targeted analyte was obtained. Calibration with standard solutions was used for quantification by the least-square method and means comparison was made by ANOVA test ($p < 0.05$).

The accuracy was tested by the determination of the recoveries of the OTA in clean wheat, wheat products (wheat-based noodles), rice, rice products (rice-based noodles), and corn samples spiked with 20, 40.0 and 100.0 µg/kg of the OTA standards and analyzed in triplicates (Table 6.1). The recoveries obtained ranged from 85.2 ± 1.2% to 109.8 ± 2.9%, with a relative standard deviation (RSD) of less than 12%. The sensitivity was determined by estimating the limit of detection (LOD) and limit of quantification (LOQ). LODs and LOQs were estimated experimentally as the lowest concentration giving a response of three- and ten-times, respectively, the base-line noise. The LOQ were from 0.60–2.08 µg/kg (see the details in Table 6.1).

Table 6.1
Mean of recoveries and RSD values (n=5)[a], LOD and LOQ of OTA

Matrix	Recovery			LOD (µg/kg)	LOQ (µg/kg)
	20 µg/kg	**40** µg/kg	**100** µg/kg		
	Mean (%) ± RSD (%)				
Wheat	99.6±0.4	90.8±5.6	85.2±1.3	0.41	1.37
Wheat products	109.3±3.3	102.1±3.4	96.5±2.5	0.62	2.07
Rice	109.8±2.9	104.1±2.2	98.2±2.4	0.62	2.08
Rice products	106.6±2.8	100.6±3.4	86.4±1.7	0.18	0.60
Corn	100.0±1.6	98.0±6.0	86.4±1.0	0.47	1.57

[a] n: is number of replicates

Intra-day precision was evaluated by assaying five replicates of blank wheat sample at a spiked level of 10 µg/L OTA on the same day. For the inter-day precision, five replicates of blank wheat sample at a spiked level of 10 µg/L OTA were analyzed on three consecutive days. The intra-day precision and inter-day precision were calculated, tabulated and shown in Table 6.2. The intra-day precision (n = 5) are between 0.8 and 1.7%, while the inter-day variation (n=15) values are between 2.4 and 4.6%. These values are much lower than the permitted acceptable maximum level, confirming the good reproducibility and repeatability of this technique. By taking into consideration the results for method validation, the current HPLC-FLD method and sample preparation procedures employed can be regarded as selective, precise and robust.

Table 6.2
The intra-day precision and inter-day precision of OTA expressed as RSD values

Matrix	Intra-Day Precision (n = 5)[a] (10 µg/kg)	Inter-Day Precision (n = 15)[a] (10 µg/kg)
Wheat	1.2	2.5
Wheat products	1.7	2.4
Rice	0.8	3.4
Rice products	0.4	4.6
Corn	1.0	2.1

[a] n: is number of replicates

6.3.4. Sample analysis

The developed method was applied to the determination of OTA in 25 samples of cereals and cereal products (wheat, wheat based noodles, rice, rice based noodles and corn), which were randomly obtained from groceries and stores in Kuala Lumpur, Malaysia. OTA were not detected in these randomly selected samples (Table 6.3).

Table 6.3
Occurrence of OTA in cereals and cereal products samples in the month of December 2010

Sample category	Total Number of Samples of the Particular Category	Number of Positive Samples	Incidence (Positive/Total Number of Samples of the Particular Category)	Incidence (Positive/ Total Number of Samples)	OTA (µg/kg)
Wheat	5	0	0.0%	0.0%	–[a]
Wheat product	5	0	0.0%	0.0%	–[a]
Rice	5	0	0.0%	0.0%	–[a]
Rice product	5	0	0.0%	0.0%	–[a]
Corn	5				
Total	**25**	**0**	**0.0%**	**0.0%**	

[a]: Values were below the LOD of the adopted method

6.4. Conclusions

The method proposed for OTA determination in cereal is based on a QuEChERS technique followed by HPLC-FLD analysis. This study shows that the coupling of a simple, rapid and low-cost QuEChERS method with HPLC-FLD together with a mixture methodology and RSM can result in a powerful tool for solving complex sample matrices especially at the very low levels of OTA found in food. Chromatographic separation was achieved within 4 minutes to allow the rapid determination as well as to reduce the consumption of mobile phase. Sample preparation was carried out in less than 10 min, without employing a prior dSPE clean up. The percentage recovery of the OTA exceeded 85%, with the LOD and LOQ all within the legal limits set by the European Union (EU). Finally, the entire QuEChERS HPLC-FLD method appears suitable for application to other types of mycotoxins in different food matrices, in addition to avoiding the use of the expensive immunoaffinity columns and the LC-MS method. However, for the final confirmation of the results, the LC-MS with accurate mass measurements would still be necessary.